THE METABOLISM OF COUMARIN BY A PSEUDOMONAS SP.

by

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A THESIS

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

A new pathway for the decomposition of coumarin by a Pseudomonas sp. has been established. This pathway serves as the major route for the metabolism of coumarin by the one culture (Mac 291) studied in detail, while the previously described pathway for the decomposition of coumarin by other soil organisms plays only a minor role in total metabolism by this bacterium. The enzymes involved in either pathway are not constitutive, but are induced through the formation of adaptive enzymes.

Optimum conditions for the decomposition of coumarin have been determined.

INTRODUCTION

Microorganisms play a major role in the decomposition of complex organic materials formed in the course of the development of higher plants and animals. Even complex high polymers that are products of the modern synthetic laboratory are known to serve as microbial substrates under appropriate test conditions (Zobell, 1946; Quastel, 1959). The failure of the accumulation of organic materials, except under conditions not favourable for decomposition, is ample evidence for the turnover of elemental substances. Thus the occurrence of an organic material either in a natural or synthetic condition of necessity makes it subject to biological transformations for an effective balance of the world's supply of available elements.

Coumarin, the lactone of o-coumarinic acid, is a natural constituent of some plants including the tonka bean, lavender, and sweet clover. It can also be prepared readily by synthetic processes in the laboratory. Its fragrant aroma and sharp unique flavor is used in perfumes, tobacco, butter, and various medicines, and as a masking agent for the offensive odors of substances such as iodoform.

Stahmann et al. (1941) identified dicoumarol as the substance in improperly cured hay which causes a hemorrhagic condition in cattle. Roberts and Link (1937) believed that dicoumarol was formed from coumarin because of the structural relationship between these two substances. This

serious feed problem resulted in the development through a plant breeding program of a sweet clover low in coumarin content. The assumption was that a low coumarin level would reduce or eliminate the formation of dicoumarol in sweet clover hay under storage conditions. Results of this program have been reported by Greenshields (1958). However, the origin of dicoumarol has never been satisfactorily explained.

A number of strains of a Pseudomonas sp. which are able to decompose coumarin were isolated by A. C. Blackwood from spoilt sweet clover hay. The aim of this investigation was to study the factors affecting the utilization of coumarin by these bacteria, to identify the intermediates involved in the pathway of the degradation of coumarin, and to assess the role which these organisms might play in the formation of dicoumarol from coumarin.

GENERAL REVIEW OF THE LITERATURE

The Metabolism of Aromatic Compounds

Investigations on the chemical composition of biological systems reveal significant quantities of aromatic compounds which are thought resistant to microbiological attack. Yet, many microorganisms possess inherent properties which allow them to carry out the decomposition of these resistant compounds, curbing an imbalance in the availability of carbon, hydrogen, nitrogen, and other inorganic elements. The tremendous scope of the degradative properties of microorganisms places them in a unique position with respect to the cyclic transformations of biologically-important materials. A stimulating review on the transformation of carbon compounds by microorganisms is presented by Nickerson (1956) who points out the significant role played by these biological agents in the release of carbon from lignin in peat, from coal, and from hydrocarbons in petroleum.

The Rupture of the Aromatic Nucleus

The number of aromatic compounds is multitudinous considering that naturally occurring aromatic systems include compounds with single and multiple rings, and each carbon atom subject to position substitutions of hydrocarbon groups, carbohydrate groups, and inorganic elemental groups, and combinations thereof. On the basis of these wide structural variations, diversities in the pathway of microbial attack are expected. However, just as investigations in the metabolism of aliphatic compounds have led to the postulation of only a few major biological routes, e.g., the

Embden-Meyerhof scheme and the TCA cycle, for metabolic transformation in biological systems, investigations of the degradative processes of aromatic compounds have led to the discovery that only a few mechanisms for cyclic ring rupture exist. Dagley et al. (1960) divide these reactions into two groups:

1. Oxidative fission of the bond between carbon atoms bearing the hydroxyl groups of an o-dihydroxyphenol, e.g., catechol ⟶ cis-cis muconic acid by pyrocatechase (Hayaishi and Hashimoto, 1950; Evans et al., 1951); protocatechuic acid ⟶ cis-cis -β -carboxymuconic acid through the action of protocatechuic acid oxidase (MacDonald et al., 1954; Gross et al., 1956). β -Ketoadipic acid is formed eventually in both cases (Kilby, 1948; Stanier et al., 1950); this compound is then enzymatically split to form acetyl-coenzyme A and succinyl-coenzyme A which are then metabolized by reactions of the TCA cycle (Katagiri and Hayaishi, 1957; Ottey and Tatum, 1957). This mechanism of ring cleavage appears to play little or no role in mammalian metabolism.

2. Rupture of the bond between the carbon atoms bearing a hydroxyl and an adjacent carbon atom carrying a carbon side-chain or carboxyl, e.g., homogentisic acid ⟶ maleyl-acetoacetate by homogentisicase (Knox and Edwards, 1955; Chapman et al., 1960); gentisic acid ⟶ maleylpyruvate by

gentisicase (Lack, 1959; Sugiyama et al., 1960a,b; Douglass et al., 1959). This method is common in both microorganisms and mammals.

One of the most significant contributions to the field of bacterial intermediate metabolism in recent years is the mechanism proposed by Dagley et al. (1960) for rupture of aromatic rings by mechanisms not previously considered. They proposed the splitting of dihydroxyphenolic compounds at a site adjacent to rather than across the oxygenated carbon atoms with the formation of a semialdehyde. The product of protocatechuic acid rupture is α-hydroxy-β-carboxymuconic semialdehyde; the product of catechol ring rupture is α-hydroxymuconic semialdehyde. The latter product had previously been encountered in mammalian systems by Wiss et al. (1956) and Mehler (1958) who had shown that an enzyme from liver oxidizes 3-hydroxyanthranilic acid to a derivative which is converted non-enzymatically to the semialdehyde. These reports on the enzyme of mammalian origin probably played a major role in the postulation of new ring cleavage mechanisms in bacteriological systems.

A summary of the mechanisms known for the cleavage of aromatic rings follows. All of the ring cleavage mechanisms involve compounds of the C_6-C_0*, C_6-C_1, and C_6-C_2 types. No mechanism for the rupture of the aromatic ring of C_6-C_3 compounds has been proposed.

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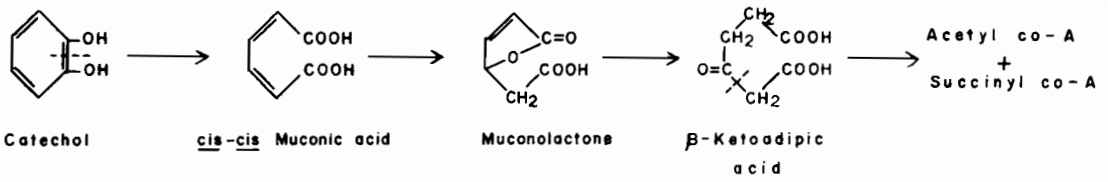
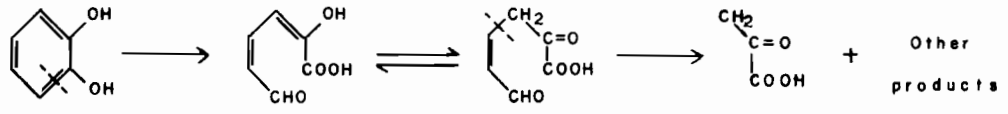
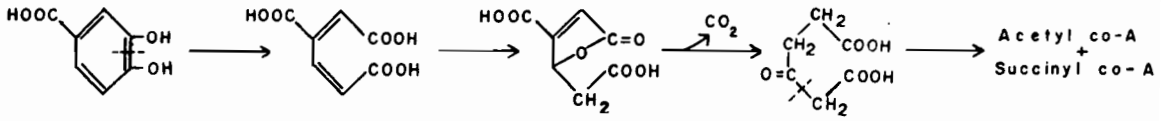
* This notation refers to groups of single-ringed aromatic compounds belonging to different classes based on structural considerations. In the notation C_6-C_n, C_6 refers to the benzene ring while C_n refers to the number of carbon atoms in the side chain. For example, phenylacetic acid can be designated as a member of the C_6-C_2 group of compounds.



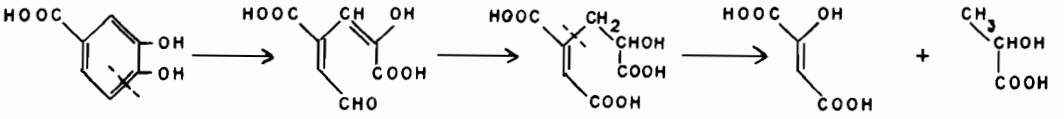
Catechol — cis-cis Muconic acid — Muconolactone — β-Ketoadipic acid



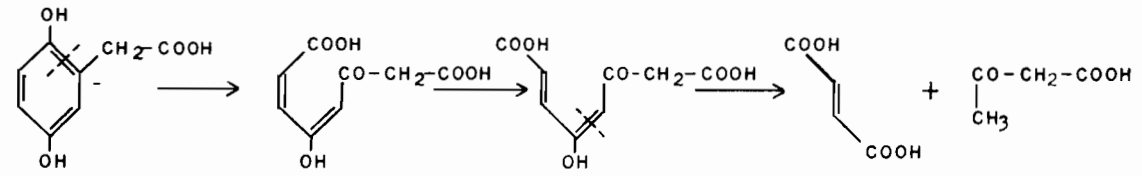
Catechol — α-Hydroxymuconic semialdehyde — [keto form] — Pyruvate



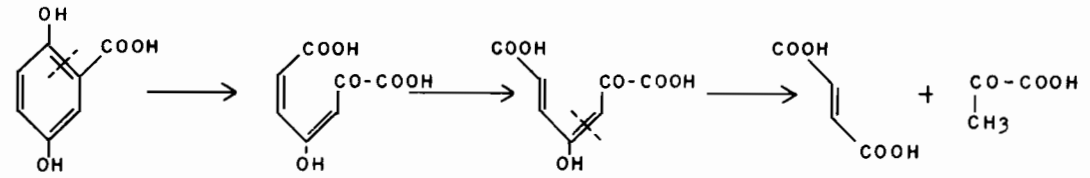
Protocatechuic acid — cis-cis-β-Carboxy-muconic acid — β-Carboxy-muconolactone — β-Ketoadipic acid



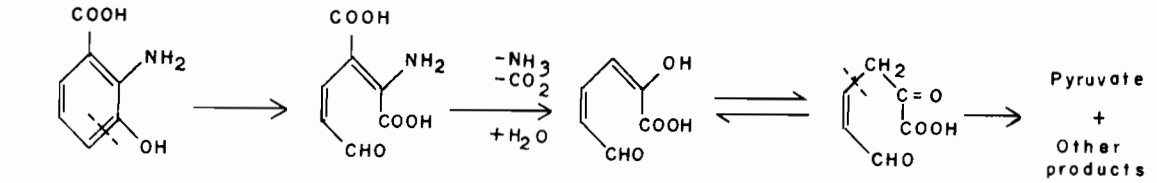
Protocatechuic acid — α-Hydroxy-γ-carboxy-muconic semialdehyde [enol form] — 2-Hydroxy-4-carboxy-2,3-dihydromuconate — Oxaloacetate — Lactate



Homogentisate Maleylacetoacetate Fumarylacetoacetate Fumarate Acetoacetate



Gentisate Maleylpyruvate Fumarylpyruvate Fumarate Pyruvate



3-Hydroxyanthranilic acid

∝-Hydroxymuconic semialdehyde

[keto form]

Pyruvate + Other products

The Intermediate Metabolism of Aromatic Compounds

Most investigations on substances not able to undergo direct ring cleavage deal with transformations of compounds which allow them to be channeled into known rupturing mechanisms. In the decomposition of coumarin, it is probable that the lactone ring is the initial site of attack, forming a benzene derivative with a hydroxyl group ortho to the side chain. Therefore, the microbial transformation of o-hydroxylated compounds will be considered preferentially although m-, and p- hydroxylated compounds are included if the reaction has not been reported for the ortho isomer. As well, preference will be given to reports in which the reaction has been described in bacteria, and not necessarily where the reaction was first described. Where mechanisms have not been recognized in bacteria, references will be given for type reaction in fungi as well as in higher plants and animal systems.

Below are some biological modifications of aromatic compounds which fit into known patterns of ring cleavage:

Metabolism of C_6-C_0 compounds:- Marr and Stone (1961) suggested the bacterial oxidation of benzene by Pseudomonas aeruginosa and Mycobacterium rhodochrous is brought about through catechol via 3,5-cyclohexadiene-1,2-diol. Phenol and o-benzoquinone were excluded as possible intermediates in the metabolism of benzene by these organisms. This indicates that the bacterial oxidation of benzene is different from mammalian systems from which phenol has been isolated (Porteus and Williams, 1949).

Phenol is metabolized through catechol in both animal and bacterial

systems (Evans and Happold, 1939; Sleeper and Stanier, 1950; Parke and Williams, 1953).

Metabolism of C_6-C_1 compounds:- This group of compounds includes benzoic acid, salicylic acid, saligenin, toluene, and hydroxytoluenes. The first reaction in the metabolism of benzoic acid is the introduction of a hydroxyl group into the benzene ring. Three hydroxylated isomers of benzoic acid exist; o-, m-, and p-hydroxybenzoic acid. The metabolic relationship between benzoic acid and its hydroxylated derivatives is quite complex. Investigations show that all three isomers can be formed in biological systems (see review by Dagley et al., 1960).

Stanier (1948) using the technique of sequential induction suggested that the oxidation of benzoic acid by several strains of Pseudomonas fluorescens involved the simultaneous introduction of two hydroxyl groups leading to the formation of catechol. He ruled out the possibility of a single step hydroxylation on the basis of either no utilization of monohydroxylated isomers of benzoic acid or the presence of a lag period before utilization of these compounds when cells were grown on benzoic acid. Bhat et al. (1959) using acetone-dried cells of Pseudomonas convexa var. hippuricum strain HPP_3 showed that salicylic acid is an intermediate of benzoic acid metabolism. They attributed differences in results from that of Stanier on the basis of cell impermeability of salicylate to Pseudomonas fluorescens. Both these Pseudomonas spp. metabolize catechol through β-ketoadipic acid.

Benzoate is also metabolized by mechanisms which do not appear

to involve monohydroxylated intermediates (Proctor and Scher, 1960; Evans, 1947). Fina and Fiskin (1960) used sewage effluents and rumen fluid to show that decomposition of benzoic acid may also take place under anaerobic conditions. The mechanism of ring rupture in none of these cases is known.

Walker and Evans (1952) showed that salicylic acid is metabolized through catechol by soil bacteria; this reaction was also observed in microorganisms by Bhat et al. (1959) and Rogoff and Wender (1957). Shepherd and Villanueva (1959) showed that salicylic acid can be converted by Aspergillus nidulans to 2,3-dihydroxybenzoic acid which is further metabolized to catechol, while Yano and Arima (1958) showed that salicylic acid can also proceed through gentisic acid in several Pseudomonas spp.

Compounds produced by the introduction of a methyl side chain into the benzene ring or into a phenol ring appear to be metabolized in a regular sequential pattern until the homologous acid is formed (the degradation of the acid produced was previously discussed)(Happold, 1950; Evans et al., 1951; Sato et al., 1956; Nickerson, 1956). A good example is p-cresol which is oxidized through p-hydroxybenzyl alcohol to p-hydroxybenzaldehyde and then to p-hydroxybenzoic acid (Dagley and Patel, 1957). Tausson (1927) studied the degradation of naphthalene and suggested the scheme proceeded through saligenin (o-hydroxybenzyl alcohol) to salicylaldehyde and hence to salicylic acid. Sato et al. (1956) demonstrated the formation of saligenin from o-cresol in rat liver but only in trace amounts. Bray et al. (1950) detected 2,5-dihydroxytoluene in the urine of rabbits administered o-cresol, but further decomposition was not shown. However,

toluene was oxidized to benzoic acid (Bray, 1951). Thus oxidation of o-cresol through the corresponding alcohol, aldehyde, acid sequence is probably correct.

o-Cresol is also metabolized anaerobically at a very slow rate by sewage effluents (Ettinger et al., 1951) but the mechanism of breakdown is not known.

Metabolism of C_6-C_2 compounds:- This group of compounds is not widely distributed in nature and very little attention has been paid to their metabolism. Kluyver and van Zipj (1951) using Aspergillus niger showed that phenylacetic acid is metabolized through homogentisic acid. This shows that C_6-C_2 compounds can undergo a hydroxylation ortho to the side chain. However, the individual steps in this system were not studied in detail and no intermediate steps have been established for any of the monohydroxylated derivatives of phenylacetic acid.

Metabolism of C_6-C_3 compounds:- These compounds are widely distributed and many studies have been reported on the synthesis and degradation of several members. Numerous phenylpropanoids including phenylpyruvic acid, phenylpropionic acid, p-coumaric acid, caffeic acid, ferulic acid, and cinnamic acid are readily incorporated into the lignin fraction in excised shoots of various plants (Neish, 1960). The structure of lignin has not been satisfactorily defined and the term "lignin" is now used as a collective term for a group of high molecular weight amorphous compounds of C_6-C_3 units rather than for a chemically defined compound. The polymer is bound in an unknown manner with carbohydrate.

Henderson and Farmer (1955) have suggested that the decomposition of C_6-C_3 compounds by the combined action of different fungi may constitute a stage in the decomposition of this biologically resistant material.

Two of the three aromatic amino acids, viz., phenylalanine and tyrosine, are C_6-C_3 compounds; both of these aromatic amino acids are present in large amounts in the protein fraction of plant and animal tissue (Fruton and Simmonds, 1953). Mechanisms for the synthesis and degradation of these amino acids have been satisfactorily explained (see recent review of Neish, 1960).

The first step in the degradation of both aromatic acids is one of deamination to form the corresponding keto acid. Dagley et al. (1953) showed that a Vibrio sp. (Vibrio 0/1) was able to deaminate both phenylalanine and tyrosine to form phenylpyruvic acid and p-hydroxyphenylpyruvic acid respectively.

Cinnamic acid is a C_6-C_3 compound having an unsaturated group between the α-, and β-carbon. Whiting and Carr (1959) showed that the side chain of cinnamic acid is reduced by Lactobacillus pastorianus var. quinicus to form dihydrocinnamic acid. Further decomposition of dihydrocinnamic acid is not known, but its o-hydroxylated derivative, melilotic acid, is metabolized by a Pseudomonas sp. to form 2,3-dihydroxyphenylpropionic acid (Coulson and Evans, 1959); further reactions have not been established. Thus, hydroxylation of the benzene ring can occur without modification of the side chain structure.

p-Coumaric acid is oxidized to phloretic acid by Lactobacillus

pastorianus var. quinicus, and as well, p-ethylphenol was identified as another product of p-coumaric acid oxidation (Whiting and Carr, 1959). This shows that following the reduction of the side chain with the formation of a terminal methyl group, a decarboxylation step occurs. The authors claim that the extent of decarboxylation decreases as the number of phenolic hydroxyl groups in the molecule decreases. Thus, dihydrocaffeic acid undergoes a considerable degree of decarboxylation, while dihydrocinnamic acid is decarboxylated to a much less extent.

o-Coumaric acid (o-hydroxy-trans-cinnamic acid) undergoes many transformations. Booth et al. (1959) showed that the side chain of o-coumaric acid is reduced by rats and rabbits to form melilotic acid. The same workers found that o-coumaric acid is also hydrated with the hydroxyl group residing either at the α-carbon or β-carbon site. Location of the hydroxyl group at the α-carbon of o-coumaric acid forms o-hydroxyphenyllactic acid. o-Hydroxyphenylacetic acid was detected in the urine of rats and rabbits, and Booth et al. (1959) proposed that o-hydroxyphenyllactic acid is oxidized to form o-hydroxyphenylpyruvic acid which undergoes decarboxylation to form o-hydroxyphenylacetic acid. This shows a decarboxylation step results in the formation of an additional terminal carboxyl group. Location of the hydroxyl group at the β-carbon of o-coumaric acid results in the formation of o-hydroxyphenylhydracrylic acid. This compound undergoes reduction and cyclization (the sequence of reactions is not known) to form 4-hydroxycoumarin (Mead et al., 1958).

All three hydroxylated derivatives of cinnamic acid undergo β-oxidation along the classical lines of β-oxidation as proposed by

Knoop (1904). Evans (1961) found that a Pseudomonas sp. degraded o-coumaric acid (rather o-coumarinic acid), by β-oxidation reactions while Booth et al. (1957;1960a) showed that β-oxidation of m-, and p-coumaric acid also occurs in rats and rabbits.

A rather unique mechanism exists in the formation of homogentisic acid from tyrosine. Following deamination to form the keto acid, the oxidation of the benzene derivative is accompanied by an intramolecular rearrangement and the side chain of p-hydroxyphenylpyruvic acid is shifted with respect to the hydroxyl group of tyrosine. The product, 2,5-dihydroxyphenylpyruvic acid, undergoes decarboxylation to form homogentisic acid, which enters the aromatic ring cleavage system (Fruton and Simmonds, 1953). The "shift" mechanism is not clearly understood.

Metabolism of coumarin:- Coumarin, the aromatic lactone of o-hydroxy-cis-cinnamic acid, is widely distributed in many plant families (Geissman and Hinreiner, 1952), and is particularly abundant in Umbelliferae and Rutaceae. It has been detected as a constituent in tonka bean, lavender oil, most varieties of sweet clover (Melilotus spp.) (Clayton and Larmour, 1935; Roberts and Link, 1937), and in the uredospores of wheat stem rust (van Sumere et al., 1957).

The presence of coumarin in sweet clover is considered to be the cause of "Melilot taint", a hemorrhagic condition in cattle indirectly responsible for the loss of livestock after eating 'spoilt' sweet clover hay. Coumarin itself is not the active principle. Stahmann et al. (1941) isolated the hemorrhagic agent from spoilt sweet clover hay and showed

that dicoumarol, and not coumarin, is the cause of the disease. Although the origin of dicoumarol has not been satisfactorily explained, Roberts and Link (1937) suggested that it is formed from coumarin. Recently, however, Bellis (1958), using two Penicillium spp., isolated large quantities of 4-hydroxycoumarin and traces of dicoumarol when o-coumaric acid was used as a sole carbon source; neither of these products formed when o-coumaric acid was replaced by coumarin. He suggested that dicoumarol is formed from o-coumaric acid and not from coumarin itself. Dicoumarol interferes with the blood clotting mechanism; investigations show that vitamin K is required for the synthesis in the liver of prothrombin, essential for blood coagulation, and that dicoumarol interferes with the action of this vitamin (Link, 1945). More recently, Ernster et al. (1960) have reported the presence in liver of a highly dicoumarol-sensitive diaphorase although the presence of this enzyme has not been linked with prothrombin formation.

This serious problem has led to the development at the Dominion Forage Crop Laboratory in Saskatoon under the direction of Dr. J. E. R. Greenshields of a "coumarin-free" variety of sweet clover termed 'Cumino'. The coumarin level of Cumino variety ranges from 0% in most plants to 0.004% in a small proportion of the population. This level is not considered significant since plants of the Artic and Erector varieties regularly show 0.2% to 1.0% coumarin. The test used measures coumarin, o-coumaric acid, and "bound" coumarin (unidentified glycosides) content, and hence the actual precursor of dicoumarol is more of an academic problem than an agricultural problem. This breeding program is justified only if all the compounds related to coumarin are eliminated by genetic control, for any

one of them could possibly be a precursor of dicoumarol.

As well as its possible role in the adverse action in animal tissue, coumarin also possesses selective phytocidal actions in very low concentrations. Audus and Quastel (1947) showed that coumarin exerts a dual influence on the development of germinating seeds. Firstly, it inhibits germination, and secondly, it inhibits root growth. A species variation in plants was also observed. This phytocidal action was lost when the coumarin configuration was modified by substitutions. On the other hand, Neumann (1959) showed that coumarin at very low levels should be regarded as an auxin on the basis of its ability to stimulate elongation of sunflower hypocotyl segments. This effect was similar in pea epicotyls and oat coleoptiles (Neumann, 1960) although species differences and degrees of effectiveness were noted. The nature of this auxin-like action, while different from that of indoleacetic acid, is not understood.

Coumarin at levels of 10-100 µgm per ml markedly stimulated the germination of the uredospores of Puccinia graminis var. tritica (van Sumere, 1957).

Mead et al. (1958), in a series of investigations on the mode of detoxication of aromatic compounds in animals fed coumarin to chinchilla rabbits and identified 3-, 7-, and 8-hydroxycoumarin as metabolites which are excreted in the urine in conjugated forms. Similar results occurred with ferret, guinea pig, rat, and mouse, the first three of which also excreted 5-hydroxycoumarin. No evidence was obtained for ring rupture. o-Coumaric acid, however, appears to be largely excreted unchanged in the rabbit. The formation of small amounts of the glucuronide of

4-hydroxycoumarin and free 7-hydroxycoumarin from coumarin shows, however, that cyclization *in vivo* does occur. The rabbit converts melilotic acid to 4-, and 7-hydroxycoumarin. Hydroxylation mechanisms in animals are common detoxication reactions in which the resulting phenols are excreted in conjugated forms. Thus, conjugation serves as a model for initial site of biological attack in systems where conjugation does not occur. Booth *et al.* (1959) reported the urinary metabolites of coumarin and related compounds in rats and rabbits. They identified a large number of coumarin derivatives which demonstrated clearly the rupture of the lactone ring. Three phenolics identified readily when coumarin was administered to rats were o-coumaric acid, melilotic acid, and o-hydroxyphenylacetic acid, as well as conjugated forms of o-coumaric acid, melilotic acid, and o-hydroxyphenyllactic acid (trace). Unidentified products were also present indicating further unresolved biochemical steps. When o-coumaric acid and melilotic acid were used, in addition to the same products formed from coumarin, o-hydroxyphenylhydracrylic acid was identified. But when o-hydroxyphenyllactic acid was administered, the only product detected was o-hydroxyphenylacetic acid. When coumarin was administered to rabbits, the major product detected was o-hydroxyphenylacetic acid, while melilotic acid, o-coumaric acid (trace), and o-hydroxyphenyllactic acid were present at lower levels. From o-coumaric acid, o-hydroxyphenylhydracrylic acid was found in addition to the same products formed from coumarin. A species difference was noted between rats and rabbits in that rabbits excreted 3-, and 7-hydroxycoumarin while rats did not. The identification of C_6-C_3 products shows that the lactone ring is ruptured, and that the side chain can be reduced in animals, and can also undergo a decarboxylation step.

Audus and Quastel (1947) were the first to report the breakdown of coumarin in the soil. A solution of coumarin containing 100 ppm was circulated through soil by means of a soil perfusion apparatus, and the rate of disappearance of coumarin followed colorimetrically. Under experimental conditions, coumarin was broken down completely in 48 hours, but no attempt was made to speculate on the intermediate steps.

Bellis (1958) was the first to use coumarin as a carbon source in pure culture studies. He used coumarin as a sole carbon source in modified Czapek medium, and was able to show that two molds identified as Penicillium jenseni and P. nigricans could reduce the level of coumarin, but at no time was it possible to isolate any coumarin derivatives. In the presence of a medium containing sucrose and coumarin, a small amount of umbelliferone (7-hydroxycoumarin) was found.

Fernley and Evans (1958) identified coumarin as a metabolite in the decomposition of naphthalene by a soil pseudomonad. The product was identified by smell, by chromatography, by absorption spectrum, and by mixed melting point. They suggested that o-coumarinic acid is the actual metabolite present and not coumarin itself. These authors, then, considered that coumarin was formed as a result of non-enzymatic action. No evidence for the disappearance of coumarin on continued culture was presented at that time. Evans (1961) suggested later, that coumarin is metabolized through to salicylic acid via o-coumarinic acid.

Synthetic mechanisms of coumarin formation in plants have been investigated. Kosuge and Conn (1959) have shown with radioactive tracers that o-coumaric acid is converted primarily to o-coumarylglucoside in

white sweet clover (Melilotus alba), although small amounts of coumarin, melilotic acid, melilotylglucoside, and at least two unidentified compounds were also found. They suggested o-coumaric acid undergoes a trans to cis isomerization, then is lactonized by a loss of a water molecule to form coumarin. trans-Cinnamic acid and phenylalanine were both effective precursors of o-coumaric acid. Labeled glucose and shikimic acid also gave rise to labeled o-coumaric acid which suggested that the shikimic acid pathway functions in the biosynthesis of both coumarin and o-coumaric acid. Similar results were obtained by Brown et al. (1960) using the perennial grass Hierochloë odorata, and Melilotus officinalis.

Possible Mechanisms for the Decomposition of Coumarin

A review of the literature has shown that certain biochemical reactions can be expected. The lactone ring of coumarin is readily split in vitro under mild alkaline conditions in the presence of mild heat treatment (Roberts and Link, 1937). As well, the detection of phenolic compounds arising as a result of cleavage of the lactone ring by plants and animals strongly indicates that the lactone ring is the initial site of attack by bacteria. Cleavage of the lactone ring results in the formation of a C_6-C_3 compound possessing an unsaturated side chain ortho to a hydroxyl group. In only one instance is a "shift" mechanism known whereby the hydroxyl group changes its position relative to the side chain, viz., the formation of homogentisic acid from p-hydroxyphenylpyruvic acid accompanying the introduction of the second hydroxyl group (Fruton and Simmonds, 1953); the p-hydroxyl group of p-hydroxyphenylpyruvate acquires the meta position in homogentisate. On the assumption that

the first step is cleavage of the lactone ring, and that a shift mechanism altering the position of the hydroxyl group from its ortho position does not occur, a number of theoretical possibilities can be considered:

A. Mechanism involving ring rupture:
   1. Modification of the side chain to the catechol configuration
   2. Modification of the side chain accompanied by benzene ring hydroxylation to the homogentisic acid configuration
   3. Ring rupture through a pathway by unknown reactions

B. Mechanisms involving side chain modification:
   1. Side chain isomerization
   2. Cleavage of a C_2 unit by β-oxidation
   3. Reduction of the double bond to form an alkane side group
   4. Removal of a carbon atom(s) by a decarboxylation process
   5. No modification prior to ring cleavage

Experimental Approaches to Intermediate Metabolism

A number of experimental methods have been used in investigations designed to elucidate steps in intermediate metabolism. Some of these methods will be briefly outlined and their limitations commented upon.

The detection of end products:- The detection of end products permits conjecture, on the basis of structural considerations, of the steps involved in the formation of one product from another. This approach

has led to the successful solution of many problems in both aliphatic and aromatic metabolism, but is dependent upon the intracellular or extracellular accumulation of products at levels sufficiently high for detection and identification. With the rapid development of chromatographic techniques, where quantities less than 10 µgm are often adequate for positive identification, this problem is less acute. However, one must interpret results obtained by this method with caution. An identified product may not be a true intermediate in a series of reactions, but only a side reaction.

The use of resting cell suspensions:- The technique of using resting cell suspensions was introduced by Quastel who felt it was necessary to distinguish between the reactions of proliferating cells and cells studied under conditions where growth was small or negligeable. The use of resting cells in manometric experiments is now common practice. Quastel (1959) writes:

> There was much antagonism, at first, particularly among the orthodox bacteriologists, to the introduction of the term "resting cells" or even "non-proliferating cells. The criticisms were rarely expressed other than verbally, but there was an opinion that "resting cells" were "dying cells" whose reactions represented post-mortem phenomena that had little to do with the phenomena of the growing cell .... the so-called biological criticisms were soon silenced by observations that the reactions of resting cells were indeed representative of normal cell life and were basal to our understanding of normal cell metabolism and growth. Nearly 30 years have now passed since the introduction of the resting cell technique. The papers being published at the present time, in the field of microbial chemistry, that utilize resting cells or washed suspensions of cells, used under conditions where there is but little or no proliferation, are as great as at any time in the history of this subject. The shortcomings and difficulties inherent in the technique are known, but they are surpassed by the advantages of working with a method that is showing itself most fruitful ...

The use of cell-free extracts:- The preparation of cell-free extracts, and the isolation of single enzymes in a reasonably pure state, aids identification of intermediates; one can measure either the decrease of substrate, or the increase of the end product of enzyme reaction. More frequently this precedure is used to confirm steps suggested by experiments with whole cells. The preparation of sub-cellular particles by suitable techniques, such as differential centrifugation, dissociates enzymatic steps. This approach has been used extensively in animal cells, but more recently (Wachsmann et al., 1960; Abrams et al., 1960), techniques have become available for the preparation of sub-cellular bacterial particles which can be associated with specific enzymatic reactions. Frequently, however, the instability of an enzyme, or inadequate co-factor considerations, place limitations on this technique.

Biological mutants:- The isolation of organisms having deficiencies in their metabolic pathways has also become a research tool of great value. Organisms of this nature arise as a result of malfunctioning of, or absence of, gene-controlled enzymes in a reaction series. Such a deficiency may permit the accumulation of products preceeding the block, or exact growth requirements for cell development. Davis (1955) has applied this technique very successfully using mutants of Escherichia coli to establish the synthesis of phenylalanine and tyrosine from carbohydrates. Isolation of mutants is usually done by treatment of cells with a mutagenic agent. Mutation is a chance occurrence, and consequently, elaborate screening programs are required in order to obtain material suitable for investigation. Frequently mutants cannot be found. On the

other hand, one has no guarantee that induced mutation does not have side effects peculiar to the normal pattern of the cell.

Radioactive tracers:- Radioactive tracers have been applied to biological problems only since the mid '40's, and in this short interval they have proven themselves most useful. With appropriately labeled substrates, the path of each atom in a sequence of reactions can be traced. It becomes possible to follow in extremely complex systems biochemical reactions which would be completely masked by normal cellular activities. The availability of numerous active isotopes, and the improvement in methods of detection, is an added impetus to investigations using this technique.

While each of these techniques has proven useful, none can be used as a sole means of experimental approach. Evidence obtained by one line of approach complements information derived from another. Several of the above techniques were used to study the pathway through which coumarin is degraded by bacteria. For greater clarity, the experimental work will be presented in several sections. The first section is concerned with the influence of environmental conditions on coumarin degradation. In the second section, results obtained with the 'sequential induction' technique are presented. In the third section, chromatographic evidence for the steps in coumarin breakdown is reported, while the last section gives results obtained with radioactive substrates.

PART I

FACTORS AFFECTING THE UTILIZATION OF COUMARIN BY BACTERIA

INTRODUCTION

Physical factors play an important role in the functioning ability of organisms. A favourable temperature, favourable pH, and a favourable degree of aeration are of prime importance for growth and metabolism. Bergey's Manual (Breed, et al., 1948) uses temperature as an important taxonomic tool for classifying Pseudomonas organisms beyond the genus level. One group has an optimum temperature at 37° C while a second group has an optimum temperature over a range of 20° to 30° C. A strain of Pseudomonas fluorescens has been recorded to undergo growth at -4° C (Porter, 1946).

Pseudomonas aeruginosa has a pH spectrum from 5.6-8.0 (Porter, 1946), a spectrum not uncommon for many bacterial species. Sugiyama et al. (1958) found that gentisic acid oxidase has an optimum pH of 8.0, a value equal to that of pyrocatechase reported by Hayaishi et al. (1957) and somewhat higher than the optimum pH of 7.0-7.2 reported for homogentisic acid oxidase (Crandall, 1955). The activity of protocatechuic acid oxidase, however, increases as the pH increases to pH 9.0; beyond this value, autooxidation occurs (Stanier and Ingraham, 1954). The optimum pH of a purified enzyme cannot be compared with that of a living cell, for the optimum pH of the latter is a summation of inumerable enzymatic reactions.

Gentle agitation, or aeration, is favourable to the growth of aerobic and facultative microorganisms in liquid medium since it aids in breaking up clumps of cells, and aids the removal from the cells immediate environment of toxic products excreted during the course of metabolism. As well, atmospheric oxygen is known to be directly involved in the cleavage of the aromatic ring. Stanier et al. (1950) showed that the oxidation of catechol by enzymatically-active dried cells of Pseudomonas fluorescens involves an oxygen uptake of one mole per mole substrate with the formation of β-ketoadipic acid. Similarly, the oxidation of protocatechuic acid by the same preparation involves an oxygen uptake of one mole per mole substrate to form β-ketoadipic acid and the release of one mole of CO_2. Sugiyama et al. (1960a) showed that one mole of oxygen is consumed per mole substrate in the formation of maleylpyruvic acid from gentisic acid. Crandall et al. (1960) using O_2^{18} showed that atmospheric oxygen is incorporated into maleylacetoacetate, the product of homogentisate cleavage by rat liver.

A medium for the growth of an organism must have three essentials. Firstly, it must have a suitable amount of a utilizable substrate from which the organism acquires its energy for synthesis of protoplasm and other cell constituents, and for the repair and maintenance of cellular components. Secondly, it must have an adequate supply of growth factors required for the proper functioning of the synthetic and degradative mechanisms. Thirdly, it must have ionic requirements in adequate supply. The first and third essentials are usually met with not too much difficulty but the second requirement may present a problem. James (1958) points

out that soil extract has long been used both as the main source of nutrients and as additives in media containing known organic materials and mineral salts for the quantitative determination of soil populations. Lochhead and Chase (1943) devised a series of media of varying complexity in an attempt to classify organisms on the basis of their nutritional requirements. The advantages of a chemically defined medium are obvious. With an increased knowledge of "growth factor" chemistry, it is possible to prepare chemically-defined media for many organisms (Brownlow and Wessman, 1960; McDonald, 1960; Baker et al., 1960; Grula et al., 1961).

Benns (1958) showed that the organisms used in this investigation could be differentiated on the basis of physiological characteristics. However, no detailed study of the environmental or nutritional factors which influence coumarin decomposition were undertaken. Consequently, a series of investigations was carried out to determine the growth characteristics of the organisms, and to assess the effect of alterations in the environment on the rate of decomposition.

MATERIALS AND METHODS

Isolation and Taxonomy of the Organisms

The organisms used in this investigation were transfers from stock cultures isolated by A. C. Blackwood, with the assistance of J. E. R. Greenshields, from a stack of sweet clover treated to induce spoilage conditions. Isolation was carried out on an agar medium containing 1.0% glucose, 0.2% N.Z. Case peptone, 0.5% coumarin, and a mineral salts mixture. The stack was sampled at varying depths. Stem cuttings were taken under conditions as aseptically as possible and dropped onto the surface of the agar plates. The plates were incubated at varied temperatures and were examined daily for indications of coumarin breakdown. Decomposition is readily observed by using a medium containing a sufficiently high concentration of coumarin (0.4%) such that crystallization of the coumarin will occur on cooling. This property was quite useful in selecting organisms with degradation properties in that organisms which possess metabolic activity produce a halo surrounding the colony.

After incubation of the stem cuttings for several days, zones of clearing were observed on several plates. While the stems themselves were heavily contaminated with mold growth, no molds developed on the plate itself. This is not surprising in view of the phytocidal properties of coumarin, and no doubt mold growth is a secondary effect taking place when the level of coumarin falls below phytocidal levels. Several attempts were made without success to isolate microorganisms which would utilize coumarin from other sources.

After preliminary isolation, the bacteria were streaked onto an agar maintenance medium containing 0.5% tryptone, 0.5% yeast extract, 0.5% coumarin, and mineral salts. A number of colonies were obtained in pure culture, given accession numbers, and placed in the Prairie Regional Laboratory Culture Collection. A preliminary study (Benns, 1958) on the taxonomic position of the isolates indicated that all were members of the genus Pseudomonas although species names as listed in Bergey's manual (Breed, et al., 1948) could not be assigned because of discrepancies in their physiological patterns.

The organisms used in this investigation were those having PRL accession numbers 1387-1394 inclusive (Macdonald College Culture Collection accession numbers 289-296 inclusive).

Preparation of Media

A coumarin base medium (CYT medium) was prepared with 0.3% coumarin, 0.5% yeast extract (Difco), and 0.5% tryptone (Difco), and concentrated Mineral Salts 1 (see TableII) incorporated aseptically 10% by volume into the base medium after sterilization at 121° C for 15 minutes. CYT semisolid medium was prepared by adding agar (Difco) to a final concentration of 0.1% to the CYT medium; CYT solid medium was prepared by adding agar to a final concentration of 1.5% to the CYT medium.

Growth and Maintenance of Cultures

Cultures were grown on CYT solid medium at 30° C throughout the course of this investigation. Frequently, transfers from stock cultures failed to grow on fresh CYT medium. In this event, cultures were transferred at 24 hour intervals firstly, onto nutrient agar slopes, secondly,

into CYT semisolid medium, and thirdly, onto CYT solid medium. CYT medium at 30° C was used to grow the cultures in the liquid state. Stock cultures were maintained on CYT solid medium at 7° C, and rejuvinated periodically.

Factors Affecting the Utilization of Coumarin

The effect of temperature, aeration, pH, inorganic ions and nutrition on the utilization of coumarin was studied intensively by modifying the physical and chemical constitution of CYT medium. Detailed procedures for each condition are presented more favourably along with the results.

Preparation of Inoculum

The importance of standardization of inoculum in nutritional experiments with fungi has been stressed by Ward and Colotelo (1960). Age of cells and quantity of inoculum gave the widest variation in response to test conditions. Vitamin carry-over was of importance in nutrition studies. In order to obtain results that were comparable during fermentation trials, the inoculum was standardized before use by a systematic series of transfers at 24 hour intervals in the following manner: From a fresh CYT slant, a transfer was made into five ml CYT semisolid medium; one ml was transferred to a 125 ml Erlenmeyer flask containing 40 ml CYT medium and was incubated at 30° C on a rotary shaker having a one inch eccentricity at a rate of approximately 250 oscillations per minute. The resultant culture served as inoculum using a 0.25% level in all trials.

The Quantitative Determination of Coumarin

Coumarin content was measured quantitatively by the colorimetric method of Clayton and Larmour (1935) using diazotized p-nitroaniline as the developing reagent; a stoichiometric response over a range of 0-40 µgm coumarin was obtained when a slight modification was made. A reduction in the quantity of sodium carbonate over that used by Clayton increased the sensitivity of the test. As the optical density is dependent on the time allowed for color formation, a developing time of 30 minutes was arbitrarily chosen as a convenient interval. The test was carried out as follows:

To an optically-calibrated test tube, add an aliquot of fermentation liquor containing less than 40 µgm coumarin and one ml 1% aqueous solution of sodium carbonate and make the volume up to nine ml with water. Heat the contents at 75° C for a period of 15 minutes (temperature and time are not critical). After cooling, add one ml freshly prepared diazotized p-nitroaniline as color developing reagent and mix thoroughly. Read in a Coleman colorimeter using a 470 mµ filter after 30 minutes. Simultaneously, prepare a blank for standardization of the instrument by omitting coumarin from the test.

With aliquots of 0.05 ml or less, determinations were carried out in triplicate; with aliquots larger than 0.05 ml, one determination was satisfactory.

The diazotized p-nitroaniline was prepared as follows:

Solution A. Dissolve 3.5 gm p-nitroaniline in 45 ml 37% hydrochloric acid and dilute to 500 ml with distilled water. Filter. This solution is stable.

Solution B. Dissolve 5.0 gm sodium nitrite in 100 ml distilled water. This solution is not stable and should be renewed periodically. Place both solutions on ice before use.

Into a chilled 100 ml volumetric flask, place 3.0 ml of each solution and leave on ice bath for five minutes. Add an additional 12.0 ml of Solution B and leave on ice bath for an additional five minutes. Make the flask contents up to volume with ice-cold distilled water. Leave on ice and use after one hour. The reagent remains stable for four hours after preparation.

RESULTS

The Effect of Alterations of the Physical and Chemical Environment on the Utilization of Coumarin

Effect of temperature:- After inoculation of 40 ml CYT medium in 125 ml Erlenmeyer flasks, incubation was carried out statically at temperatures of 20° C, 30° C, 37° C, and 45° C. Five ml samples were withdrawn from each flask after 18, 48, and 72 hour incubation times. The samples were acidified with one drop 6 N HCl and centrifuged. The effect of temperature was determined by measuring residual coumarin in the supernatant liquid. In these and all subsequent samples, the pH and turbidity were determined as a routine practice.

The response of one culture (Mac 291) to variations in temperature is shown graphically in Fig. 1; Appendix Tables I-IX contain the results obtained with all cultures. It was found that all of the organisms grow moderately at 20° C, which was the lowest temperature at which quantitative determinations were made. Unacidified sample supernatant stored in the cold at 7° C still continued to develop turbidity accompanied by pellicle formation. None of the organisms grew at 45° C. A difference in optimum temperature was found among the organisms; some decomposed coumarin most rapidly at 30° C while others gave the best response at 37° C. Cultures Mac 289, Mac 291, Mac 292, Mac 293, and Mac 295b utilized coumarin most rapidly at 30° C while cultures Mac 290, Mac 294, and Mac 295a utilized coumarin most rapidly at 37° C. Culture Mac 296 utilized coumarin equally well at both 30° and 37° C.

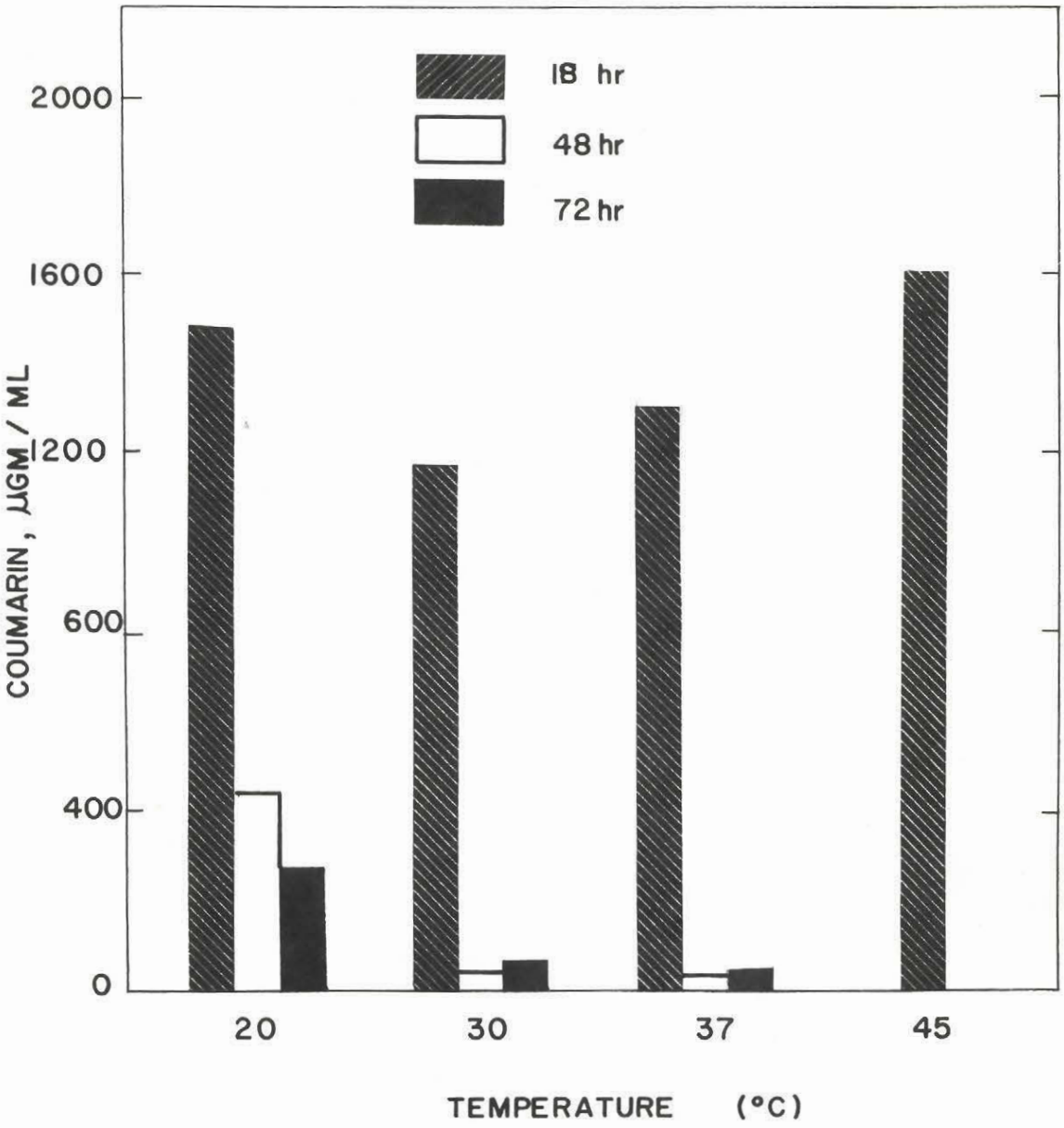


Figure 1. Effect of temperature on the utilization of coumarin by a Pseudomonas sp. (Mac 291). Initial coumarin concentration equals 3,000 µgm/ml.

Measurement of turbidity and pH:- Turbidity determinations were carried out with a Coleman Nephelo-colorimeter on all fermentation samples in order to compare different growth conditions. The instrument was standardized against a Coleman nephelos standard. A fermentation sample, usually 0.01 ml, was suitably diluted to give a scale reading of 30 scale units or less, and the turbidity calculated in nephelos units. The results are shown in Appendix Tables I-IX.

Turbidity was very similar in all cultures in the presence of the same growth factor. But growth factors which increased the turbidity did not necessarily increase the rate of coumarin utilization. Thus, turbidity is not a true reflection of the degree of coumarin decomposition, and obviously, must not be used to interpret the results.

In samples where pH was measured, the determination was carried out prior to analysis for residual coumarin. Appendix Tables I-IX show that an alkaline pH usually results, and values as high as pH 8.5 were frequently found. While the pH rose as the level of coumarin fell, the degree of pH change reflects the composition of the medium, and not the degree of metabolism. The rise in pH was thought to be due to the undetermined end products of protein metabolism.

Effect of aeration:- Aeration effects were measured by altering the volume of CYT medium in 125 ml Erlenmeyer flasks. Flasks were prepared containing 20 ml, 40 ml, 70 ml and 100 ml CYT medium. After inoculation, the cultures were grown at 30° C on a rotary shaker at approximately 250 r.p.m. (eccentricity = 1 in). One flask containing 100 ml was incubated

statically. Aeration effects were determined by measuring the residual coumarin in supernatant liquid of samples obtained at 18, 48, and 72 hour intervals which were treated as previously described.

The response of Mac 291 to variations in aeration is shown graphically in Fig. 2; Appendix Tables I-IX contain the results obtained with all cultures. It was found that coumarin utilization is strongly enhanced by a high oxygen tension, and a high degree of aeration is necessary for rapid coumarin breakdown.

Effect of pH:- CYT medium was buffered with 0.05 M KH_2PO_4-NaOH mixtures (Clark, 1922). The inoculum was added to 125 ml Erlenmeyer flasks containing 40 ml of buffered CYT medium to give various initial pH's ranging from 6.0-8.0, and incubation was carried out statically at 30° C. Samples were taken after 18, 48, and 72 hours. After acidification and centrifugation, residual coumarin was determined on the supernatant.

The effect of pH was assessed with only one organism (Mac 291) because of its favourable response in other trials. The results of this experiment are shown in Table I. It was found that an alkaline pH favours slightly the decomposition of coumarin although good utilization is observed over the entire pH range tested. The organisms in unbuffered CYT medium produce a strong alkaline reaction which accounts for the increase in pH at the acid range in buffered medium.

Effect of inorganic ions:- Three different mineral salts solutions were compared as a source of inorganic ions for the decomposition of coumarin either as the sole source of carbon or in the presence of

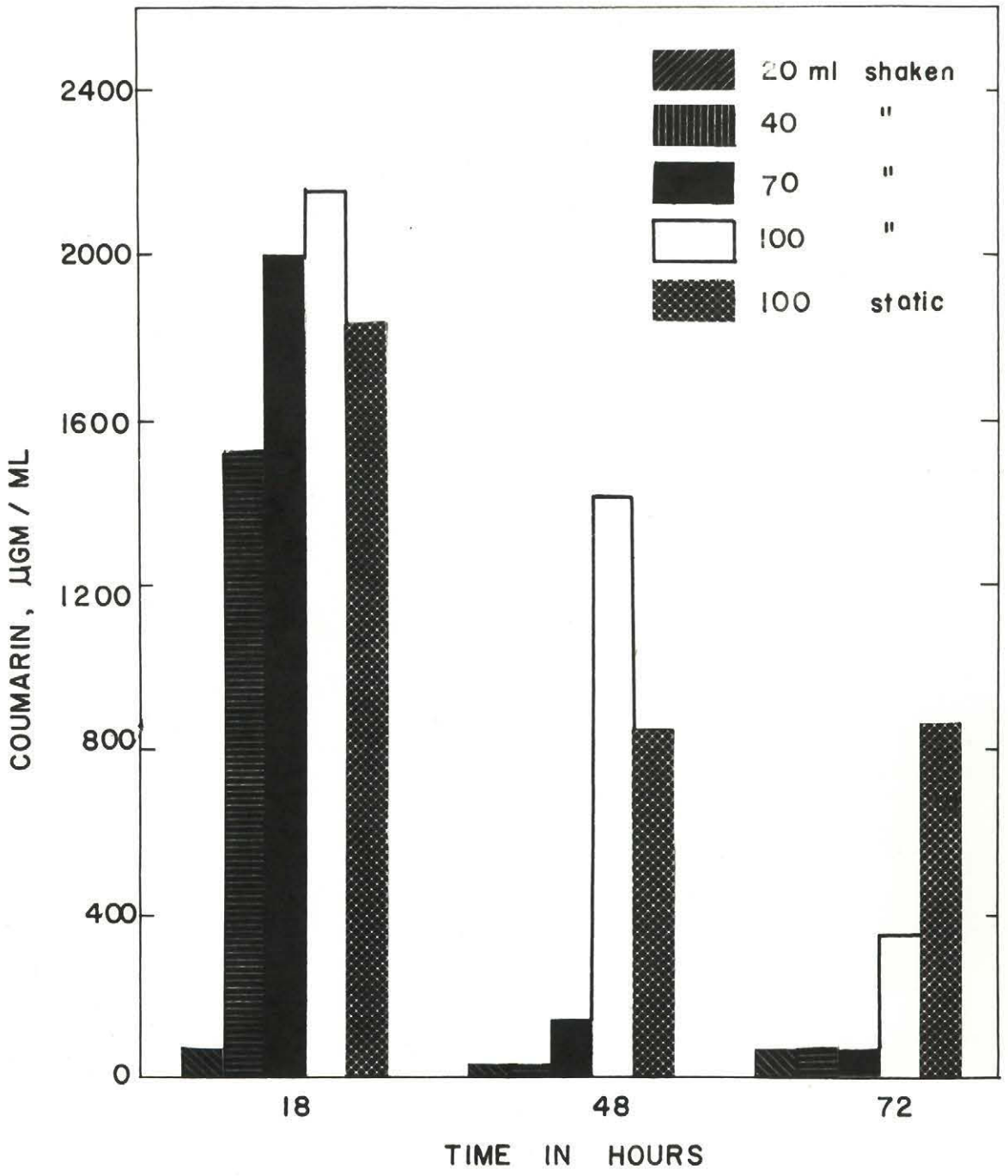


Figure 2. Effect of aeration on the utilization of coumarin by a Pseudomonas sp. (Mac 291). Initial coumarin concentration equals 3,000 µgm/ml. Numbers correspond to the volume of CYT medium contained in a 125 ml Erlenmeyer flask.

TABLE I. Effect of pH on the utilization of coumarin in buffered CYT medium by a Pseudomonas sp. (Mac 291).

pH		Coumarin, µgm/ml	
0 hrs	18 hrs	0 hrs	18 hrs
6.0	6.4	Approx. 3000	1971
6.4	6.7	"	2018
6.8	7.0	"	2018
7.0	7.2	"	2042
7.2	7.4	"	1971
7.6	7.6	"	1821
8.0	7.9	"	1900
6.8*	8.3	"	2208

* Unbuffered.

additives. Table II shows the composition of each mineral salts solution.

Inoculum was added to a 125 ml Erlenmeyer flask containing 40 ml of test medium and the proper mineral salts solution. Incubation was carried out statically at 30° C.

In a second series of experiments, the effect of decreasing concentrations of Mineral Salts I in combination with different growth factor substances was tested. The quantity of Mineral Salts I and additives is shown in Table III. A 125 ml Erlenmeyer flask, containing 40 ml of medium and inoculum, was incubated at 30° C on a shaker as previously described. The level of residual coumarin in samples drawn at suitable intervals was again used to assess the response of the organism to growth conditions.

The results of the first series of experiments are shown in Appendix Tables I-IX. No growth was obtained with any of the cultures using coumarin as a sole carbon source in the presence of all mineral salts solutions tested. With added growth factors, however, Mineral Salts I and Mineral Salts II were of equal value in supplying the inorganic requirements of the organisms. Mineral Salts III was not tested in conjunction with additives.

The results of the second series of experiments are shown in Table III. Differences in the quantity of mineral salts added did not affect the rate of breakdown in CYT medium. This indicates that 0.5% yeast extract and 0.5% tryptone, growth factor sources in CYT medium, contain sufficient quantities of inorganic ions to meet the requirements

TABLE II. The composition of three mineral salts solutions used as a source of inorganic ions for the decomposition of coumarin by a Pseudomonas sp. (Mac 291).

Solution	Mineral Salt	Gm/liter dist. H_2O	Remarks
I	$Na_2HPO_4 \cdot 12H_2O$	13.0	Autoclave separately and
	KH_2PO_4	9.0	incorporate into medium
	KCl	10.0	10% by volume.
	$MgSO_4 \cdot 7H_2O$	10.0	
II	KH_2PO_4	10.0	Autoclave separately and
	$MgSO_4 \cdot 7H_2O$	5.0	incorporate into medium
	KCl	5.0	10% by volume.
	$FeSO_4 \cdot 7H_2O$	0.015	
III	KH_2PO_4	1.0	Add nutrients directly
	K_2HPO_4	1.0	to mineral salts solution
	NH_4NO_3	1.0	prior to sterilization.
	$MgSO_4 \cdot 7H_2O$	0.2	
	$CaCl_2$	0.02	
	$FeCl_3$	0.003	

TABLE III. Effect of varying concentrations of Mineral Salts I as a source of inorganic ions on the utilization of coumarin by a Pseudomonas sp. (Mac 291) grown in three different media.

	Medium	Mineral Salts I (% by volume)	Coumarin, µgm/ml 0 hrs	Coumarin, µgm/ml 18 hrs
A	Coumarin + 0.5% tryptone (Difco)	10.0	2964	2208
	+ 0.5% yeast extract (Difco)	5.0	2993	2185
		2.5	2803	2161
		1.0	2803	2147
		0.0	2755	2332
B	Coumarin + 0.5% peptone	10.0	2850	482
		5.0	2993	904
		2.5	2803	908
		1.0	3040	1448
		0.0	2803	-
C	Coumarin with no additives	10.0	2755	-*
		0.0	2755	-

* - No growth.

of the organism. When 0.5% peptone was used as a source of additives, however, the full complement of 10% Mineral Salts I gave the best conditions for decomposition.

Effect of nutrition:- The effect of nutrition on decomposition was assessed by modifying both qualitatively and quantitatively the growth factor substances in CYT medium. Modifications of the medium are shown in Appendix Tables I-IX. A second series of experiments was carried out using a number of different protein hydrolysates as a source of growth factor substances; only Mac 291 was used in this trial. After inoculum was added to 40 ml medium in a 125 ml Erlenmeyer flask, incubation was carried out at 30° C on the rotary shaker. Samples were withdrawn after 18, 48, and 72 hour intervals unless otherwise indicated and treated as described previously. In both series of experiments, nutrition effects were based on the level of residual coumarin in fermentation liquor.

The results of these experiments are shown in Appendix Tables I-IX. The rate of utilization of coumarin was better with added tryptone than with yeast extract (Difco or BYR), or with tryptone and yeast extract (Difco or BYR) combined. This shows that large quantities of growth factor substances contain energy substrates that can be used for cell development, for good growth occurred in these media without added coumarin.

The competitive nature of the substrate(s) involved is demonstrated readily by adding glucose to CYT medium. In Fig. 3, a comparison is made between residual coumarin, pH, and turbidity in the presence of

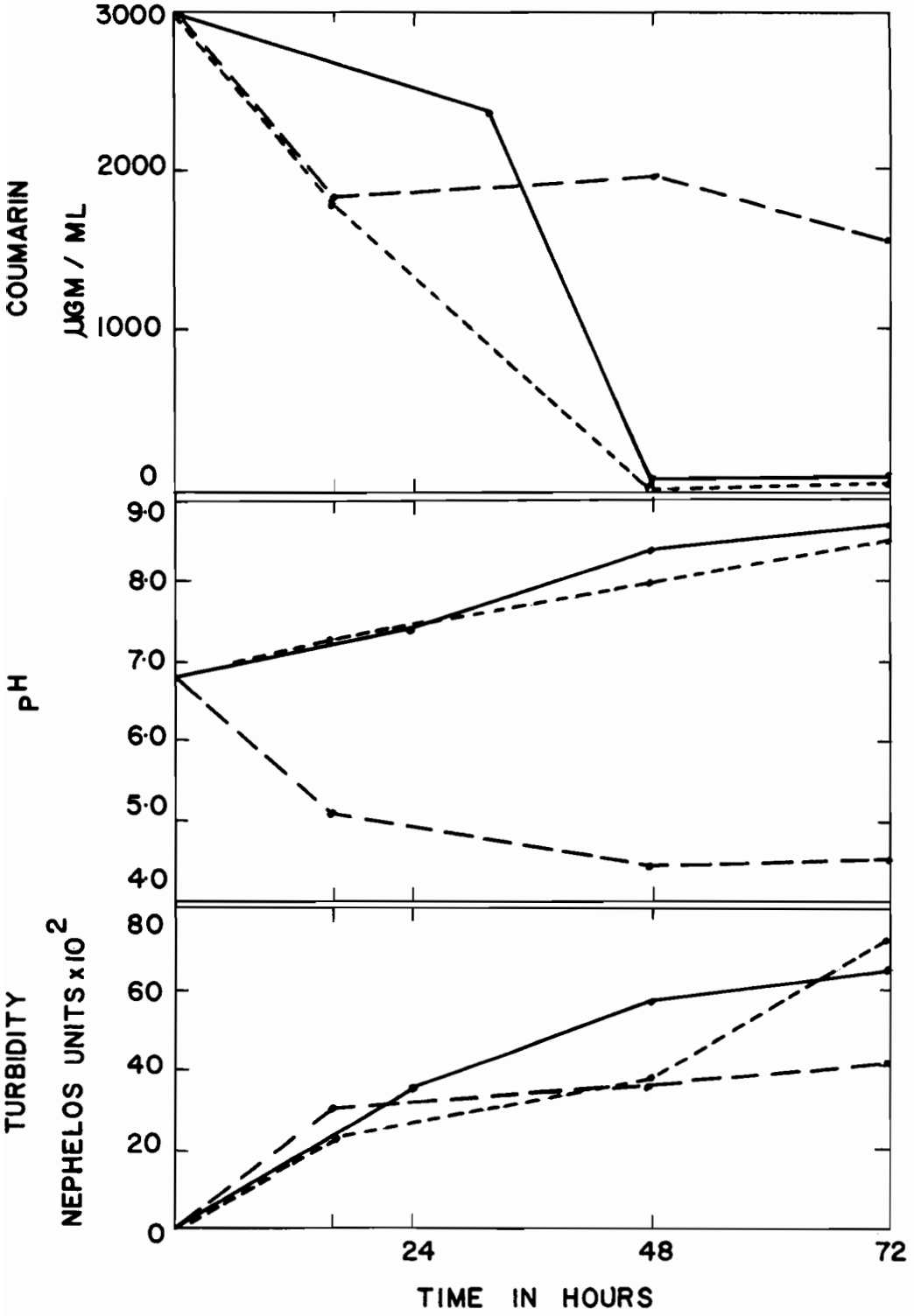


Figure 3. Effect of glucose added to CYT medium on the utilization of coumarin, on the pH, and on the turbidity by a Pseudomonas sp. (Mac 291). — — — 1.0% Glucose. - - - - 0.01% Glucose. ——— 0% Glucose.

different quantities of glucose. With increasing concentrations of glucose, there was a decrease in the utilization of coumarin indicating a preferential utilization of glucose over coumarin. Glucose undergoes fermentation with the production of large amounts of acid, e.g., in an unbuffered medium without added glucose, the final pH is 8.5, while with 1.0% glucose added, the final pH is 4.5. This high acidity inhibits growth and metabolism, for no further degradation of coumarin or no further increase in turbidity was found after the pH had dropped to 5.0.

The results of the second series of experiments are shown in Table IV. Peptone proved to be the best source of additives. At a level of 0.3% peptone, utilization of coumarin was as efficient as 0.5% peptone; 0.1% peptone supported good growth but residual crystalline coumarin on the walls of the flask prevented a quantitative determination of the level of residual coumarin in this trial. In the case of 0.5% peptone, 99.0% of the coumarin added was utilized at 18 hours; in the case of 0.3% peptone, 97.2% of the coumarin added was utilized in the same time interval.

TABLE IV. Effect of several protein hydrolysates as a source of growth factor substances on the utilization of coumarin by a Pseudomonas sp. (Mac 291).

Protein hydrolysate (Difco)	Gm/100 ml medium	Coumarin, µgm/ml		
		0 hrs	18 hrs	24 hrs
Casein hydrolysate	0.5	2356	788	5
"	0.3	2470	712	-**
"	0.1	2418	X*	X
Peptone	0.5	2413	23	9
"	0.3	2432	68	-
"	0.1	2237	X	X
Tryptone	1.0	-	-	33
"	0.5	2631	646	18
"	0.3	2527	342	12
"	0.1	2594	X	X
Vitamin-free Casamino acids	0.5	2518	X	1429
Casitone	0.5	2446	328	17
Casamino acids	0.5	2496	X	X
Neopeptone	0.5	2461	551	21
Proteose-peptone	0.5	2408	X	12
Tryptose	0.5	2470	X	21
Control	0.0	X	X	X

* Crystals of coumarin on the walls of the fermentation flask prevented measurement of coumarin content.

** Determinations were not made.

DISCUSSION

The effect of alterations in environmental conditions was assessed by means of a quantitative test for coumarin. Several methods for the estimation of the residual level of coumarin are available. The colorimetric test employed involves cleavage of the lactone ring by mild heat at an alkaline pH and coupling the free hydroxyl group with an aniline salt. Clayton et al. (1935) found that of a number of aryl compounds tested for color formation, saligenin, guiacol, phenol, and melilotic acid gave red colors at concentrations of 1:125,000 as well. Consequently, the color test is not specific for coumarin. Values given for "residual coumarin" are outside limits, and the actual decomposition of coumarin probably occurs at a rate more rapid than shown in the tables.

Frequently, paper chromatography has been used for the identification of coumarin in plant and animal tissue. Most reports, however, are only of a qualitative nature. Booth et al. (1959) have estimated the quantity of coumarin in the urine of rats and rabbits by the paper chromatographic technique on the basis of color intensity using a series of coumarin standards. But the method does not lend itself to rapid and critical analysis of numerous samples.

Pocaro and Johnston (1955) have described a quantitative method for the determination of small amounts of coumarin. This method is based on measurement of the ultra-violet absorption in a spectrophotometer at 335 mμ after treatment with alkali. However, the method was not considered because absorption interferences could lead to large errors.

When coumarin is dissolved in dilute alkali, a brilliant fluorescence results after exposure to ultra-violet light, due to the formation of o-hydroxy-trans-cinnamic acid (Feigl et al., 1955). Sensitivity to 0.005 µgm coumarin can be achieved. Hydroxylations into the benzene ring interfere with the fluorescence. Haskins and Gorz (1959) stated that an equilibrium is established between the cis and trans forms of o-hydroxycinnamic acid, and that the lactone ring of coumarin undergoes cleavage under alkaline conditions in the presence of light. Thus, the fluorescent method of Feigl could be used to measure residual coumarin in fermentations only where acid conditions prevailed. This, of course, would limit experimental scope.

Similar responses by all organisms to the factors tested suggests that the overall metabolic pattern is identical or very similar. While variations in response to temperature were noted, almost identical results were obtained in studying the effects of aeration, pH, and nutrition, factors more likely to reveal differences in metabolism. None of the organisms grew in the absence of added growth factors, indicating the inability to synthesize organic materials essential for cell proliferation.

The nature of the essential growth factor substance or substances is not known. A good response was obtained with all of the protein hydrolysates tested at levels of 0.3% or higher with the exception of casamino acids and vitamin-free amino acids. No suitable peptone can be equally suitable for each microbiological situation (Difco Manual, 1953). While the gross analysis of peptones shows only slight chemical variation, wide responses of organisms to the addition of these nitrogenous additives

are known. Bacto-peptone contains a high peptone and amino acid content and only a negligeable quantity of proteoses and more complex nitrogenous constituents. The organisms may have a vitamin requirement. At a level of 0.3% protein hydrolysate or higher, only casamino acids responded unfavourably. However, such a criticism may not be valid, for crystal formation of the walls of the fermentation vessel prevented an analysis for residual coumarin although growth was good. While no growth factors were identified, the investigation does, however, confine further growth factor investigations.

The preferential utilization of glucose or other fermentable materials strongly suggests that the degradation of coumarin is brought about by adaptive enzyme formation. Difficulty was encountered frequently in initiating growth on CYT slants when transfers were made from nutrient agar slopes. This, too, suggests adaptive enzyme formation. Passage from nutrient agar through CYT semisolid medium prior to transfer to CYT solid medium was effective in producing satisfactory cultures.

A number of apparent discrepancies in the Appendix Tables I-IX merit comment. Frequently, when coumarin utilization was poor or the organisms were slow to adapt to new growth conditions, some of the flasks contained crystals of coumarin either in the medium or on the wall of the fermentation vessel, and the level of residual coumarin could not be determined. Where the tables contain the symbol "X", crystals are present, and where no values are given, the test was not carried out. The tests for temperature, aeration, and nutrition were carried out as separate experiments with fresh lots of media as well as newly prepared inoculum.

Variations in initial coumarin level, and of more significance, variations in the activity of the inoculum could lead to experimental variations with each group of experiments. When cultures were incubated at 45° C, no growth ensued; yet the quantity of residual coumarin after 18 hours was far below the quantity added initially. The reason for this is not known. Although the vapor pressure of coumarin is high, a sufficient amount of coumarin should not be volatolized to give such a drastic reduction of the coumarin level. A polymerization or some other alteration of the coumarin molecule could be involved.

Benns (1958) reported variation in morphology occurs when incubation is carried out at different temperatures. Mac 288 (not used in the present study) and Mac 289, when grown at 37° C, were long and flexible with motility absent or weak. But when incubation was carried out at 30° C, rods were much shorter and much more strongly motile. A detailed study of morphological responses to alterations in environment conditions was not undertaken in the present study.

Harle and Lyons (1950) reported that coumarin exists entirely in the lactone form at pH less than 6.8, and entirely as o-coumaric acid at pH greater than 11.2; an equilibrium exists between the two components at pH values intermediate between these two limits. They were able to calculate on the basis of spectral considerations the degree of pyrone hydrolysis. All of the cultures grew well at pH values considerably below pH 6.8. Preliminary studies on the fermentation liquor revealed the presence of phenolic compounds. This indicates that lactone ring cleavage is a true enzymatic reaction.

The rate of decomposition of coumarin is in sharp contrast to the findings of Bellis (1958) who, using two Penicillium species, found the rate of mold growth extremely slow. Similarly, Henderson et al. (1955) reported that a large number of molds from several different genera were able to decompose a number of aromatic acids and aldehydes. Using an initial concentration of 0.01% (w/v) of phenolic compound, decomposition rates reached 90% after 21 days incubation at 21.5° C. Frequently, levels of decomposition are given at only 20% or less. They suggest that the combined activities of these different fungi may be concerned in the breakdown of lignin under natural conditions. The presence of bacteria in the soil would on the basis of rate of decomposition of aromatic compounds exemplified by coumarin play a far more significant role in the turnover of organic material than would fungi.

Reference has been made of the phytocidal properties of coumarin. As well, Audus and Quastel (1947) quote references for the toxicity of coumarin towards algae, and of its inhibitory effect on the development of wheat. The inhibitory effect of coumarin on bacteria has never been reported. During this investigation freedom from bacterial contamination was soon apparent. CYT medium in Petri plates exposed to the air at room temperature for four days failed to develop any bacteria or mold contaminants. It is certain that the phytocidal properties of coumarin extend to many bacterial species as well as the biological systems previously reported.

CONCLUSIONS

1. Nine organisms, all members of the genus Pseudomonas, were tested for differences in rate of breakdown of coumarin. It was found that all organisms responded very similarly to the same test conditions.

2. Some of the cultures degraded coumarin most rapidly at 30° C while others favoured 37° C.

3. High aeration enhanced utilization.

4. Alkaline pH favors the utilization of coumarin slightly.

5. Peptone at 0.5% concentration was most satisfactory of the nutritional adjuncts tested.

6. Under optimum conditions 0.25% coumarin is utilized completely in 18 hours.

PART II

MANOMETRIC EVIDENCE FOR THE INTERMEDIATES OF COUMARIN DECOMPOSITION

INTRODUCTION

Constituents of all living cells contain high-molecular-weight proteins which function by catalyzing the biochemical reactions necessary for cellular activity. These biological catalysts or enzymes have been recognized for many years, and while the scope of enzymology is very diversified and continually expanding, our discussion will be confined exclusively to that of the induced formation of enzymes.

The enzyme level in a system is not constant, but varies depending on the genetic constitution, physiological condition and environment of the cell. Such information is applied in microbiological systems in order to raise the enzyme level to a maximum prior to enzyme purification. Karström (1930) was the originator of the classification of enzymes into two groups, the first being defined as 'adaptive enzymes', those which are present when required, and whose formation is dependent upon the presence of a specific substrate which causes its formation, and 'constitutive enzymes', those which are always present in the cell independent of substrate presence. The term 'adaptive' has been superceeded by inducible (Dixon and Webb, 1958) to avoid confusion with the use of 'adaptation', a term frequently used in biology to imply a selection of individuals by mutation giving rise to genetically transferable characters which were previously not part of the genotype of the parent cell. In this discussion the terms 'adaptation' and 'induction' are used interchangeably

and bear no relation to enzymes formed by mutation.

The early history of enzyme induction has been presented by many workers of whom Stephenson (1939) and Pollock (1959) are most notable. In brief, adaptation results from either natural selection or by exposure to chemical environment. Adaptations belonging to the first group are now considered mutations and affect only small numbers of individuals in a population. Because of the aquisition of a more favourable genotype, these mutants soon become the dominant population. Adaptation belonging to the second group are alterations in the enzyme constituency of the entire population. Such alterations in the enzyme pattern occur only in certain organisms, and the ability for such modification to take place resides in the genetic makeup of the system. The widely held 'one-gene-one-enzyme' theory of Beadle (1945) postulates a separate gene for the formation of each enzyme; the simplest interpretation is that the gene is actually a part of the mechanism which forms the corresponding enzyme. Where no constitutive enzyme exists, the absence of adaptive enzyme formation can be interpreted as a lack of, or non-functioning of, the gene-controlled enzyme formation mechanism.

Adaptive enzyme formation is widespread in nature. Knox and Mehler (1950; 1951) reported that tryptophan peroxidase activity in rat liver increased in activity 10 fold 4-10 hours after intraperitoneal injection of DL-tryptophan and returned to normal after 15-20 hours. The half-life of induced enzymes is considerably shorter than that of constitutive enzymes. The half-life of induced tryptophan peroxidase <u>in vivo</u> was found to be 2.5 hours while the half-life of total liver protein

was found to be about 15 hours. Gordon and Roder (1953) produced up to a five fold increase in adenosine deaminase activity of chick embryos after injection of adenosine into the air sac of fertilized eggs. Knox et al. (1956) has presented a very good review on metabolic adaptation in animals, and defines an adaptation as a response to a stimulus which causes an alteration in the enzyme level of animal tissue. Numerous examples and references quoted in this article, along with tables showing changes in enzyme level under various treatments, include arginase, hexokinase, cytochrome oxidase, and lipase, which shows that adaptations occur in all phases of metabolism.

A number of adaptive enzymes are known to occur in plants. Pollock (1959) gives references for several reports to the effect that glycolic acid oxidase, indoleacetic acid oxidase, nitratase, and triphosphopyridine-linked glyceraldehyde dehydrogenase fall into this group. Adaptive enzymes have also been reported in fungi. Henderson (1956) showed that a Hormodendrum sp. oxidized p-hydroxybenzaldehyde to p-hydroxybenzoic acid via adaptive enzyme formation. Similar results were found with a Haplographium sp. and a Penicillium sp. Cellulase is also attacked by adaptive enzymes in Trichoderma viride (Mandels and Reese, 1959).

The phenomenon of enzyme induction has been studied more intensively in bacteria. The decomposition of pyrimidines by Nocardia corallina (Batt and Woods, 1961), the dissimilation of indoleacetic acid by a Pseudomonas sp. (Proctor, 1958), and the permease of succinic acid in Azotobacter (Repaske et al., 1960) are only several of the avalanche of recent references showing the presence of induced enzymes using

techniques eliminating the objection of cell mutation which can be interpreted into the earliest references. In fact, induced enzyme synthesis of β-galactosidase in cell-free preparations of Escherichia coli has been demonstrated (Reiner, 1960).

Whether 'inducible enzymes' are completely lacking in an organism or whether levels are present below methods of detection, but which increase in the presence of inducers, has not been satisfactorily answered. Monod (1956) reaches the following conclusions concerning the nature and mechanism of induced enzymes: "Induced enzyme formation involves the complete de novo synthesis of the enzyme protein molecule, from its elements or elementary building blocks (amino acids)." Frequently, however, in manometric experiments, small amounts of oxygen uptake are observed over and above endogenous activity at time intervals too short to account for enzyme synthesis per se (e.g., see data of Stanier, 1947). This could be due to low levels of enzyme prior to the synthesis of new enzyme units.

Adaptations in enzyme patterns have been known for many years. Dubos (1931) isolated an organism capable of hydrolysing the capsular polysaccharide of Pneumococcus type III. The hydrolyzing enzyme was adaptive and specific for the polysaccharide, being unable to attack the specific polysaccharides of Pneumococcus types I, and II, or of Friedlander's bacillus types A, B, and C (Klebsiella pneumoniae), or gum arabic. This application could be interpreted as evidence for differences in chemical composition, although the authors made no such suggestion.

One of the most significant contributions that has been made to the field of aromatic metabolism was the development by Stanier (1947) of a technique for the elucidation of metabolic pathways by the use of adaptive enzyme formation. Stanier summarized the principles of the technique as follows:

> (1) If the dissimilation of a given substance A proceeds through a series of intermediates B, C, D, E, F, G ...., and if the individual steps in this chain of reactions are under adaptive enzymatic control, then growth on a medium that contains A will produce cells that are simultaneously adapted to A, B, C, D, E, F, G .....
>
> (2) If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain.
>
> (3) Growth on E will adapt the cells for F, G, ... but not necessarily for A, B, C, and D. The probability growth on E will adapt the cells to precursors decreases with the number of intervening steps; i.e., adaptation to D is more probable than adaptation to A.

The sequential induction principle has been applied successfully by several workers to solve several problems in intermediate metabolism (Stanier, 1948; 1950; Rogoff and Wender, 1957; Sugiyama et al., 1958; Yano and Arima, 1958; Cohen, 1949). Indeed, Stanier himself was puzzled as to why, in view of the knowledge available concerning adaptive enzyme formation, the method came into use after such a long delay.

Preliminary experiments strongly suggested that glucose was used preferentially over coumarin and that perhaps an induction period was responsible for this effect. Consequently, the technique of sequential induction was applied to this problem to discover firstly, whether coumarin is metabolized through induced enzymes, and secondly, whether any additional aromatic compounds could be regarded as intermediates on the basis of manometric considerations.

MATERIALS AND METHODS

Preparation of Cells

Resting cell suspensions of Mac 291 were prepared from cells grown on glucose on a medium containing 0.1% glucose, 0.5% yeast extract, 0.5% tryptone, plus Mineral Salts I. Cells adapted to coumarin were obtained with CYT medium. Although the organism grows well in 0.3% coumarin plus 0.3% peptone and mineral salts, yields were rather small, and CYT medium with its additional supply of oxidizable substances gave much higher yields of cells. Cells adapted to o-coumaric acid were grown on a medium containing 0.1% o-coumaric acid (limit of easy solubility), 0.5% yeast extract, 0.5% tryptone, plus Mineral Salts I, and with the initial pH adjusted to 7.0. In all trials, cultures were incubated on the rotary shaker at 30° C, and cells were harvested from the medium after 24 hours by centrifugation at 10,000 x gravity for 10 minutes. After washing twice in physiological saline, the harvested cells were resuspended in 10 volumes of saline and allowed to respire statically overnight, or occasionally were incubated instead on a rotary shaker for two hours.

Cells adapted to melilotic acid were prepared by incubating cells grown on glucose in a medium containing melilotic acid under conditions designed to allow induction to occur. This technique was necessary, for only limited quantities of melilotic acid were available.

Standardization of Cells

The concentration of cells was standardized by adjusting the suspensions with physiological saline to a cell density equal to 3,750 nephelos units per 1.0 ml.

Preparation of Substrates

Twenty-three aromatic compounds were tested in this investigation. In all trials substrates were used at a level of four μM per Warburg flask. With the exception of o-coumaric acid, all of the substrates are water-soluble at one μM per 0.1 ml and stock solutions were prepared at this concentration. o-Coumaric acid was prepared with one μM per 0.025 ml in the phosphate buffer, pH 7.0 (see page 35), and a correction was made when the Warburg vessels were prepared to compensate for the quantity of buffer added along with this substrate.

All of the compounds were available commercially with the exception of 2,3-dihydroxyphenylpropionic acid, which was obtained through the courtesy of Dr. C. E. Evans, University College of North Wales.

Manometric Conditions

To each Warburg vessel was added 1.0 ml standardized cell suspension, four μM substrate, 0.3 ml Mineral Salts I, phosphate buffer pH 7.0 to a final concentration of 0.067 M, and saline to a volume of 3.2 ml. Standard manometric techniques (Umbreit et al., 1957) using air as the gas phase at 30° C were employed; 0.2 ml 20% potassium hydroxide was used as the CO_2 absorbant. All trials were duplicated at least, and Warburg readings made usually at 10 minute intervals.

RESULTS

The Manometric Response of Several Aromatic Compounds to Cells Grown on Coumarin

Substrates utilized with ease:- A number of substances in addition to coumarin were utilized with ease by cells grown on coumarin, i.e., following a period of rapid and steady increase in oxygen uptake the rate returned again to the endogenous level. Substances in this group were benzoic acid, melilotic acid, 2,3-dihydroxyphenylpropionic acid, o-coumaric acid and protocatechuic acid. Of the six compounds in this group benzoic and protocatechuic acids showed readily detectable lag periods when cells adapted to coumarin were used. These compounds thus fail to meet Stanier's second requirement as intermediates of coumarin decomposition, while the four remaining compounds were considered as possible intermediates. Fig. 4 shows exogenous plots obtained with these compounds.

Substrates utilized with difficulty:- A number of the substrates tested were not so readily metabolized. Oxygen uptake values were in reasonable excess over that of endogenous cells, yet failed to meet the requirements of intermediate status on the grounds of rate and quantity of oxygen consumed. The minimum limit arbitrarily permitted was 20 µl per hour for two consecutive one-hour intervals. Compounds falling into this group were catechol, o-cresol, 3-methylcatechol, salicylic acid, saligenin, salicylaldehyde, 2,3-dihydroxybenzoic acid, homogentisic acid lactone and shikimic acid. Table V shows the quantities of oxygen consumed for each time interval for this group of compounds.

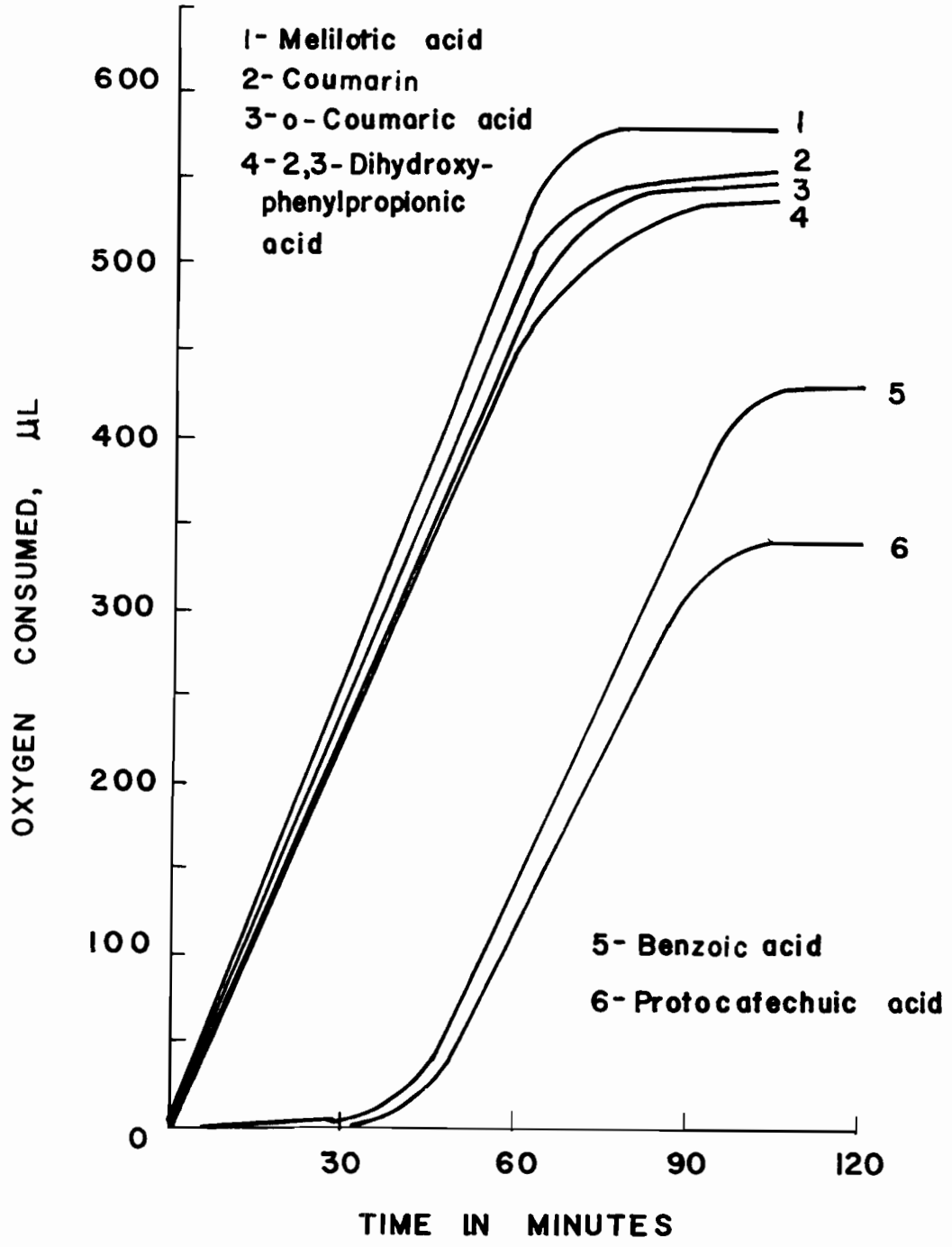


Figure 4. Oxidation of several aromatic compounds utilized with ease by resting cell suspensions of a Pseudomonas sp. (Mac 291) grown on coumarin. Warburg flask contains 1 ml standardized cell suspension, 4 µM substrate, 0.3 ml Mineral Salts I, PO_4^{---} buffer to 0.067 M final concentration, 0.2 ml 20% KOH in the center well, and saline to a final volume of 3.2 ml. Curves are exogenous plots representative of several trials.

TABLE V. Oxygen consumption for the first two consecutive one-hour intervals in the presence of several aromatic compounds used with difficulty by a Pseudomonas sp. (Mac 291) grown on coumarin.

Substrate	Trial	Oxygen consumed in µl	
		Time interval	
		0-60 min	60-120 min
Catechol	A	96.5	42.8
	B	76.7	45.0
o-Cresol	A	63.1	52.6
Salicylic acid	A	59.2	33.8
	B	45.7	22.0
Saligenin	A	20.0	17.2
	B	25.0	23.9
Salicylaldehyde	A	218.7	-4.6
	B	58.9	1.4
3-Methylcatechol*	A	22.0	23.7
	B	26.5	32.5
2,3-Dihydroxybenzoic acid	A	57.9	23.2
	B	40.4	24.0
Homogentisic acid lactone	A	195.1	23.7
	B	184.4	16.2
Shikimic acid	A	27.9	51.1
	B	32.9	45.4

* Cells grown on glucose. A short but perceptible lag period is present.

While salicylaldehyde in both tests does not undergo oxidation in the second hour interval, and the level of saligenin falls outside the limits set for this group of compounds, the responses in both cases in the first hour interval in all trials were satisfactory; thus these compounds are included here.

With the exception of shikimic acid, all of the compounds in this group failed to show the presence of a lag period when grown on coumarin as a substrate. Therefore, shikimic acid does not meet the second requirement laid down by Stanier for induced enzyme patterns and is excluded as a possible intermediate.

All of these compounds are phenolic in nature, and by some conceivable mechanism, coumarin could be degraded to each one of these substances. However, the demonstration of the complete or partial utilization of a compound is, in itself, insufficient evidence to consider it as an intermediate, and the mechanism of degradation could be totally unrelated. Because utilization occurs, the compound could be considered as an intermediate.

Substrates not utilized:- A number of substances were not utilized, and little or no oxygen uptake was found over that of endogenous cells. The maximum limit arbitrarily permitted was an oxygen uptake of 20 µl per hour for two consecutive one-hour periods. This group of substances included benzene, cinnamic acid, hydrocinnamic acid, 4-hydroxycoumarin, mandelic acid, phenol, o-hydroxyphenylacetic acid and toluene. Quantities of oxygen consumed for each time interval are shown in Table VI.

TABLE VI. Oxygen consumption for the first two consecutive one-hour intervals in the presence of several aromatic compounds by a Pseudomonas sp. (Mac 291) grown on coumarin.

Substrate	Trial	Oxygen consumed in µl	
		Time interval	
		0-60 min	60-120 min
Benzene	A	10.0	3.5
	B	0.1	-3.4
Cinnamic acid	A	5.7	15.2
	B	25.0	-1.3
Hydrocinnamic acid	A	5.0	-9.9
	B	4.1	-0.9
4-Hydroxycoumarin	A	0.9	1.2
	B	3.6	1.1
Mandelic acid	A	6.4	1.3
	B	-4.9	-1.8
Phenol	A	28.6	13.3
	B	13.3	5.9
o-Hydroxyphenylacetic acid	A	18.7	6.2
	B	21.2	5.7
Toluene	A	16.9	24.0
	B	9.5	8.6

Results of this nature can be interpreted in either of two ways. Firstly, the compounds are not potential intermediates on the basis of insufficient oxygen consumed. Secondly, the compounds are potential intermediates, but fail to cross the cell membrane due to impairment or lack of a transport system. If in this organism permeases are lacking, and if the presence of the substance fails to induce permease formation, it appears unlikely that under any situation using resting cell suspensions a more positive manometric response will be obtained. Consequently these substances were excluded from further trials. This does not necessarily exclude them from being intermediates in the system; it merely reduces their likelihood of playing a significant role in any metabolic relationship with coumarin.

The Manometric Response of Several Aromatic Compounds to Cells Grown on Glucose

Substrates utilized with ease:- All of the compounds utilized with ease were exposed to resting cells grown on glucose. It was found that the metabolism of all compounds is under induced enzyme control; all showed a strong lag period of approximately one hour duration. Thus o-coumaric acid, 2,3-dihydroxyphenylpropionic acid, and melilotic acid still meet the requirements of Stanier for adaptive enzyme control.

The results of this experiment are shown graphically in Fig. 5.

Substrates utilized with difficulty:- Six of the eight compounds showing partial utilization with no detectable lag period when tested with coumarin-grown cells were tested with glucose-grown cells. Only o-hydroxyphenylacetic acid failed to show an increase in oxygen

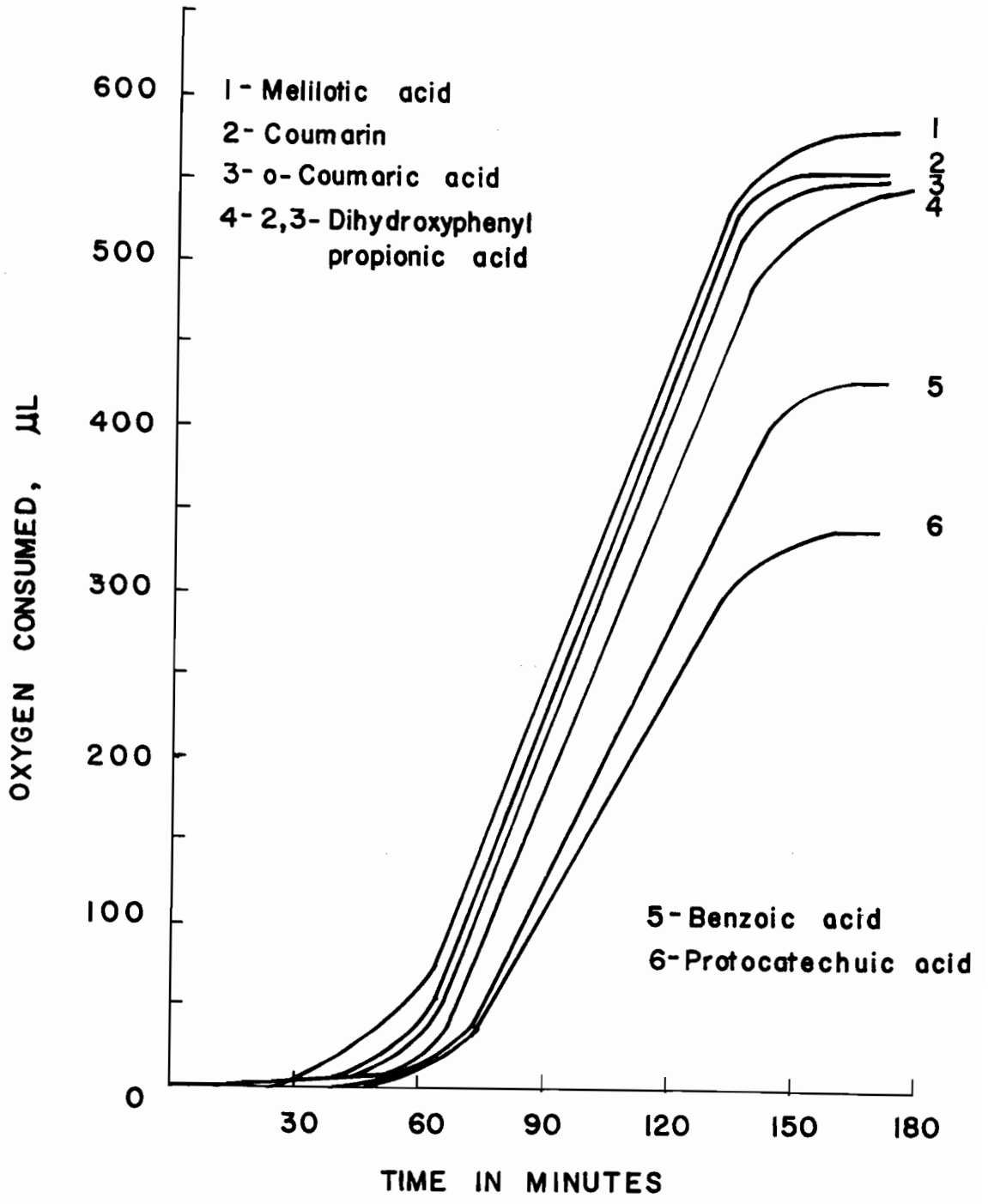


Figure 5. Oxidation of several aromatic compounds utilized with ease by resting cell suspensions of a Pseudomonas sp. (Mac 291) grown on glucose. Warburg flask contents as in Fig. 4. Curves are exogenous plots representative of several trials.

consumption over that of normally respiring cells in an interval of two hours. Of the remaining compounds only homogentisic acid lactone was utilized without the presence of a lag period; with all other compounds a lag period was detected. The lag periods were peculiar to each compound and ranged from greater than 15 minutes with o-cresol to 90 minutes with salicylic acid. In spite of their low rates of oxidation, on the basis of appropriate induction periods, all compounds were considered as potential intermediates in the decomposition of coumarin.

The results of this experiment are expressed graphically in Fig. 6.

Confirmation of Manometric Utilization

The complete or partial utilization of various substances suggested that these substrates could serve as substrates for growth. CYT medium was modified by substituting the aromatic compounds for coumarin at levels of 0.10% and 0.01% in the case of substances undergoing partial utilization, and 0.3% for those showing complete utilization. All media were adjusted to pH 7.0 with sodium hydroxide prior to sterilization. With o-coumaric, melilotic, and benzoic acids, growth was carried out on the rotary shaker at 30° C in 500 ml flasks containing 200 ml medium. Very good growth was obtained with these compounds in as little as 12 hours, a property they share with coumarin. A qualitative test for phenols showed that the substrate had markedly been reduced at 24 hours and had been removed completely by 30 hours. Preliminary trials with o-coumaric acid and benzoic acid were negative when the pH of the medium was not controlled. Buffering the medium to neutrality, or adjusting to pH 7.0

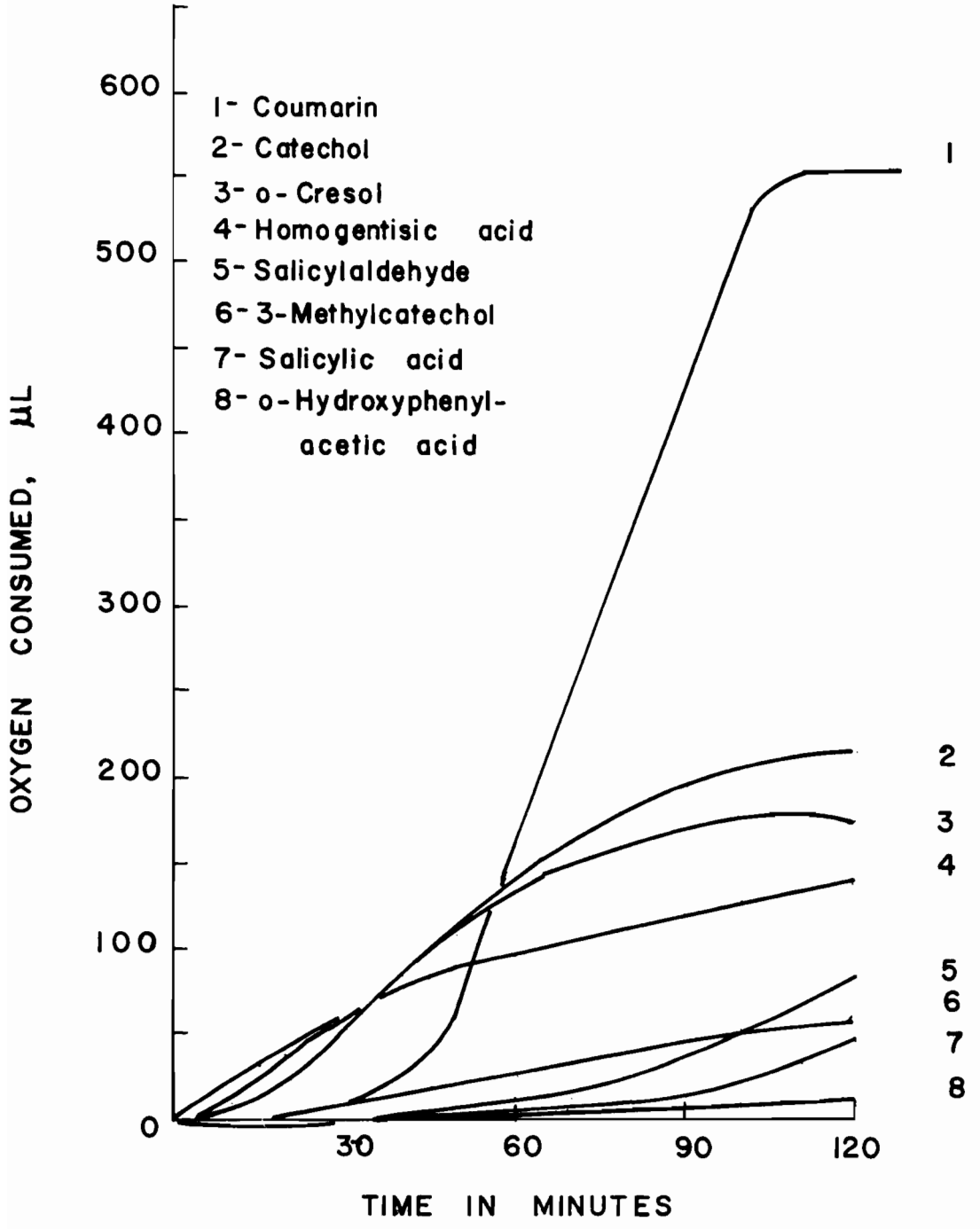


Figure 6. Oxidation of several aromatic compounds utilized with difficulty by resting cell suspensions of a Pseudomonas sp. (Mac 291) grown on glucose. Warburg flask contents as in Fig. 4. Curves are exogenous plots representative of several trials.

with sodium hydroxide and a glass electrode eliminated this effect.

With o-cresol, saligenin, salicylaldehyde, and salicylic acid, the medium was dispensed in 10 ml quantities into 50 ml flasks to which were fused calibrated test tubes serving as side arms. A flask containing tryptone, yeast extract and mineral salts served as a control. At intervals the contents of the flasks were tipped into the side arms and the optical density measured in a Coleman instrument with a 625 mμ filter. A correction was made for extraneous carbon by calibrating the instrument to read 0.30 O.D. Units with the blank flask. The results of this experiment are shown in Figs. 7 and 8, where Fig. 7 shows substrate levels of 0.10% and Fig. 8 shows substrate levels of 0.01%. It was found that at 0.10% only saligenin and salicylic acid support growth; o-cresol and salicylaldehyde were inhibitory at this level. At 0.01%, salicylaldehyde is effective as a growth substrate; o-cresol still has an inhibitory effect, while saligenin and salicylic acid are present at levels too low to influence the cell level. Samples of the fermentation liquor at the end of the experiment were retained for chromatographic studies. Catechol was not used in these experiments because it undergoes spontaneous oxidation in the presence of air; 2,3-dihydroxybenzoic acid and 3-methylcatechol were not used because quantities of these compounds were not available. Thus, only saligenin, salicylic acid, and salicylaldehyde support the growth of cells.

The Oxidation of Catechol by Cells Grown on Coumarin

The key intermediate in the group of substances undergoing only partial decomposition is catechol. Stanier et al. (1950) showed that

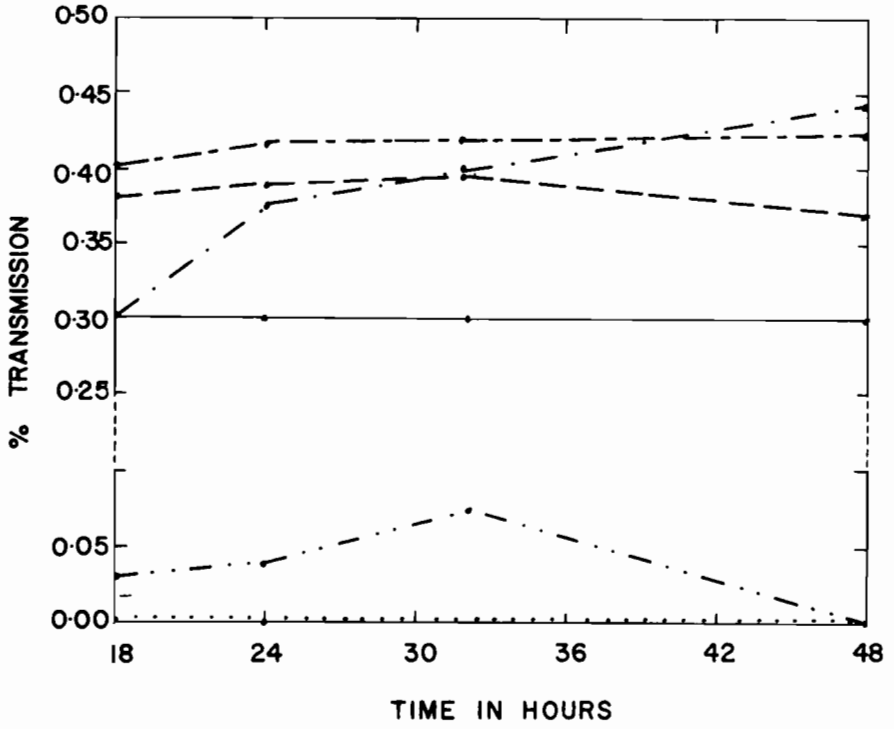


Figure 7. Effect of several aromatic compounds at a level of 0.1% in the presence of additives on the growth of a Pseudomonas sp. (Mac 291).

— · — · Saligenin
— — — — Salicylic acid
— — — Coumarin
— · · — o-Cresol
· · · · · Salicylaldehyde
——— Blank

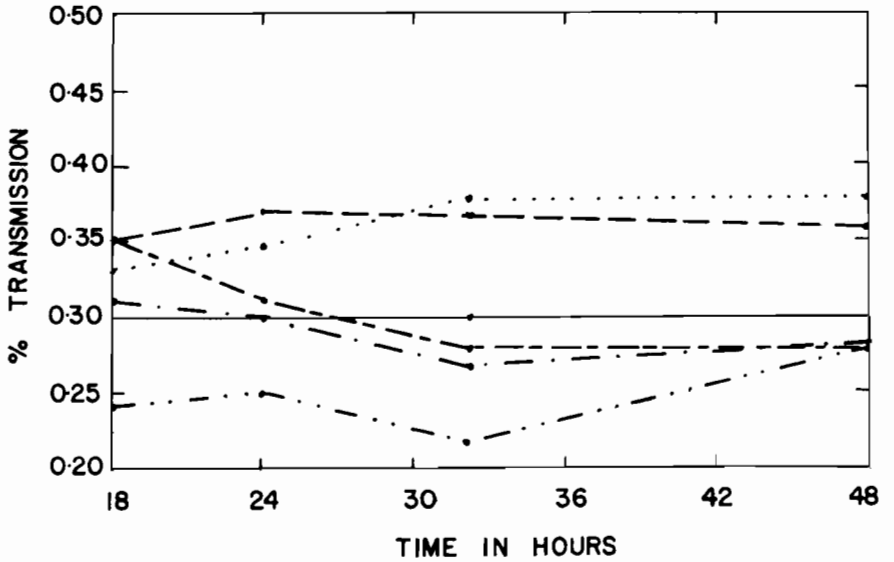


Figure 8. Effect of several aromatic compounds at a level of 0.01% in the presence of additives on the growth of a Pseudomonas sp. (Mac 291). Key as in Fig. 7.

pyrocatechase from Pseudomonas fluorescens strain A.3.12 has a requirement for Fe^{++}. A similar requirement is known for homogentisicase (Tokuyama, 1959), gentisic acid oxidase and protocatechuic acid oxidase (Sugiyama et al., 1958), three additional ring-splitting enzymes. It was thought that ferrous ions may be a limiting factor in the oxidation of catechol by Mac 291 as no ferrous salt had been included in the growth medium. The effect of ferrous ions on catechol oxidation was determined by incorporation ferrous sulfate to a final concentration of 1×10^{-3} M into Warburg trials but the addition of ferrous ions did not increase the oxygen uptake over that of control cells.

Edwards and Knox (1956) showed that the activation of homogentisicase by ferrous ions can be enhanced by the addition of glutathione. Therefore, the oxidation of catechol was carried out in the presence of glutathione at a final glutathione/catechol molar ratio of 1.25. The presence of glutathione did not increase the oxygen uptake over that of control cells.

The curves presented by Stanier et al. (1950) show that growth on one aromatic compound may reduce the induction period of another physiologically unrelated aromatic compound. Thus growth on mandelic acid reduced the lag period of protocatechuic acid from that obtained on asparagine, yet protocatechuic acid is not an intermediate of mandelic acid metabolism. It was plausible that the same phenomenon was occurring in the system under investigation, and that a lag period, while present, was not detected. Readings previously taken every 10 minutes were reduced to one minute intervals and the results are shown in Table VII. The

TABLE VII. Oxygen consumption for the first three consecutive ten-minute intervals in the presence of catechol as a substrate by a Pseudomonas sp. (Mac 291) grown on coumarin.

	Oxygen consumed in µl		
	Time interval		
Trial	0-10 min	10-20 min	20-30 min
A	18.7	19.5	16.7
B	26.6	24.2	-

quantity of oxygen consumed in the first 10 minute interval was compared to that of subsequent intervals of the same duration. No lag period was found.

The Sequence of Intermediates in Coumarin Decomposition

According to Stanier's third principle, if a series of compounds are under adaptive enzyme control the growth of cells on one compound in the chain is likely to produce a lag period in the compounds preceeding it, while those compounds following will be used with no induction period. For manometric studies cells were grown on coumarin and o-coumaric acid as previously described. Because the quantity of melilotic acid available was limited, cells were grown on glucose and exposed to melilotic acid under conditions identical to those used in the respiration studies for 90 minutes. This time interval, as shown in Fig. 5, is sufficient for induction and for approximately 50% utilization of the substrate. The remaining unused substrate was then washed free with saline, and the cells restandardized for manometric trials. (Only mgm quantities of 2,3-dihydroxyphenylpropionic acid were available, so induced enzymes by this compound could not be included). Each substrate was tested with each cell preparation and records were taken at five minute intervals to insure the detection of a period of induction.

No lag periods were found (Fig. 9), i.e., cells induced to any one substrate used all others with ease and without the presence of a period of induction. Based on these experimental results, no sequence of reactions on these substances can be postulated, but based on structural considerations, the following scheme (Fig. 10) is presented showing the

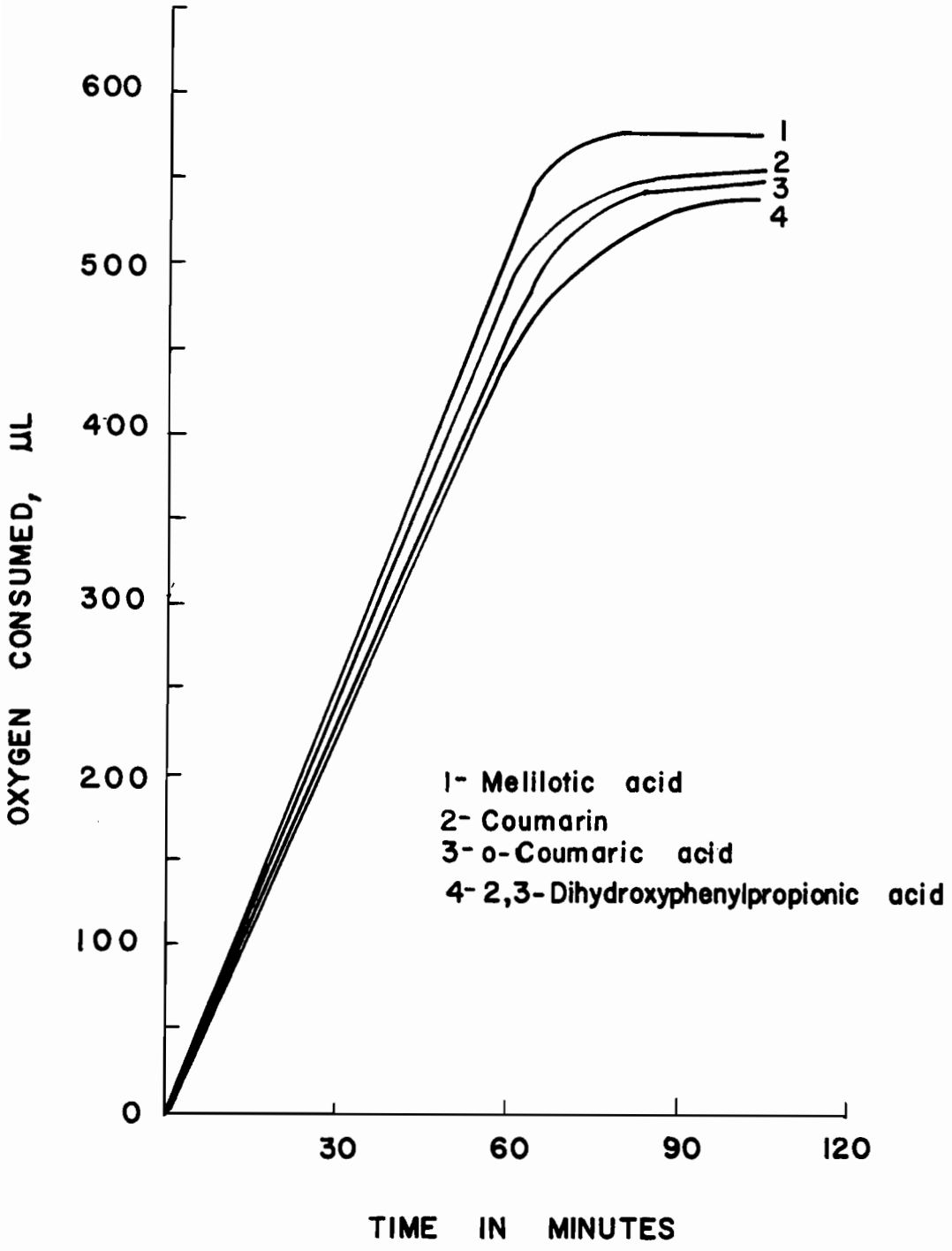


Figure 9. Oxidation of several aromatic compounds utilized with ease by resting cell suspensions of a Pseudomonas sp. (Mac 291) containing induced enzymes for coumarin, o-coumaric acid, or melilotic acid. Warburg flask contents as in Fig. 4.

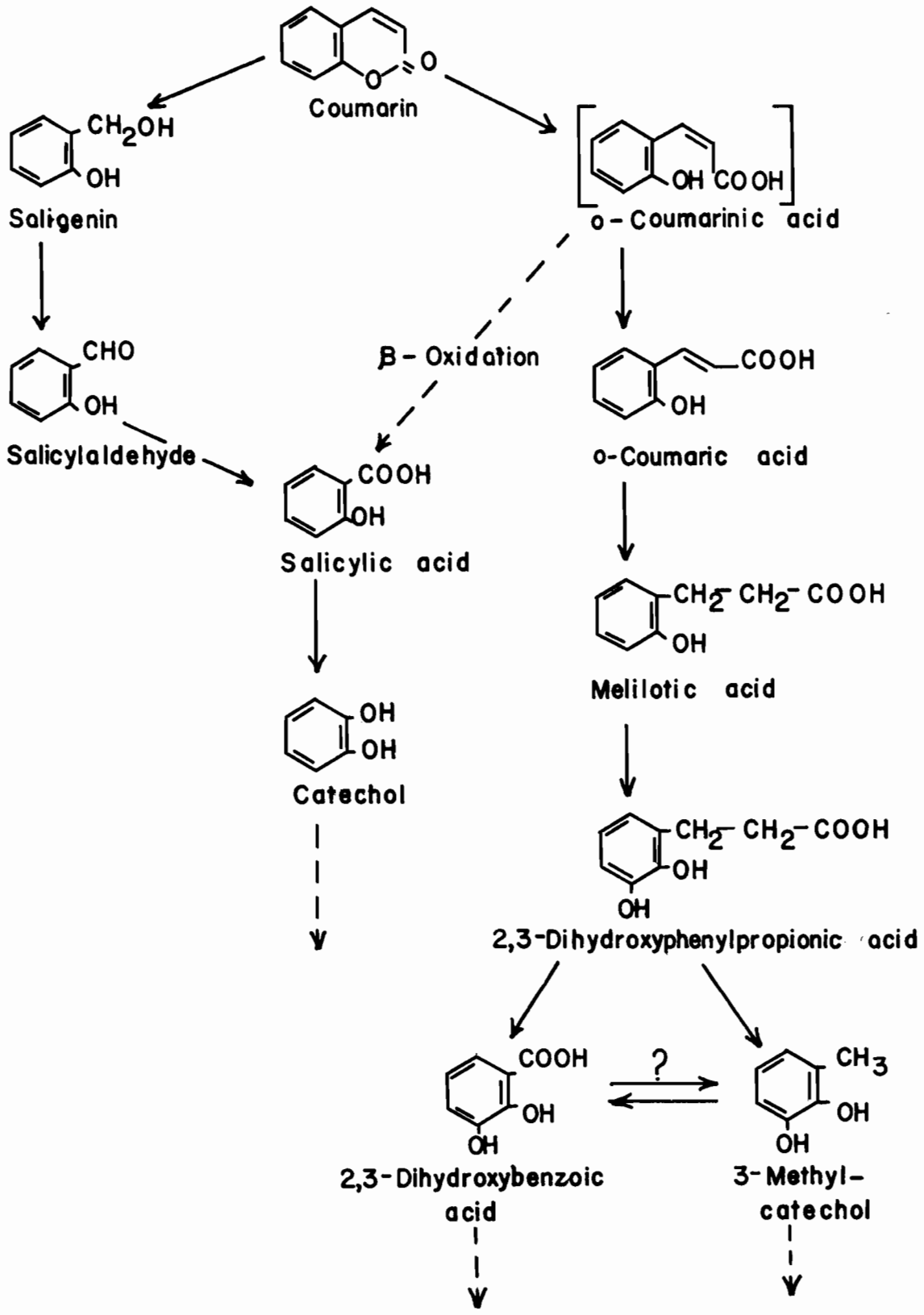


Figure 10. Tentative scheme for the decomposition of coumarin by a Pseudomonas sp. (Mac 291).

interrelationship between those compounds possibly involved in the degradation of coumarin.

The Relationship Between Oxygen Consumption and Substrate Utilization

While the existance of a functioning TCA cycle in bacteria was in doubt for some time (Ajl, 1951), it is now accepted that the TCA cycle is the chief pathway of terminal respiration of most microorganisms including those in the genus Pseudomonas (Kornberg, 1959). Evidence for the occurrence of a TCA cycle in Ps. aeruginosa, Ps. ovalis, and Ps. fluorescens has been reported (Kornberg, 1959; Campbell and Stokes, 1951). Gaby and Harley (1957) found an active electron transport mechanism involving the cytochrome system and used the presence of high concentrations of cytochrome oxidase as a taxonomic tool for the identification of Ps. aeruginosa. Oxygen serves as the final hydrogen acceptor in the cytochrome system, and hence, plays an important role in terminal respiration.

The oxidation of aromatic compounds proceeds through the TCA cycle or substances readily introduced into the TCA cycle (see Literature Review), and these intermediates are in a dynamic state. TCA cycle acids are able to spark energy release through the formation of reduced DPN or TPN which may be further oxidized ultimately through the cytochrome system with the release of energy and CO_2 or they can also serve as precursors for the synthesis of needed cell substances.

Marr and Stone (1961) have calculated that with cells of Pseudomonas aeruginosa grown on benzene, sufficient oxygen uptake was observed to account for 90% of the benzene being transformed to CO_2 and water, while Mycobacterium rhodochrous consumed oxygen for only 54%

oxidation of benzene to CO_2 and H_2O. This shows that a large part of TCA substance is in a dynamic state and can either be diverted to energy production as with Pseudomonas or diverted to the formation of cell organic products as with Mycobacterium. Obviously the organization of these two organisms is fundamentally different.

In all manometric trials equi-molar quantities of substrate were used in the presence of equal cell densities, and consequently, both rate and quantity of oxygen uptake among all compounds are comparable on a molar basis. Table VIII shows the oxygen consumption for the substances undergoing oxidation with ease. In spite of frequent attempts, it was not possible to obtain agreement between replicates with only a few µl differences in oxygen uptake; this variation was believed the result of variations in the endogenous respiration of the cells, where duplicates gave differences of the same order as those found between duplicate tests with the substrates for a number of trials. Table VIII shows the oxygen uptake of the compounds utilized with ease for a number of trials. In Table IX the molar ratio of oxygen consumption to substrate utilization and the relative oxidation of each of the compounds is calculated. Coumarin, o-coumaric acid and 2,3-dihydroxyphenylpropionic acid, all at the same oxidation level, required an equivalent quantity of oxygen, namely 6.0 moles per mole substrate, while melilotic acid required 6.5 moles per mole substrate. The oxidation of benzoic acid to protocatechuic acid required 1.0 moles per mole substrate, a figure identical to values previously reported in the literature (Stanier et al., 1950). The oxygen consumption in all trials was approximately two-thirds that required for

TABLE VIII. Oxygen consumption in the presence of several aromatic compounds used with ease by a Pseudomonas sp. (Mac 291) grown on coumarin.

	Oxygen consumed in µl						
Trial	Coumarin	o-Coumaric acid	Melilotic acid	2,3-Dihydroxy-phenylpropionic acid	Benzoic acid	Protocatechuic acid	Catechol
A	547.2	522.6	602.6	552.3	395.1	340.2	135.6
B	578.5	510.9	583.9	538.2	474.9	333.8	168.8
C	547.2	551.2	589.4	494.1	392.1	348.8	138.3
D	529.5	553.9	603.2	589.0	451.5	336.7	163.6
E	562.7	(465.3)*	549.2	(484.1)*	477.4	352.7	162.2
Mean	552.3	534.6	585.7	543.4	439.2	342.4	153.7

* Incomplete oxidation.

TABLE IX. Relative oxidation of a number of aromatic compounds used with ease by a Pseudomonas sp. (Mac 291).

Compound	Theoretical O_2 uptake in µl for complete oxidation	Mean observed O_2 uptake in µl	µM O_2/mole substrate		
			Theoretical	Observed	% Oxidation
Coumarin	851.2	552.3	9.50	6.16	64.8
o-Coumaric acid	851.2	534.6	9.50	5.97	62.8
Melilotic acid	896.0	585.7	10.00	6.54	65.4
2,3-Dihydroxyphenyl-propionic acid	851.2	543.4	9.50	6.06	63.8
Benzoic acid	672.0	439.2	7.50	4.90	65.3
Protocatechuic acid	582.4	342.6	6.50	3.82	58.7
Catechol	582.4	153.7	6.50	1.71	26.3

complete oxidation to CO_2 and H_2O. This indicates that one-third of the substrate used is incorporated either into cell substance or is converted to non-oxidizable end products.

DISCUSSION

Stanier's 'sequential induction' technique has led to the solution of many enigmas of intermediate metabolism. In this investigation, substances utilized with ease can be interpreted with reasonable clarity. But what of those substances where oxygen consumption is low or negligeable? Are these values significant?

A permeability factor may account for these findings. Considerable work has been done on the permeases of bacteria. Cohen (1949) reported that at least eight permeases are present in Escherichia coli and suggested, on the basis of specificity, it would not be unlikely for at least 30-50 different permeases to be synthesized pertinent to biochemical reactions. Permeases have also been reported in Pseudomonas spp. Kogut and Podoski (1953) showed that a fluorescent Pseudomonas oxidized citrate and other intermediates of the TCA cycle with a lag period when whole cells were used, but that cell-free extracts contained constitutive enzymes for these same substances and obviously, the induction period is required for permease formation. Campbell and Stokes (1951) showed that Pseudomonas aeruginosa oxidized several TCA cycle intermediates only after a lag period, whereas dried preparations oxidized the compounds without any lag, and the limiting condition was attributed to a permeability factor. Bhat et al. (1957) showed that Pseudomonas convexa var. hippuricum metabolized benzoic acid to catechol through salicylic acid. While Stanier excluded salicylic acid as an intermediate in this reaction, Bhat suggested this ambiguity arises from a permeability factor.

It has never been shown that the two mechanisms are, in fact, identical or mutually exclusive, and the criticism is not valid until this additional study has been executed.

On the strength of impermeability of a host of TCA cycle intermediates, one need not be disturbed at the wide range of compounds undergoing oxidation at such a low rate.

The sequential induction technique has also failed to demonstrate a sequence of reactions in the compounds most readily oxidized. Stanier predicted that the degree of adaptation should increase as the number of intervening steps from the precursor to substrate increased. The low level of substrate required for induced enzyme formation could readily be supplied by reversibility of enzyme action.

Campbell et al. (1949) has severely criticized Stanier's technique on the grounds that it is valid only for reactions in which the intermediate in question was separated from the parent compound by only one reaction. He writes:

> ----cells grown on glucose medium could attack gluconic acid without a period of adaptation but there was a period of adaptation before they attacked 2-ketogluconic acid. Cells grown on pyruvic acid would attack acetic acid without a period of adaptation but cells grown on glucose have a period of adaptation before either pyruvic or acetic acids were oxidized.

While such a criticism was perhaps sound at the time, it is not in keeping with current knowledge. Permease formation through the use of whole cells and cell-free extracts would invalidate Campbell's views. The function of permeases may serve not only as a mechanism of transport but also as a

defense mechanism of the cell which shields it from a harsh and heartless environment. The selective nature of the cell membrane will never cease to be a source of amazement to the physiologist.

Campbell also invalidates Stanier's theory on the grounds that compounds in enzymatic equilibrium fail to produce appropriate induction periods where anticipated. This limitation is surely written into the principles laid down by Stanier. Campbell's evidence, as well as the results obtained in this investigation, clearly indicates not that Stanier's principles are invalid, but merely that results should be interpreted with caution.

No sequence of intermediates could be established by the 'sequential induction' technique. Mead et al. (1958) showed that melilotic acid, when fed to rabbits, gave rise to o-coumaric acid which was detected in the urine. o-Coumaric acid, however, when administered in the same manner did not form melilotic acid. Booth et al. (1959) showed that the enzyme reducing o-coumaric acid to melilotic acid is reversible. The failure of appropriate lag periods to occur is probably due to reversibility of enzyme action though it is most surprising that this reversibility should extend back as far as coumarin which involves a lactonizing reaction.

The failure of shikimic acid to undergo decomposition at a rapid rate is of some interest. This acid is metabolized through catechol via quinic acid in mammalian systems (Booth et al., 1960) and through protocatechuic acid in bacteria (Rushton, 1959; Yano and Arima, 1958) which

is decomposed with ease by the Pseudomonas sp. studied here. Consequently a reversal of the shikimic acid pathway appears unlikely.

Homogentisic acid is apparently oxidized by cells grown on glucose without the presence of an induction period. The medium used to grow these cells contains 0.5% tryptone. Both phenylalanine and tyrosine are known to be metabolised through homogentisic acid in bacteria classified in the genera Pseudomonas and Vibrio (Jones et al., 1951; Dagley et al., 1953). It is plausible that the tryptone added to the medium contains sufficiently high quantities of these aromatic amino acids to maintain the induced enzyme state. Consequently the role of homogentisic acid in the metabolism of coumarin has unfortunately not be adequately assessed.

The biochemical transformations of benzoic acid have been studied in many organisms; a summary of most of the known reactions has been presented by Dagley and Evans (1960). In brief, it may form all three isomers of the monohydroxylated derivatives. The o-hydroxy acid (salicylic acid) is metabolized through catechol, the m-hydroxy derivative is metabolized through either gentisic acid or protocatechuic acid while the p-hydroxy derivative is metabolized through protocatechuic acid. The oxidation of benzoic acid and protocatechuic acid by induced enzymes, as well as a balanced oxygen consumption, is ample evidence that benzoic acid is metabolized by the protocatechuic acid pathway in this organism.

Previous reports show that the introduction of each hydroxyl group into the aromatic ring requires one-half mole of atmospheric oxygen

per mole substrate. Evans (1947) showed that the oxidation of phenol to catechol required one atom oxygen per molecule substrate, and that the oxidation of benzoic acid to p-hydroxybenzoic acid to protocatechuic acid required one atom oxygen per molecule substrate for each step. Saito et al. (1957) found that the introduction of one hydroxyl group into kynurenine to form 3-hydroxykynurenine required two atoms of atmospheric oxygen, but that one mole of TPNH per mole substrate is also required. They suggested that one atom of oxygen is required for aromatic hydroxylation, while the second atom is required for the oxidation of TPNH to form TPN and H_2O. Mac 291 required one-half mole atmospheric oxygen for the oxidation of melilotic acid to 2,3-dihydroxyphenylpropionic acid; this strongly suggests that the oxygen required for the second hydroxyl group is of atmospheric origin, and that the step is TPN-independent.

CONCLUSIONS

1. The 'sequential induction' technique of Stanier showed that the oxidation of a number of aromatic compounds by Mac 291 takes place through induced enzyme formation.

2. The technique was applied to 23 compounds; only o-coumaric acid, melilotic acid, and 2,3-dihydroxyphenylpropionic acid were identified as intermediates of coumarin degradation. Like coumarin, all showed a lag phase of approximately one hour when grown on glucose as a substrate. The technique, however, failed to reveal a reaction sequence for these compounds.

3. The oxidation of coumarin, o-coumaric acid and 2,3-dihydroxyphenylpropionic acid required 6.0 moles oxygen per mole substrate while melilotic acid required 6.5 moles oxygen per mole substrate.

4. The oxidation of benzoic acid to protocatechuic acid required one mole oxygen per mole substrate.

5. In all compounds readily utilized, a sufficient quantity of oxygen was consumed to account for the oxidation of only 60-65% of the substrate to CO_2 and H_2O. Catechol consumed oxygen to account for the oxidation of only 26% of CO_2 and H_2O.

6. A group of compounds which include catechol, salicylic acid, saligenin, salicylaldehyde, 2,3-dihydroxybenzoic acid and 3-methylcatechol are still regarded as potential intermediates in the degradation of

coumarin.

7. A group of compounds which include benzene, benzoic acid, cinnamic acid, o-cresol, hydrocinnamic acid, 4-hydroxycoumarin, o-hydroxyphenylacetic acid, mandelic acid, phenol, protocatechuic acid, shikimic acid, and toluene have been eliminated as possible intermediates of coumarin degradation.

PART III

CHROMATOGRAPHIC EVIDENCE FOR THE PATHWAY OF COUMARIN DECOMPOSITION

INTRODUCTION

Paper chromatography, from a modest beginning, has mushroomed into a laboratory workhorse. This simple technique has provided answers to problems whose solutions would be inconceivable prior to its development. Its prime virtues are the low quantity of material required for a positive identification, a factor of the utmost importance where large amounts of material are rare, and the high efficiency of separation when suitable solvents are used.

Chromatographic separation has been used extensively for the detection of phenols in plant tissue, for the characterization of phenolic metabolites in the urine of animals, and for the identification of intermediates in bacterial systems involving aromatic transformations (Block et al., 1958). It has been applied to some rather unique problems, such as the purification of specific radioactive products formed by biological agents (Perkins and Arnoff, 1959), and the diagnosis of physiological disorders (Smith et al, 1959). Very frequently problems have been solved using no other technique. Booth et al. (1957; 1959; 1960) have used it extensively to determine the fate of aromatic compounds in animals, and have shown species differences exist when the same compounds are administered to different animals.

Paper chromatographic methods have been used successfully to identify metabolites of coumarin in plant, animal, and fungal systems

(Kosuge and Conn, 1959; Mead et al., 1958; Bellis, 1958). Manometric experiments have shown clearly that phenolic substances are involved in the decomposition of coumarin by the organism under investigation. The positive identification of one or several compounds in combination with Warburg results, would be ample evidence for the operation of mechanisms previously suggested for coumarin decomposition. Of major interest are those compounds which did not respond favourably under manometric conditions. The identification of any compound not readily oxidized would confirm the pathway of coumarin breakdown through catechol, and would show that the interpretation of results based on a permeability factor is a valid one.

MATERIALS AND METHODS

Solvent Systems

Several solvent systems were used to determine the presence of suspected phenolic compounds, and the composition of the solvent systems and the conditions under which chromatograms were run was as follows:

n-Butanol:pyridine:water:- To 50 parts n-butanol was added 20 parts pyridine and 18 parts distilled water by volume. Chromatograms were run on Whatman No. 1 filter paper at room temperature for a period of 18 hours using the ascending method.

This solvent has not previously been applied to aromatic compounds, although it has been used successfully for aliphatic compounds (Blakley and Blackwood, 1960).

Isopropyl alcohol:ammonium hydroxide:water:- To eight parts isopropyl alcohol was added one part concentrated ammonium hydroxide and one part distilled water by volume (Smith et al., 1959). Chromatograms were run on Whatman No. 1 filter paper at room temperature for a period of 24 hours using the ascending method.

Chloroform:acetic acid:water:- To two parts chloroform was added two parts glacial acetic acid and one part distilled water by volume (Booth et al., 1957). The mixture was shaken for 15 minutes and the organic phase used as the mobile phase. Chromatograms were run on Whatman No. 1 filter paper at room temperature for a period of 16 hours

using the ascending technique.

20% KCl:- Twenty grams potassium chloride were dissolved in distilled water to a final volume of 100 ml (Booth et al., 1957). Chromatograms were run on Whatman No. 1 filter paper at room temperature for a period of three hours using the ascending technique.

Benzene:ethylmethylketone:formic acid:water:- To 900 ml benzene and 100 ml ethylmethylketone was added 100 ml 2% formic acid (two ml formic acid plus 98 ml distilled water) by volume. The mixture was agitated for one hour. After separation, the organic phase was used as the mobile phase, while the aqueous phase was used as the stationary phase (Reio, 1958). Chromatograms were run on Whatman No. 1 filter paper in a thermostatically controlled room at 27° C for a period of three hours using the descending method. Papers were equilibrated for one hour before development with the solvent.

The chromatography tanks used were of all glass construction. Reagent grade solvents were used without further purification.

Color Developing Reagents

Diazotized p-nitroaniline (DPA):- DPA was made as previously described (p. 30) and used one hour after preparation. Chromatograms were oversprayed with 20% sodium carbonate while still moist for maximum color development.

This solution gives a purplish-red to deep blue color with phenols. The background deepens with the age of the reagent.

Diazotized sulfanilic acid (DSA):- DSA was prepared from stock solutions as follows:

Solution A. Dissolve 0.9 gm sulfanilic acid in nine ml concentrated hydrochloric and dilute to 100 ml.

Solution B. Prepare a 5% aqueous solution of sodium nitrite.

Chill both solutions in an ice bath. To five ml solution A add 25 ml solution B. Store in ice bath for a period of 2-4 hours before use (Bray et al., 1950a). After spraying with DSA spray, chromatograms were oversprayed with 20% sodium carbonate while still moist.

This spray gives a light yellow to orange to deep red color with a pale yellow background with phenols. The background deepens with the age of the reagent. It was used preferentially over all other sprays because of the wide spectral range it gives with phenolic compounds.

Gibbs' reagent:- This reagent was prepared by dissolving 2.0 gm 2,6-dichloroquinonechloroimide in 100 ml ethyl alcohol (Reio, 1958). The chromatograms were oversprayed with a saturated aqueous solution of sodium bicarbonate (Bray et al., 1950a).

This solution gives blue to grey colors on a blue background with phenols. While the spray is more sensitive than the diazotized sprays the background darkens considerably on standing limiting its usefulness. Where the response of diazotized sprays was not satisfactory, for example with phenol and o-ethylphenol, this spray was used.

1 N NaOH:- Because of the absence of phenolic groups, none of the reagents described above will react with coumarin; this property was most useful where samples contained high levels of residual coumarin which would mask metabolites. When coumarin was to be detected, papers were sprayed with 1 N sodium hydroxide which served to break the lactone ring, and detected by fluorescence under uv or by spraying with DPA spray.

Some dihydroxyphenolic compounds turn dark greenish black when sprayed with sodium hydroxide.

2,4-dinitrophenylhydrazine:- To 100 ml hot 1 N hydrochloric acid was added 50 mgm 2,4-dinitrophenylhydrazine. The solution was filtered after 48 hours through Whatman No. 1 filter paper (Reio, 1958).

This reagent gives a yellow to orange color with aldehydes and ketones.

Chromatogram Standards

Stock solutions prepared from Warburg trials having one μM substrate per 0.1 ml using water as the solvent were employed as chromatogram standards. o-Coumaric acid was prepared at this concentration with ethanol as a solvent. Compounds not used for manometric trials were prepared at the concentration of one μM per 0.1 ml with the exception of dicoumarol which was prepared at levels of 10 mgm per ml with ethanol as a solvent. Usually 10 lambda of these standards were spotted as controls, i.e., equimolar concentrations of each phenol were applied to the paper. Quantities, then, are proportional to their molecular weight

and standards ranged from 9.4 μgm for phenol, the compound having the lowest molecular weight, to 18.2 μgm for 2,3-dihydroxyphenylpropionic acid, the compound having the highest molecular weight.

Preparation of Samples for Chromatography

Samples of fermentation liquor from different growth conditions were withdrawn at one hour intervals, acidified with hydrochloric acid (which served as a preservative) and centrifuged at 10,000 x g for 10 minutes to remove the cellular material. Where material was available in 100 ml quantities or greater the liquor was cleared of protein by adding an aqueous solution of zinc sulfate (25% $ZnSO_4 \cdot 7H_2O$) one-tenth by volume, and raising the pH to 7.6-7.8 using a glass electrode (Neish, 1952). The protein is removed along with zinc hydroxide by centrifugation. The resulting liquor was saturated with sodium chloride and shaken vigorously for 10 minutes in a separatory funnel with 0.5 volumes of peroxide-free ether. The ether extraction process was repeated a second time to insure adequate extraction. The ether phase was evaporated to dryness and redissolved in water to a final volume one-tenth that of the original sample. When fermentations were carried out on a small scale and sample sizes were of magnitude 50 ml or less, the solution was concentrated on a rotary evaporator to a volume of approximately five ml, salt-saturated and ether-extracted in a continuous ether extractor having a sample capacity of 5-7 ml. Extraction was carried out for four hours with a rate of ether reflux of approximately five ml per minute. The ether phase was evaporated to dryness and taken up in a volume of water one-tenth that of the original sample.

RESULTS

Assessment of Chromatographic Methods

Samples of untreated fermentation liquor obtained from CYT medium developed with n-butanol:pyridine:H_2O and sprayed with DSA reagent showed the presence of a host of colored areas ranging in shades of pink to yellowish-brown. Interestingly, the majority of these colored areas could not be detected in control medium in which no coumarin was added. Labouring under the misapprehension that the DSA spray was specific for phenols, these colored areas were interpreted as intermediates in the decomposition of coumarin which accumulated in detectable amounts. An example is shown in Fig. 11. The Rf values of standards varied considerably, and detailed migration scores were not kept. Other solvent systems failed to give as good a separation as n-butanol:pyridine:water and were not used frequently. The majority of the standards gave Rf values in excess of 0.70; consequently, DSA-positive substances of lower Rf values remained unidentified while compounds of higher Rf value were too close together for positive identification. However, several compounds with Rf values different from DSA-positive substances were eliminated as standards in solvent systems used subsequently.

Considerable variation was observed in Rf values obtained with standards using n-butanol:pyridine:water. As the number of times the solvent was used increases, the closer do the standards run to the solvent front. This solvent, then, is not satisfactory as a general solvent for aromatic compounds.

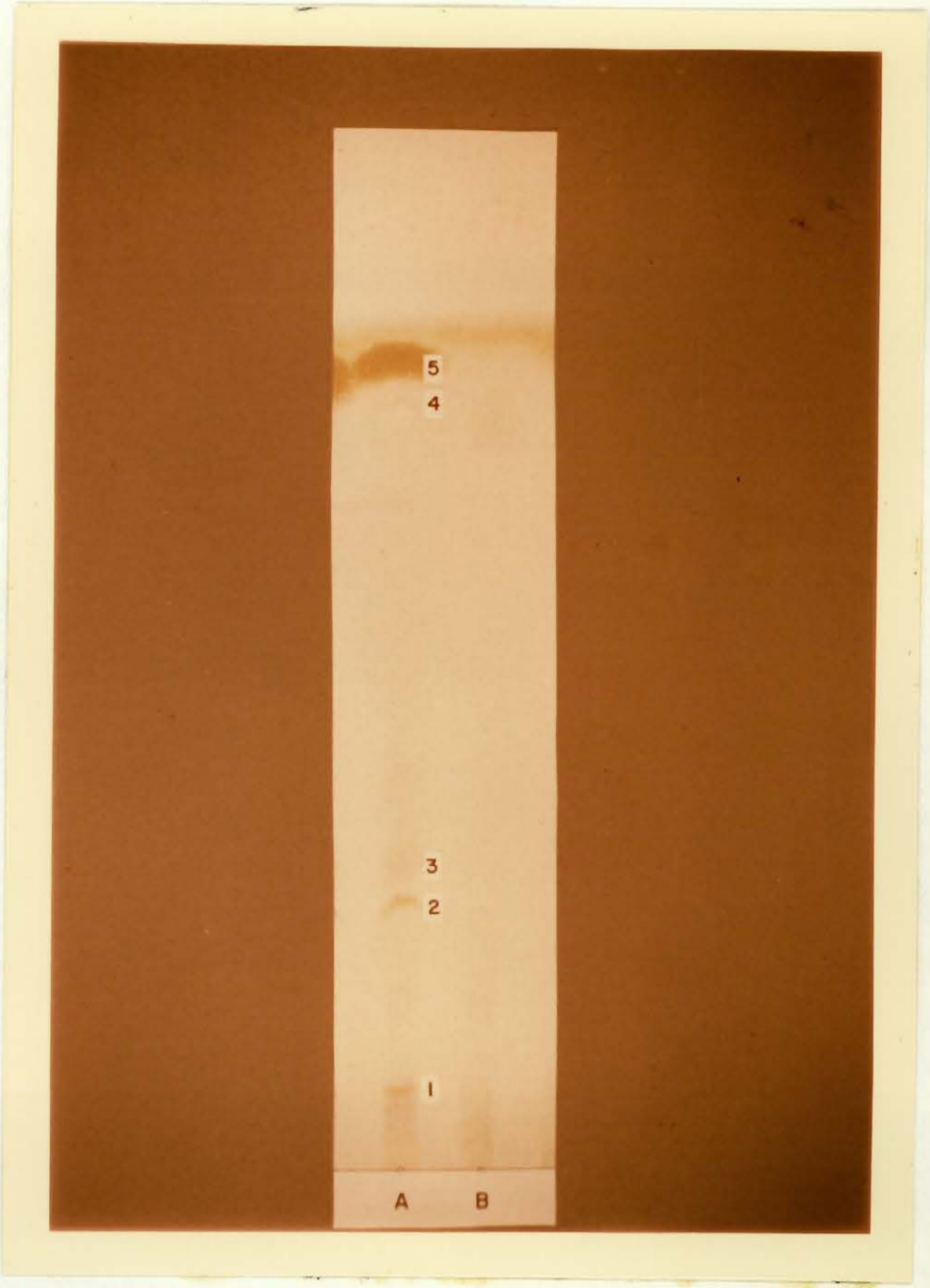


Figure 11. Chromatogram showing at least five DSA positive areas in untreated sample (A) of 18 hr fermentation liquor from CYT medium not present in control sample (B).

The method of protein removal outlined by Neish (1952) was tested against 10 arbitrarily chosen phenols, and the resulting precipitate washed once with distilled water. The precipitate was suspended in water and redissolved by adding hydrochloric acid until complete solution was achieved. Chromatography of the liquid failed to reveal any phenols indicating that the method did not remove phenolic substances from the liquor.

The DSA spray gives color reactions with compounds other than phenols, and the slower running compounds were found not to be phenolic in nature. Some of superfluous matter was removed by protein removal; aromatic phenols were removed from the remainder by ether extraction, and the results of sample treatment are shown in Fig. 12.

The use of CYT medium for growth gave higher yields of detectable phenolic substances than did conditions devised for optimum utilization, and this indicated the adaptive nature of the enzyme system. Attempts to increase the yield of intermediates which included malonic acid inhibition, controlled pH, controlled oxygen tension, removal of mineral salts from medium or, the use of liquor from resting cell suspensions under manometric conditions, all were unsuccessful.

Identification of Intermediates

Samples from CYT medium prepared for chromatography gave only two compounds which were identified with certainty. Melilotic acid was identified readily. When catechol was used as a chromatogram standard and sprayed with DSA spray, a light pink color developed which turned deep cherry-red on overspraying. The spot became deeper in color with

Figure 12. Effect of sample treatment on removal of non-phenolic DSA positive substances in CYT medium.

A - Untreated fermentation liquor
B - Fermentation liquor following protein removal
C - Ether extract of B

age as did catechol. A similar spot with Rf values identical to catechol was found in four different solvent systems; a pronounced difference in Rf value was obtained in ethylmethylketone:benzene:formic acid:water and the compound was identified as 2,3-dihydroxyphenylpropionic acid.

Confirmation of the identity of the compounds was made spectrophotometrically. A 0.1 ml sample was applied one inch from the base of the filter paper over a distance of fifteen centimeters and after drying, was developed. A one-quarter in strip was cut from the edge of the paper and sprayed with DSA spray to locate the phenolic compounds. The corresponding bands were cut from the chromatogram and eluted, using the descending method, in a chromatography tank with water as the eluting agent. The first few drops were caught in a small beaker, and the spectrum obtained in a Zeiss spectrophotometer over the whole ultra-violet range (200-400 mμ) were compared with that of standards. The spectra obtained with unknowns was identical to authentic samples as shown in Figs. 13 and 14.

Factors Affecting the Accumulation of 'Catechol Substance'

Quantitative determination of 'catechol substance':- Evans (1947) developed a colorimetric test for the detection of dihydroxyphenolic compounds in fermentation liquor, and used it to follow the formation of catechol from phenol by a Vibrio sp. A positive test is given by all dihydroxyphenolic compounds and is not specific for catechol, but it does not react with monohydroxyphenolic compounds. The test was carried out as follows:

To 1.0 ml of neutral test solution was added 1.0 ml 10% sodium molybdate, 0.5 ml 0.5 N HCl and 1.0 ml 0.5 N sodium nitrite. After the

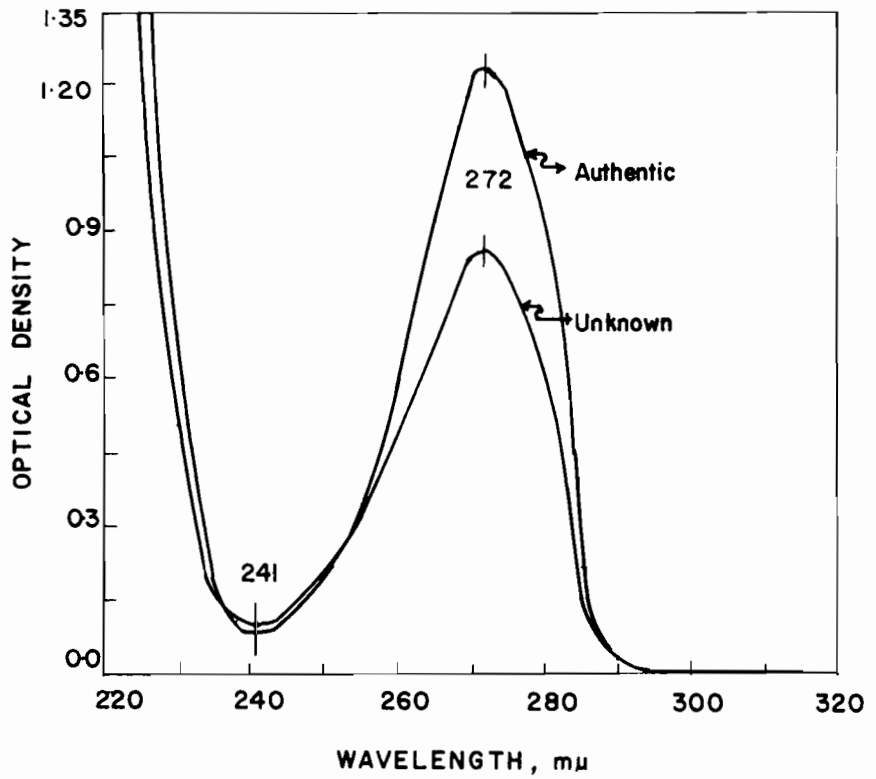


Figure 13. Comparison of ultra-violet absorption spectrum of authentic sample of melilotic acid and chromatogram eulate.

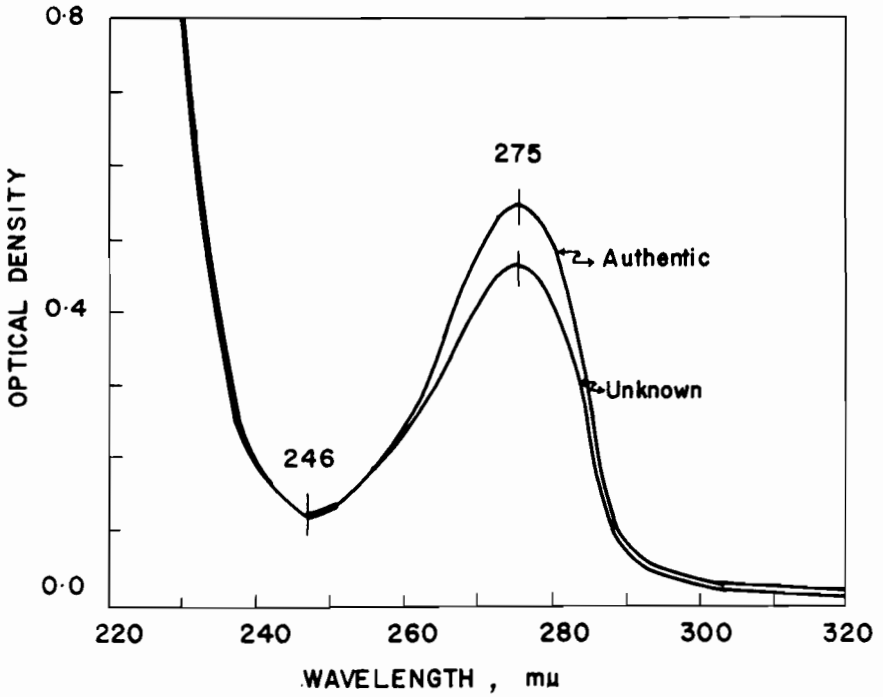


Figure 14. Comparison of ultra-violet absorption spectrum of authentic sample of 2,3-dihydroxyphenylpropionic acid and chromatogram eulate.

yellow color which formed reached a maximum, 1.0 ml 0.5 N sodium hydroxide was added. The color turned cherry red. The volume was made up to 10 ml and the optical density read in a Coleman colorimeter with a 470 mμ filter.

The test applied to catechol gave a stoichiometric response from 10 to 100 μgm provided a five minute interval and thorough mixing were employed between each addition. The test could also be used qualitatively as a spot test with small portions of reagents. This test was used to follow the formation of 'catechol-substance' in fermentation liquor accompanying the utilization of coumarin in CYT medium, and the data are shown in Fig. 15 and Appendix Table X.

Maximum accumulation of 'catechol-substance' occurred when the level of residual coumarin was reduced considerably. Maximum production was 350 mgm per litre, but this level dropped rapidly when the level of residual coumarin fell to less than 10% of the original concentration.

Fermentation liquor in an unbuffered medium characteristically turned alkaline, a condition under which catechol is not stable and quinone formation occurs readily. To prevent autooxidation the pH was controlled at several values over a range of 6.4-7.4 by means of PO_4^{---} buffer, but no increase in the quantity of dihydroxyphenolic compound was found. Attempts to induce an accumulation by means of controlled aeration were also unsuccessful.

Partial purification of 2,3-dihydroxyphenylpropionic acid:- Evans (1947) purified catechol at levels sufficiently high for crystallization from fermentation liquor using lead acetate as a precipitant.

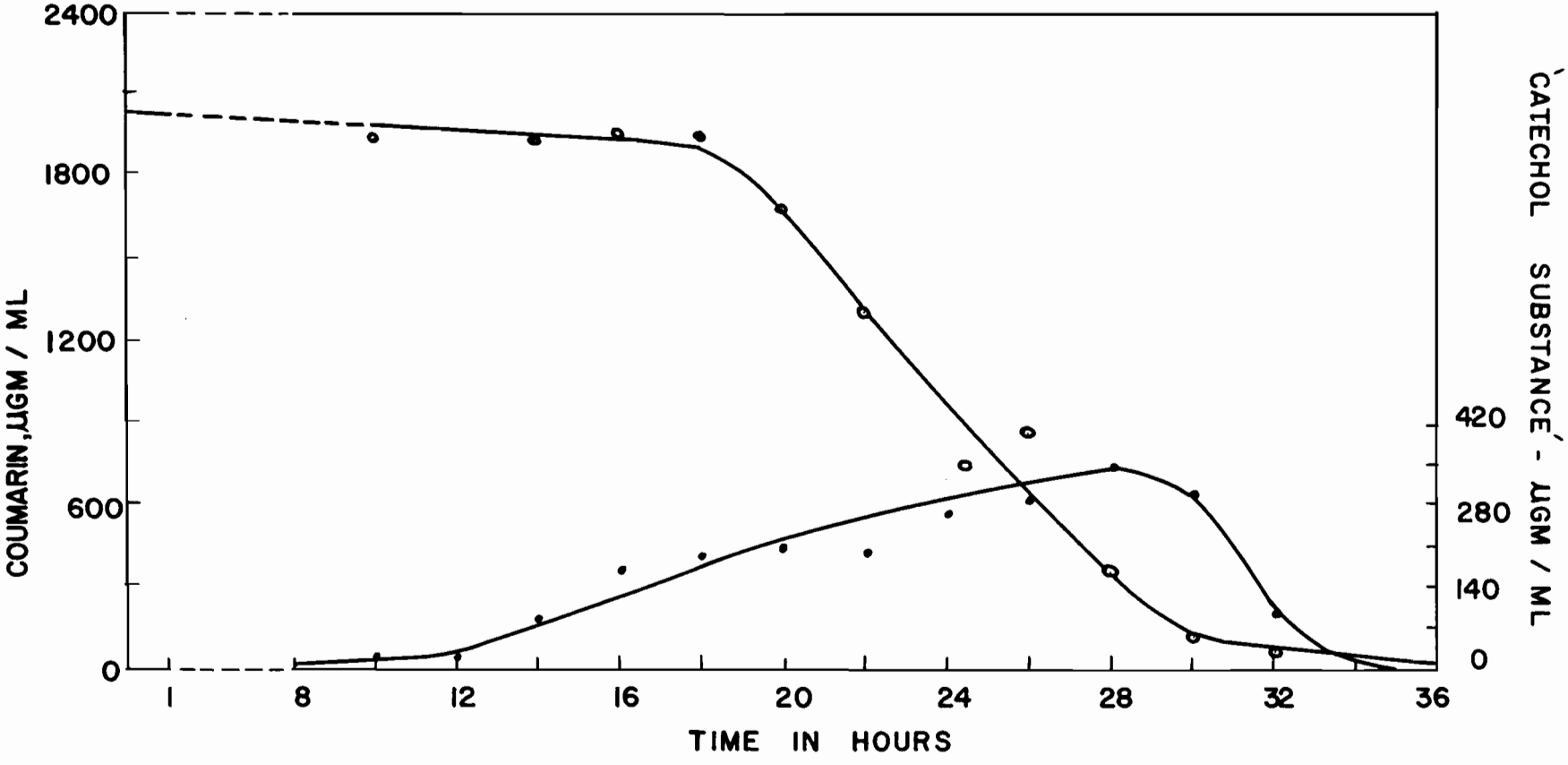


Figure 15. Formation of 'catechol substance' from coumarin by a Pseudomonas sp. (Mac 291).

This procedure was modified as follows:

To a volume of solution containing catechol in the presence of five additional phenolic compounds was added 0.1 volumes 20% lead acetate. The pH was adjusted to 6.0 with 0.5 N sodium hydroxide and the heavy white precipitate formed removed by centrifugation. The filtrate failed to give a positive 'catechol substance' test. The white precipitate was washed once in distilled H_2O and the lead acetate-catechol complex dissociated by bubbling H_2S through freely. Lead sulfide was removed by filtration and the aromatic fraction removed by ether extraction. Chromatography of the ether extract showed that catechol was removed free of any monohydroxyphenolic compounds.

Ten litres of fermentation liquor giving a color-equivalence test of 240 mgm per litre "catechol" was deproteinized, salt saturated, and ether extracted. The ether extract evaporated to dryness which left approximately 40 ml of a deep-brown oily liquid as well as a waxy crystalline material non-phenolic in nature (this waxy substance was not identified). The liquid portion was dissolved in one litre of water and the 'catechol substance' removed by the lead acetate method described above.

Chromatography of the 'catechol substance' after lead acetate purification showed at least a ten-fold concentration of 2,3-dihydroxyphenylpropionic acid although it was still not pure. Of great interest, however, was the detection of a second compound thought to be a dihydroxyphenolic compound on the basis of (a) concentration on treatment with lead acetate, (b) production of a cherry-red color with DSA reagent,

(c) darkening when chromatogram is sprayed with 1 N sodium hydroxide and (d) formation of a yellow color when chromatogram is sprayed with 20% sodium molybdate. Fig. 16 shows it is chromatographically distinct from 3-methylcatechol, 2,3-dihydroxybenzoic acid, and 2,3-dihydroxyphenylpropionic acid, the only dihydroxyphenolic compounds previously reported in biological systems in which the side chain is ortho to the dihydroxy group. This is interpreted as evidence that the side chain of 2,3-dihydroxyphenylpropionic acid is modified prior to the cleavage of the aromatic nucleus.

Chromatography of Fermentation Samples of Substrates Utilized with Difficulty

Samples from fermentations with saligenin and salicylic acid as substrates had been retained. The disappearance of each phenol was determined chromatographically by running untreated samples obtained at 24 and 48 hour intervals in butanol:pyridine:water, and using DSA spray as color developing reagent. No appreciable drop of the saligenin in fermentation liquor was found after 48 hours; salicylic acid content dropped considerably although it was detected readily even after 48 hours. These results suggest that saligenin is not an intermediate in coumarin metabolism, while salicylic acid, though used slowly, may act in an intermediate capacity. The slow rate of breakdown of salicylic acid is probably due to a permeability factor, an observation found previously in another Pseudomonas sp. (Bhat et al., 1959).

Protocatechuic Acid as an Intermediate in Benzoic Acid Metabolism

When benzoic acid is used as a substrate, the fermentation liquor gave readily a positive 'catechol substance' test, and

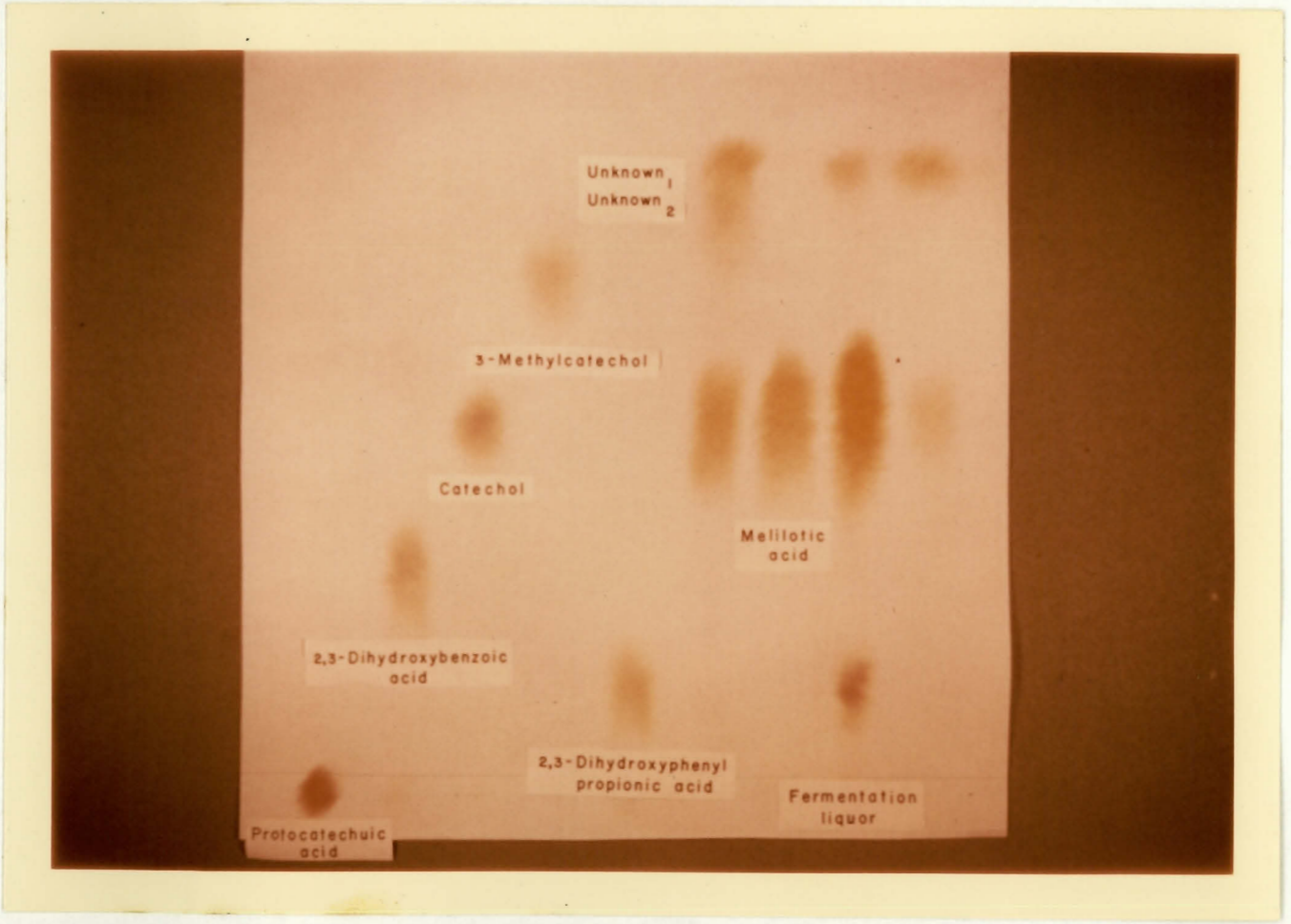


Figure 16. Chromatogram showing:
(a) Melilotic acid and 2,3-dihydroxyphenylpropionic acid in ether extract of CYT medium fermentation liquor
(b) $Unknown_1$ - an unidentified monohydroxyphenolic compound
(c) $Unknown_2$ - an unidentified dihydroxyphenolic compound chromatographically distinct from several o-dihydroxyphenolic standards

protocatechuic acid was identified chromatographically as an intermediate with ethylmethylketone:benzene:formic acid:water as the solvent system using DSA spray as color developing reagent.

DISCUSSION

Attempts to identify intermediates of the decomposition of coumarin by means of paper chromatography were rather disappointing. Both the compounds identified are utilized with ease under manometric conditions by Mac 291. The detection of these compounds does, however, confirm part of the coumarin degradation scheme previously presented.

o-Coumaric acid was not detected in any fermentation samples. Fernley and Evans (1958) suggested that melilotic acid is formed from o-coumarinic acid and not o-coumaric acid, but the data presented here does not confirm this view. The detection of melilotic acid in fermentation liquor using o-coumaric acid as a substrate suggests that after cleavage of the lactone ring, the trans isomer is formed which then undergoes reduction. o-Coumarinic acid does not fluoresce under ultraviolet light, but does form the highly fluorescent trans isomer when exposed to alkali. Only one fluorescent area was ever detected on chromatograms; o-coumaric acid was formed from residual coumarin when chromatograms were sprayed with 1 N sodium hydroxide. It appears that an accumulation of intermediates between coumarin and melilotic acid does not take place.

Dixon and Webb, (1958), in a comprehensive list of known enzyme reactions, list 10 isomerases. While an isomerization of the cis to trans form of o-coumaric acid takes place, the instability of o-coumarinic acid makes it impossible to predict whether this isomerization is under

enzymatic control.

None of the compounds utilized with difficulty by Mac 291 were detected. If a permeability factor prevents access of intermediates into the cell, perhaps the same principle prevents the passage of intermediates into the surrounding medium, and compounds cannot be detected. The results, however, do not suggest that the pathway of coumarin decomposition is through catechol.

One mechanism of decomposition of protocatechuic acid is through β-ketoadipic acid, an intermediate common to catechol degradation. Mac 291 utilized protocatechuic acid readily, but catechol very slowly. Dagley et al. (1960) suggested a new pathway for the decomposition of both protocatechuic acid and catechol involving ring fission by a rupture of the aromatic nucleus at a point adjacent to rather than across the dihydroxy group. No intermediate is common to both substances by this route, i.e., compounds are decomposed by distinct enzyme patterns. The results indicate that in the system under investigation catechol and protocatechuic acid are metabolized through separate pathways although no effort was made to determine the mechanism of metabolism beyond the aromatic stage.

CONCLUSIONS

1. In fermentation liquor derived from CYT medium only melilotic acid and 2,3-dihydroxyphenylpropionic acid were identified with confidence on the basis of Rf values in several solvent systems, on the basis of color responses to several spray reagents, and on the basis of absorption spectra.

Two additional unidentified compounds were also detected and tentatively identified as a monohydroxyphenolic compound and a dihydroxyphenolic compound.

2. Melilotic acid and 2,3-dihydroxyphenylpropionic acid were identified in fermentation liquor containing o-coumaric acid as a substrate, while in fermentation liquor containing melilotic acid as a substrate, only the dihydroxyphenolic compound was identified.

3. None of the compounds utilized with difficulty, or not at all, were found.

4. Benzoic acid is metabolized through protocatechuic acid. Steps beyond this product are not known, but the mechanism does not appear to have intermediates in common with catechol.

PART IV

RADIOACTIVE EVIDENCE FOR THE DECOMPOSITION OF COUMARIN

INTRODUCTION

The diversity of radioactive compounds, and the role they have played in scientific research is beyond reproach. Their usefulness has been demonstrated, for example, by Kaighen and Williams (1961), who showed that when coumarin is administered to rabbits, 80% is excreted into the urine within 24 hours. By isotopic dilution the metabolites were found to be coumarin (0.5%), 'an acid-labile coumarin precursor', i.e., an unidentified substance which forms coumarin on acid reflux (14.9%), 3-hydroxycoumarin (21.7%), 4-hydroxycoumarin (0.6%), 5-hydroxycoumarin (0.4%), 6-hydroxycoumarin (3.4%), 7-hydroxycoumarin (12%), 8-hydroxycoumarin (1.9%), o-hydroxyphenyllactic acid (3%), and o-hydroxyphenylacetic acid (20%). The hydroxycoumarins were excreted mainly in conjugated forms. In rats, however, only 58% of the radioactivity was excreted in the urine with just 3% of the metabolites identified as hydroxylated coumarin derivatives and 5% as an 'acid-labile precursor' of coumarin. The major urinary metabolite was o-hydroxyphenylacetic acid (20%). The results showed that coumarin was more extensively metabolized by ring opening in rats than in rabbits.

The bacterial system under study is quite different from mammalian systems. The ring does undergo extensive rupture by Mac 291. After 18 hours under rapid oxidation, it is not possible to detect any residual phenolic compounds.

There are two major areas in which experimental evidence presented in this work has failed to yield satisfactory information, and where strong criticism could be directed. Firstly, are the compounds which have been identified as metabolites of coumarin derived, in fact, from coumarin itself or could they arise from some other source? Several DSA-positive substances have been excluded as metabolites of coumarin on the basis of their removal with zinc sulfate or their failure to be extracted with ether. Might not some of the intermediates of coumarin be lost by the application of these methods? Secondly, does coumarin decomposition, in fact, proceed through catechol? Perhaps the use of radioactive tracers could adequately quench these objections.

Two experiments were carried out. In the first experiment uniformly tritiated coumarin was used as a substrate in CYT medium, and the activity of DSA-positive areas on the resultant chromatograms determined. In the second experiment coumarin-2-C^{14} was used as a substrate with resting cell suspensions, and the % recovery of radioactivity as CO_2 was found. The quantity of radioactive carbon released as CO_2 gives some insight into the mechanism under which the decomposition occurs.

MATERIALS AND METHODS

Radioactive Substrates

Tritiated coumarin:- A sample of tritiated coumarin (obtained by tritiating coumarin by the Wilzback technique (Wilzback, 1957) through the courtesy of Mr. O. Sepall of the Pulp and Paper Research Institute, McGill University), was recrystallized twice in distilled water to obtain a uniform preparation.

Coumarin-2-C^{14}:- A small quantity of coumarin labeled with C^{14} in the number two position (obtained through the courtesy of Dr. S. Brown of the Prairie Regional Laboratory, National Research Council, Saskatoon) containing approximately two uc radioactivity was diluted with 200 mgm coumarin and recrystallized twice in distilled water to obtain a uniform preparation. Approximately 60 mgm was added to a 50 ml volumetric flask and made to volume. The level of coumarin was determined colorimetrically using the DPA method described in Part I; 1.0 ml contained 1.230 mgm coumarin.

Chromatography of Fermentation Liquor using Tritiated Coumarin as a Substrate

Preparation of samples for chromatography:- Tritiated coumarin was incorporated into 40 ml CYT medium, and fermentation carried out as described previously in Part I. Five ml samples were withdrawn at 12, 18, and 24 hour intervals and the cellular material removed after acidification. A small portion (0.5 ml) was retained from each sample with no further treatment. This was designated as "untreated liquor". The

remaining portion was subjected to ether extraction, the extract evaporated to dryness, and the residue taken up in one ml distilled water. This was designated as "treated liquor". Both fractions were chromatographed in butanol:pyridine:H_2O, and phenols located with DSA spray.

Location of radioactivity on chromatograms:- Chromatograms from treated and untreated fermentation liquor were cut into strips two inches wide (one inch on either side of the point of sample application) in the direction of the solvent flow. Each strip was applied to a paper chromatography counting unit consisting of a Forro Chromatograph Scanner Model ACSE used in conjunction with a Baird-Atomic Research Ratemeter Model 432 and an Esterline-Angus Chart Recorder to locate activity. A windowless scanner was used to measure the weak beta rays emitted by tritium. The chromatogram was held firmly against the scanner by means of a hanging weight to insure a gas-tight fit and helium (0.95% isobutane) was used as the carrier gas. In the operation of the unit, a choice of variable must be made depending, for the most part, on the level of radioactivity of sample components. The following conditions were found satisfactory:

	Quantity of Radioactivity	
	Maximum < 1,000 c.p.m.	Maximum > 1,000 c.p.m. but < 10,000 c.p.m.
Scanner slit width	$\frac{1}{4}$ in	$\frac{1}{4}$ in
Rate of chromatogram flow	$1\frac{1}{2}$ in/hr	6 in/hr
Ratemeter total range	1,000 cpm	10,000 cpm
Ratemeter time constant	30 secs	10 secs

In this unit, the rate of chart flow is synchronized with the rate of passage of the chromatogram over the Geiger counter. To determine the presence of active areas, the chromatogram is alligned with the recorder chart, and quantitative values are obtained by direct reading from the chart scale.

Recovery of CO_2 Formed During Fermentation of Coumarin

Molar ratio of CO_2 to substrate:- Preliminary trials showed the amount of CO_2 derived from coumarin under the fermentation conditions described below:

Twenty-five ml of a standardized cell suspension prepared for manometric experiments as described in Part II, and 3.5 ml Mineral Salts I were added to a 125 ml Erlenmeyer flask. Fermentation was carried out with the Warburg apparatus as a water bath at 30° C with constant agitation. The flask was swept through with CO_2-free air to remove atmospheric carbon dioxide. Then, after adding 0.10 mM (14.61 mgm) coumarin, the CO_2 formed was swept with CO_2-free air into a glass bead bubbling tower of 20 ml capacity containing sodium hydroxide as absorbant. A similar unit containing no coumarin served as a blank for respiratory CO_2. Simultaneously, a Warburg flask along with its endogenous control was prepared having a ratio of substrate to cells identical to that of the large fermentation unit and the utilization of coumarin in the large fermentation vessel was followed manometrically in the comparative trial. When oxygen uptake in the manometer had ceased, the large flasks were allowed to run an additional 30 minutes to insure complete utilization of substrate (total running time was 90 minutes). The sodium

hydroxide was removed from the bubbling tower and made to volume, and a suitable aliquot was titrated against standardized hydrochloric acid. Similarly an aliquot from the blank fermentation was carried through the same procedure to correct for endogenous carbon dioxide. The quantity of CO_2 formed from the added substrate was calculated on a molar basis.

Recovery as CO_2 of radioactivity from coumarin-2-C^{14}:- The fermentation procedure was repeated using 10 ml of a solution containing 1.230 mgm coumarin-2-C^{14}/ml. After dilution to volume the CO_2 trapped in the bubbling tower was precipitated as the barium salt. The residual base was neutralized to phenolphthalein end point and the amount of barium carbonate was measured by direct titration to the brom-cresol green end point with standardized hydrochloric acid. The carbonate in the remaining portion of the base from the bubbling tower was prepared for counting by precipitating as the barium salt and preparing a slurry which was placed uniformly on glass planchets for counting. Infinitely-thin planchets were prepared using a barium carbonate weight of 0.1-0.2 mgm/cm^2 as recommended by Calvin et al. (1949). The activity of the carbonate was determined to 1000 counts in a Berkeley Decimal Geiger-Müller scaler coupled to a thin-window mica Geiger counter of approximately 5% efficiency (this gives a counting error of less than 3%). Corrections were made for background interference. Samples were prepared in duplicate, and each sample was counted twice with the mean values used for calculations.

Wet Combustion of Coumarin-2-C^{14}

Wet combustion of coumarin was carried out in a combustion screw-cap unit described by Baker et al. (1954); 5 ml Van Slyke-Folch

combustion fluid and 1.0 ml solution containing 1.230 mgm coumarin-2-C^{14}/ml were used. The contents were autoclaved at 121° C for 30 minutes, and the CO_2 absorbed by sodium hydroxide which was confined to a small beaker serving as a centre-well inside the jar. The contents of the beaker were washed out with CO_2-free distilled water and diluted to volume. The carbonate was precipitated as the barium salt and samples were prepared and counted as described above.

RESULTS

Chromatography of Radioactive Fermentation Samples

Untreated sample:- Chromatograms of untreated fermentation liquor showed the presence of three radioactive areas. One major area was found having an Rf value of 0.87, and which had been identified previously as melilotic acid. Two minor areas were detected, the first with an Rf value of 0.24 and the second remaining at the origin. The faster moving minor component was considered to be a metabolite of coumarin, while the area at the origin could be a second metabolite, or could be cellular debris. The activity of both areas was barely detectable, and appeared fairly stable during the fermentation process.

Treated sample:- Chromatograms of the treated fraction are shown in Fig. 17 where three radioactive areas were found. The active area closest to the solvent front corresponds to residual coumarin, which does not give a color reaction with DSA spray. The second radioactive area with a lower Rf value than coumarin has been identified as melilotic acid, and appears bright yellow on the chromatogram. The third active area is 2,3-dihydroxyphenylpropionic acid, and appears pinkish-red on the chromatogram. As the level of coumarin decreases, the activity of the two metabolites increases; then the metabolites in turn are utilized and are no longer detected after 24 hours incubation. While an apparently substantial quantity of radioactivity persists at 24 hours no colored areas are observed on the chromatograms, undoubtedly due to the comparative lack of sensitivity of the spray reagent.

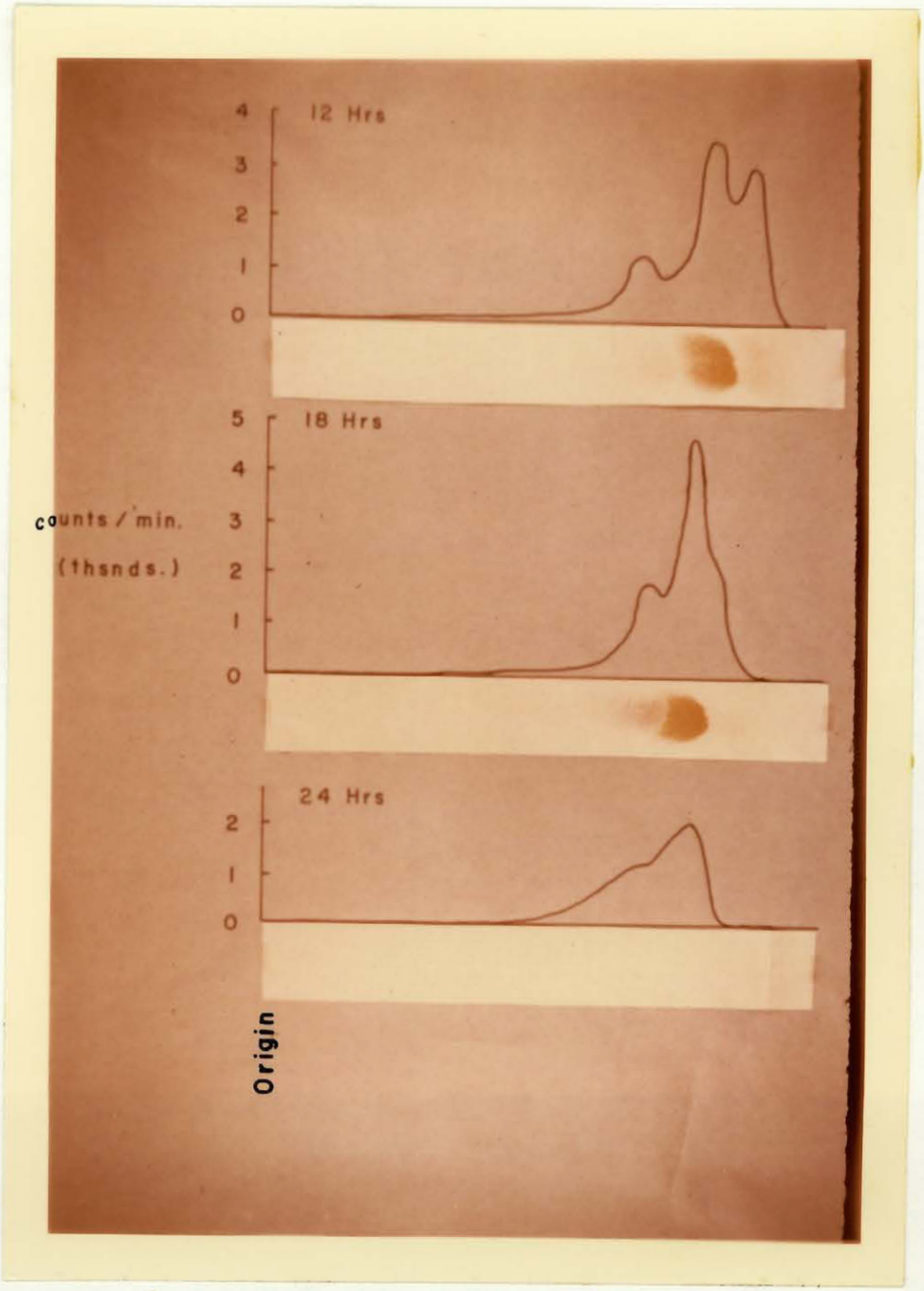


Figure 17. Chromatogram and corresponding radiogram of ether extract of CYT medium containing tritiated coumarin, using samples from fermentation liquor withdrawn at 12, 18, and 24 hr. Radioactive areas have been identified in order from the solvent front as (a) residual coumarin, (b) melilotic acid, and (c) 2,3-dihydroxyphenylpropionic acid.

The minor components in whole fermentation liquor are not extracted by ether, and this indicated the presence in very low amounts of a metabolite(s) in which the aromatic ring is no longer present.

Samples of treated fermentation liquor were run in ethylmethylketon:benzene:formic acid:water to obtain a chromatogram showing the two unidentified products of coumarin decomposition. When 20 lambda samples were applied to the paper, an aliquot identical to that used in ethylmethylketone:benzene:formic acid:water, the unidentified monohydroxycompound was faintly visible while the dihydroxycompound was not detected. When three times the level of samples was applied to chromatograms and run in ethylmethylketone:benzene:formic acid:water, the monohydroxycompound was detected readily and found to be radioactive. The dihydroxycompound was just detected, but could not be assessed for radioactive content because of the overlap of high quantities of interfering residual coumarin.

Formation of CO_2 from Coumarin

Moles CO_2 liberated per mole coumarin utilized:- Table X shows that for each mole of coumarin utilized, six moles of CO_2 are formed.

Recovery as CO_2 of radioactivity from coumarin-2-C^{14}:- To determine the % recovery of radioactivity, the total counts obtained from the fermentation were compared on a unit basis to the total counts added to the fermentation liquor as determined by wet combustion of coumarin-2-C^{14}. Table XI shows that 94.5% of the label is recovered as CO_2.

TABLE X. Quantitative recovery of CO_2 from coumarin during fermentation.

Trial	Coumarin fermented in mM	CO_2 recovered in mM/mM substrate
A	0.10	5.88
B	"	6.04
C	"	6.09
D	"	6.13
E	"	5.85
Mean	"	5.99

TABLE XI. Recovery of radioactivity as CO_2 from coumarin-2-C^{14}.

Source of radioactivity	Coumarin content/trial in mgm	Total mgm carbonate/trial	Counts /min/mgm carbonate	Total counts	Counts/unit coumarin	% Recovery (B/A)
A. Coumarin-2-C^{14}	1.23	39.32	19.8	778.53	778.53	
						94.5
B. CO_2 as barium carbonate	12.30	176.88	41.6	7358.2	735.82	

DISCUSSION

Tritiated coumarin was used successfully to show that the aromatic products identified were, indeed, intermediates of coumarin breakdown, and has eliminated several DSA positive compounds insoluble in ether as intermediates. This technique has shown also the presence of one, and possibly two non-ether soluble intermediates though present in very low amounts.

The detection of chromatographic compounds assumes that not only are suitable sprays selected for use, but that compounds are present at quantities sufficiently high to meet the sensitivity of the spray reagent. Because of the high sensitivity of detecting instruments, the use of radioactive substrates aids the location of compounds which fall outside the range of conventional chromatographic sprays. Conceivably, intermediates may also be formed in biological systems in considerable amounts which are not detected because of a limited selection of spraying reagents. The use of tritium could eliminate this hazard.

The location of radioactive areas on chromatograms has been used extensively with C^{14} substrates in spite of the disadvantage of a 1-3 week period for development depending on the quantity of radioactivity in sample components (Block et al., 1958). The assay of radioactivity on chromatograms using tritium, however, is a relatively recent inovation. The use of tritium as a label, because of its difficulty of detection, has not been encouraged until recently. Improved instrumentation is over-

coming this problem. Its short half-life, its weak beta emission property, i.e., its safety in handling, and above all, its low cost are factors increasing the application of this tracer to biological research. Kopin and Axelrod (1960) using an automatic radiochromatogram scanner, showed that radioactive 3,4-dihydroxyphenylglycol is formed in the animal body from the deamination of tritiated epinephrine. On the other hand, Alpen and Mandel (1960) found that tritiated diaminopimelic acid, a known constituent of bacterial cell walls, failed to yield satisfactory radioautograms whereas radiograms from compounds containing labeled carbon with counts considerably less pronounced than tritium produced noticeable darkening on the X-ray film used for radiography. This is no doubt a reflection of the low penetration power of the β-rays emitted by tritium.

Quantitative studies have shown that for each mole of coumarin oxidized, six moles of CO_2 are released, and six moles of oxygen are consumed. Although the tritium content of the cells was not assessed, this indicates that assimilation is occurring, for no by-products were formed in appreciable amounts.

Table XI shows that total carbonate formed is considerably in excess of the theoretical amount that could be formed from the coumarin added to the test system. In the fermentation of coumarin, endogenous CO_2 is given off by the organisms; in the wet-combustion of coumarin, carbon is added from trapped air as well as from the oxidation of the rubber gasket used to seal the combustion unit. However, these sources

of extraneous carbon serve merely as a diluent for carbonate derived from coumarin. Clearly, the total carbonate formed must be used to determine the total activity of the system.

CONCLUSIONS

1. Using tritiated coumarin as a substrate, melilotic acid and 2,3-dihydroxyphenylpropionic acid were shown to be radioactive; this confirms their role as intermediates in the metabolism of coumarin. An unidentified monohydroxyphenolic compound was also found to be radioactive. An unidentified dihydroxyphenyl compound could not be assessed for radioactive content because of interfering residual coumarin.

2. One and possibly two unidentified non-ether soluble intermediates have been detected.

3. Ether extraction of fermentation liquor treated to remove protein was a satisfactory method for the concentration of DSA-positive substances.

4. For each mole of coumarin utilized, six moles of CO_2 were liberated.

5. When coumarin-2-C^{14} was used as a substrate, 94.5% of the radioactivity was recovered as CO_2.

GENERAL DISCUSSION

Bellis, using two Penicillium spp., isolated 4-hydroxycoumarin in fermentation liquor containing o-coumaric acid as a sole carbon source; small quantities of dicoumarol were also detected. He suggested that o-coumaric acid and not coumarin itself was converted to dicoumarol. In the present work, neither 4-hydroxycoumarin, no dicoumarol, nor o-coumaric acid was detected using sensitive chromatographic methods, nor was 4-hydroxycoumarin utilized by Mac 291 under manometric conditions, i.e., no evidence was obtained to suggest any of the Pseudomonas cultures plays a role in the formation of dicoumarol in spoilt sweet clover hay.

The first step in the β-oxidation mechanism outlined by Knoop is a reduction across the α - β carbons to form an alkene; the identification of melilotic acid as an intermediate constitutes an exact reversal of the β-oxidation mechanism. On this basis, a β-oxidation mechanism was not considered initially. However, this view does not hold should a multiple pathway exist.

The side chain of melilotic acid was originally thought to be the site of biological attack, and that each carbon atom of the side chain was removed successively with the resultant formation of catechol. No evidence was obtained to favour this mechanism; in fact, evidence contrary to this view was found. The mechanism through which terminal decarboxylation occurs involves oxidation through the corresponding alcohol, aldehyde, acid, and then decarboxylation. All of the compounds

tested which are obligatory intermediates if terminal decarboxylation occurs, namely o-cresol, saligenin, and salicylaldehyde failed to react favourably under test conditions. None of these compounds was detected using chromatographic methods. Although o-ethylphenol was not tested manometrically, it too was absent from chromatograms. On the basis of inadequate evidence, the proposed pathway of successive decarboxylation of the terminal carbon of the side chain was ruled out. This does not, however, eliminate a single step decarboxylation.

Should a multiple pathway exist, and β-oxidation occur with the formation of acetyl-coA, one could expect some assimilation of the C_2 and C_3 carbons of coumarin to occur. Of major significance was the almost complete recovery as CO_2 of the number two carbon of coumarin. This strongly suggests that β-oxidation plays only a minor role in coumarin degradation, and that following lactone cleavage, most of the number two carbon is removed via a decarboxylation process.

Is one justified in stating that a β-oxidation mechanism is present? Several pieces of evidence collectively affirm this view. The presence of adaptive enzymes for both salicylic acid and catechol was demonstrated; lag periods which were present when cells were grown on glucose disappeared when cells were grown on coumarin. Salicylic acid, when used as a substrate for growth, increased the cell density and underwent a considerable decrease in quantity over a period of 48 hours. The extent of catechol oxidation was also too large to be excused lightly. And as well, the failure of complete recovery of the number two carbon of coumarin is in keeping with a β-oxidation process. As well, other

workers (Bhat et al., 1959) have shown that salicylic acid does not cross the cell membrane with ease. β -Oxidation of o-hydroxy-cis-cinnamic acid has been demonstrated in many soil organisms (Evans, 1961). These arguments are in favour of a β -oxidation mechanism although the contribution of this pathway to overall coumarin decomposition is probably very small. A more detailed investigation with cell-free preparations of Mac 291 could perhaps bring a more decisive outlook on this phase of coumarin degradation.

Booth et al. (1959) reported the formation in rats and rabbits of o-hydroxyphenylhydracrylic acid from o-coumaric acid; this is the first reaction of the β -oxidation mechanism. β -Oxidation did not go to completion, for neither the corresponding keto acid nor salicylic acid was found. Booth suggested that o-hydroxyphenylhydracrylic acid is the precursor of 4-hydroxycoumarin; no 4-hydroxycoumarin was formed by Mac 291. The charge densities of the carbon atoms of coumarin listed by Mead et al. (1958) show that position four of coumarin possesses the lowest density and is the most likely site for hydroxylation to occur. Since the production of 4-hydroxycoumarin by Mac 291 was not shown, it appears improbable that, unlike mammalian metabolism, hydroxylations at higher charge densities occur.

Kaighen et al. (1961) have found that o-hydroxyphenylpyruvic acid is quite unstable, and on keeping for 2-3 days after preparation formed the anhydride, 3-hydroxycoumarin, and the decarboxylation product, o-hydroxyphenylacetic acid. Since the latter compound was not detected on chromatograms, nor was it oxidized manometrically, this mechanism

probably does not occur in Mac 291.

While an intensive study was carried out only with one organism, similarities or differences in the remaining cultures could be established readily. It is unlikely, but not impossible, that metabolic differences in the decomposition of coumarin occur, for all cultures responded very similarly in nutritional studies.

While previous reports (Nickerson, 1956; Happold, 1950) have suggested that the metabolism of aromatic compounds is confined to limited pathways, it is now apparent that diversity as outlined by Dagley et al. (1960) for the rupture of aromatic rings also applies to steps leading to the formation of those compounds upon which ring rupture occurs. Benzoic acid may be metabolized to catechol by a single step reaction (Sleeper, 1951), or through salicylic acid and then through to catechol (Bhat et al., 1959). m-Hydroxybenzoic acid may be metabolized either through gentisic acid, or through protocatechuic acid (Yano and Arima, 1958). Cinnamic acid may be metabolized through either dihydrocinnamic acid (Whiting and Carr, 1959), melilotic acid (Coulson and Evans, 1959), or phloretic acid (Farmer et al., 1959). Strikingly, phenylalanine may be metabolized to homogentisic acid through one of two distinct routes in the same organism (Dagley et al., 1953). Scrutiny of metabolic maps shows many such choices exist. Investigators fail usually to eliminate possible alternative pathways in intermediate studies once one pathway has been established. Of interest would be a study of the efficiency of different pathways in terms of energy gain by the cell. Such a discussion, though provocative, is beyond the scope of this work.

Booth et al. (1959) suggested that o-hydroxyphenylacetic acid was formed from coumarin by two distinct routes, one pathway from coumarin directly, and the second through o-coumaric acid, o-hydroxyphenyllactic acid, o-hydroxyphenylpyruvic acid, and then to o-hydroxyphenylacetic acid. The major route proceeds from coumarin directly, for greater amounts of o-hydroxyphenylacetic acid were excreted after ingestion of coumarin than o-coumaric acid. No intermediates were suggested to account for this quantitative difference. However, if hydration across the double bond occurred prior to lactone ring cleavage, greater amounts of o-hydroxyphenylacetic acid would be formed because the o-coumaric acid step would be by-passed.

How does one account for the slight oxidation of o-cresol, saligenin, and salicylaldehyde? Perhaps it is due to a low degree of specificity of enzyme action. While the high specificity of the majority of enzymes is one of their unique properties, many enzymes are known which are specific for only a small portion of a molecule (Dixon and Webb, 1958). Unfortunately, the characterization of enzymes responsible for the transformation of aromatic compounds, with the exception of those involved in direct ring cleavage, has been neglected. Aromatic hydroxylation involving atmospheric oxygen could occur independent of the side chain structure. Should no further modification occur, the formation of a chemical analogue, or competitive inhibitor may arise which could interfere with further enzyme action. This may account for the rather abrupt termination of the oxidation of salicylaldehyde that was encountered, and for the extremely low rate of oxidation of other compounds.

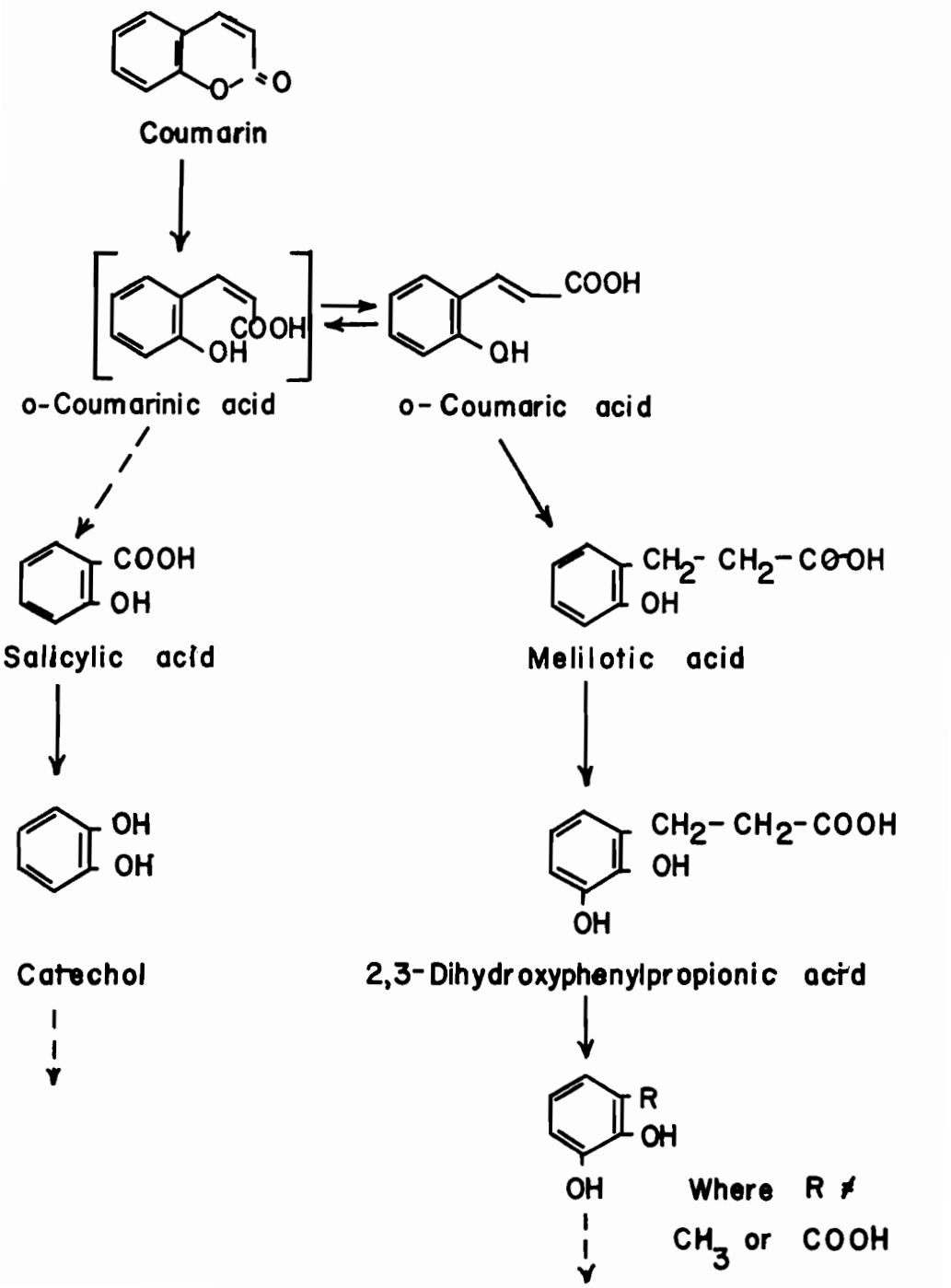
Whether the site of ring rupture is adjacent to or across the hydroxyl group is not known, but the lack of detectable aldehyde groups suggests the latter. On the other hand, 2,3-dihydroxyphenylpropionic acid and the second unidentified dihydroxyphenolic compound were present in such small amounts that semi-aldehydes would not be expected to accumulate to detectable levels. The actual site of aromatic cleavage, then, awaits further study.

The conversion of coumarin to 2,3-dihydroxyphenylpropionic acid has been demonstrated conclusively. What of the fate of this 'catechol-substance'? The detection of at least one additional dihydroxyphenolic compound, though in very small amounts, suggests that some side chain modification occurs prior to ring rupture. However, the degree of decarboxylation does not extend beyond a single carbon; neither 2,3-dihydroxybenzoic acid, nor 3-methylcatechol responded favourably under test conditions, and both standards were chromatographically distinct from fermentation products. A C_6-C_2 dihydroxyphenolic compound may be involved in ring rupture, with the cleavage occurring across the hydroxyl groups as in the case of catechol or protocatechuic acid. Although a mechanism of this nature has never been encountered, it offers the most fruitful path to pursue.

GENERAL SUMMARY

Nine organisms, all members of the genus Pseudomonas, were tested for differences in the rate of breakdown of coumarin. All organisms responded very similarly to changes in the test conditions. However, some of the cultures degraded coumarin most rapidly at 30° C while others favoured 37° C. High rates of aeration enhanced utilization in all cultures. In the only strain tested (Mac 291) an alkaline pH slightly favoured the utilization of coumarin. Unidentified growth factors were required by all organisms for satisfactory rates of coumarin utilization and of several protein hydrolysates tested, 0.5% peptone best satisfied the growth factor requirements of Mac 291 although 0.3% peptone was almost as good. Under optimum conditions 0.25% coumarin was utilized in 18 hours.

The following scheme for the decomposition of coumarin, based on manometric, chromatographic, and radioactive evidence is proposed:


Coumarin
o-Coumarinic acid
o-Coumaric acid
Salicylic acid
Melilotic acid
Catechol
2,3-Dihydroxyphenylpropionic acid
Where R ≠ CH3 or COOH

The pathway through β-oxidation however plays only a minor role in total decomposition.

The following compounds were eliminated as intermediates in the decomposition of coumarin by Mac 291: benzene, benzoic acid, cinnamic acid, o-cresol, 2,3-dihydroxybenzoic acid, hydrocinnamic acid, 4-hydroxycoumarin, o-hydroxyphenylacetic acid, mandelic acid, 3-methylcatechol, phenol, protocatechuic acid, shikimic acid, saligenin, salicylaldehyde, and toluene.

Benzoic acid is metabolized through protocatechuic acid by Mac 291.

The following quantitative data were obtained with resting cell suspensions of Mac 291:

(a) The oxidation of coumarin, o-coumaric acid and 2,3-dihydroxyphenylpropionic acid requires 6.0 moles oxygen per mole substrate, while melilotic acid requires 6.5 moles oxygen per mole substrate.

(b) The oxidation of benzoic acid to protocatechuic acid requires 1.0 moles oxygen per mole substrate.

(c) In the complete oxidation of coumarin, o-coumaric acid, melilotic acid, 2,3-dihydroxyphenylpropionic acid, benzoic acid and protocatechuic acid, a sufficient quantity of oxygen was consumed to account for the oxidation of only 60-65% of the substrate to CO_2 and H_2O. In the oxidation of catechol approaching completion, a sufficient quantity of oxygen was consumed to account for only 26% of the substrate being oxidized to CO_2 and H_2O.

(d) For each mole of coumarin utilized, six moles of CO_2 are liberated.

(e) When coumarin-2-C^{14} was used as a substrate, 94.5% of the radioactivity was recovered as CO_2.

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APPENDIX TABLES I - X

APPENDIX TABLE I. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 289).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.8	7.4	7.3	7.3	4	36	60		1216	413	77
	30° C	6.8	7.6	7.4	7.4	21	58	68		1064	209	87
	37° C	6.8	7.6	7.6	7.9	24	72	79		1104	219	91
	45° C	6.8	7.0	6.7	6.2	0	0	0		1473		
Aeration **	20 ml/125 ml flask (shaken)	6.8	8.2	8.7	8.3	30	54	64		656	22	100
	40 ml/125 ml flask (shaken)	6.8	7.6	8.5	8.4	18	43	80		1817	48	64
	70 ml/125 ml flask (shaken)	6.8	7.6	7.5	8.2	9	33	42		1853	535	67
	100 ml/125 ml flask (shaken)	6.8	7.6	7.5	7.5	8	17	36		1722	1454	
	100 ml/125 ml flask (static)	6.8	7.4	7.1	7.0	4	15	26		1332	522	605
Nutrition **												
CYT medium + 1.00% glucose		6.8	5.4	4.8	4.6	30	36	36		1639	1589	1674
CYT medium + 0.10% glucose		6.8	7.6	8.4	8.4	20	48	63		1734	42	96
CYT medium + 0.01% glucose		6.8	7.8	8.5	8.4	20	42	71		1603	24	70
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	I	6.8	7.3	8.3	8.7	11	54	51	3126	-	100	109
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	II	7.8	7.5	8.6	8.8	12	50	57	3026	2351	95	44
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts	I	7.0	7.3	7.8	8.5	7	52	51	3050	-	70	95
Coumarin - + Y.E. (Difco) + Min. Salts	I	6.6	7.4	8.2	8.5	7	36	33	2978	-	81	96
Coumarin - + Y.E. (BYF) + Min. Salts	I	6.6	7.2	7.8	8.1	5	48	57	2831	-	29	27
Coumarin + Tryptone - + Min. Salts	I	6.8	7.0	7.4	7.9	7	38	41	2978	-	29	25
Coumarin - - + Min. Salts	I	6.7	6.7	6.6	6.7	0	0	0				
Coumarin + Min. Salts	III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE II. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 290).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.5	7.5	7.3	7.0	3	33	54	3000	1119	192	166
	30° C	6.8	7.9	7.4	7.8	25	59	71	"	1050	162	86
	37° C	6.8	7.8	7.6	7.6	28	60	80	"	898	105	82
	45° C	6.8	7.0	6.5	6.2	0	0	0	"	1335	1247	
Aeration **	20 ml/125 ml flask (shaken)	6.8	8.8	8.3	8.2	30	64	69	"	323	57	78
	40 ml/125 ml flask (shaken)	6.8	7.9	8.3	8.3	23	44	57	"	1769	21	72
	70 ml/125 ml flask (shaken)	6.8	8.0	7.8	8.3	12	32	41	"	1900	597	76
	100 ml/125 ml flask (shaken)	6.8	7.9	7.4	7.6	7	15	30	"	1810	1876	67
	100 ml/125 ml flask (static)	6.8	7.5	7.2	7.1	6	20	33	"	1128	1537	745
Nutrition ** *												
CYT medium + 1.00% glucose		6.8	5.5	4.4	4.7	30	55	39	"	1496	1532	1413
CYT medium + 0.10% glucose		6.8	7.8	8.2	8.4	23	48	66	"	1781	352	67
CYT medium + 0.01% glucose		6.8	8.0	8.2	8.6	24	42	60	"	1591	266	72
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	I	6.8	7.4	8.3	8.9	11	57	49	3126	-	85	91
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	II	7.8	7.7	8.5	8.9	11	59	57	3026	2565	175	148
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts	I	7.0	7.5	7.9	8.7	9	54	56	3050	-	48	62
Coumarin - + Y.E. (Difco) + Min. Salts	I	6.6	7.4	8.0	8.4	10	36	33	2978	-	77	77
Coumarin - + Y.E. (BYF) + Min. Salts	I	6.6	7.3	7.9	7.9	10	49	48	2931	-	-	29
Coumarin + Tryptone - + Min. Salts	I	6.8	6.8	7.2	7.4	10	43	42	2978	-	28	25
Coumarin + - - Min. Salts	I	6.7	6.6	6.6	6.6	0	0	0	3020	-	-	-
Coumarin + - - Min. Salts	III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE III. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 291).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.8	7.5	7.2	7.2	10	44	57		1473	442	276
	30° C	6.8	7.5	7.3	7.5	28	58	84		1183	40	76
	37° C	6.8	7.6	7.9	8.2	25	73	83		1316	29	76
	45° C	6.8	6.6	6.5	6.2	0	0	0		1610		
Aeration	20 ml/125 ml flask (shaken)	6.8	8.5	8.0	8.4	30	62	60		64	28	77
	40 ml/125 ml flask (shaken)	6.8	7.7	8.6	8.5	20	53	58		1532	26	76
	70 ml/125 ml flask (shaken)	6.8	7.5	7.8	8.5	11	30	51		2007	156	70
	100 ml/125 ml flask (shaken)	6.8	7.5	7.6	7.7	8	16	38		2161	1418	352
	100 ml/125 ml flask (static)	6.8	7.0	7.2	7.1	6	18	29		1841	1846	874
Nutrition * **												
CYT medium + 1.00% glucose		6.8	5.1	4.5	4.6	30	36	41		1829	1995	1579
CYT medium + 0.10% glucose		6.8	7.6	8.0	8.5	22	38	73		1817	28	72
CYT medium + 0.01% glucose		6.8	7.6	8.0	8.5	18	52	63		1781	38	72
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts I		6.8	7.4#	8.4	8.7	18	57	65	3126	2375	80	100
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts II		7.8	7.5#	8.5	8.9	21	59	69	3026	2185	76	82
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts I		7.0	7.2#	8.0	8.6	10	66	63	3050	2328	38	41
Coumarin - + Y.E. (Difco) + Min. Salts I		6.6	7.1#	8.1	8.3	12	40	33	2978	2280	70	95
Coumarin - + Y.E. (BYF) + Min. Salts I		6.6	7.0#	7.8	7.3	5	43	51	2931	1876	24	25
Coumarin + Tryptone - + Min. Salts I		6.8	6.3#	7.2	7.4	14	39	38	2978	1297	18	20
Coumarin - - + Min. Salts I		6.7	6.5#	6.5	6.6	0	0	0	3002	-	-	-
Coumarin + Min. Salts III		7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

\# 24 hours.

APPENDIX TABLE IV. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 292).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.8	7.2	7.1	7.2	13	55	68		1275	413	86
	30° C	6.8	7.0	7.1	7.4	28	66	65		1168	234	100
	37° C	6.8	7.3	7.6	7.5	23	46	57		1427	798	189
	45° C	6.8	6.4	6.4	6.3	0	0	0		1499	1734	1568
Aeration **	20 ml/125 ml flask (shaken)	6.8										
	40 ml/125 ml flask (shaken)	6.8										
	70 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (static)	6.8										
Nutrition * **												
CYT medium + 1.00% glucose		6.8	5.3	5.0	5.0	17	33	24			1805	1805
CYT medium + 0.10% glucose		6.8	7.2	7.7	7.7	12	39	63		1876	76	62
CYT medium + 0.01% glucose		6.8	7.2	7.7	7.8	10	45	63		1971	87	64
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	I	6.8	7.4	8.2	8.6	10	53	53	3126	X	85	86
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts	II	7.8	7.4	8.6	8.9	11	57	57	3026	X	76	89
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts	I	7.0	7.4	8.2	8.5	11	56	54	3050	X	41	119
Coumarin - + Y.E. (Difco) + Min. Salts	I	6.6	7.3	8.2	8.4	8	33	30	2978	X	74	81
Coumarin - + Y.E. (BYF) + Min. Salts	I	6.6	7.2	7.9	8.0	9	42	41	2931	X	27	33
Coumarin + Tryptone - + Min. Salts	I	6.8	6.8	7.5	7.5	8	10	39	2978	X	37	30
Coumarin - - + Min. Salts	I	6.7	6.5	6.7	6.5	0	0	0	3002	X	X	X
Coumarin - - + Min. Salts	III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE V. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 293).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.8	6.8	7.1	7.1	4	39	62		1406		83
	30° C	6.8	6.9	7.0	7.4	17	69	72		974	157	100
	37° C	6.8	7.2	7.3	7.8	21	52	66		1351	223	98
	45° C	6.8	6.4	6.4	6.3	0	0	0		1456		
Aeration **	20 ml/125 ml flask (shaken)	6.8										
	40 ml/125 ml flask (shaken)	6.8										
	70 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (static)	6.8										
Nutrition * **												
CYT medium / 1.00% glucose		6.8	5.5	5.1	5.0	11	26	26		1757	2019	1781
CYT medium / 0.10% glucose		6.8	7.2	7.6	7.8	10	45	69		1829	74	65
CYT medium / 0.01% glucose		6.8	7.1	8.0	7.7	11	35	54		1665	57	52
Coumarin / Tryptone / Y.E. (Difco) / Min. Salts	I	6.8	7.4	8.4	8.6	11	50	50	3126	X	77	71
Coumarin / Tryptone / Y.E. (Difco) / Min. Salts	II	7.8	7.6	8.6	8.9	15	45	59	3026	X	119	76
Coumarin / Tryptone / Y.E. (BYF) / Min. Salts	I	7.0	7.4	8.2	8.6	9	58	57	3050	X	42	42
Coumarin - / Y.E. (Difco) / Min. Salts	I	6.6	7.3	8.3	8.5	9	33	33	2978	X	69	63
Coumarin - / Y.E. (BYF) / Min. Salts	I	6.6	7.3	8.0	8.0	7	48	41	2931	X	26	67
Coumarin / Tryptone - / Min. Salts	I	6.8	6.8	7.4	7.6	14	33	36	2978	X	18	15
Coumarin - - / Min. Salts	I	6.7	6.5	6.4	6.5	0	0	0	3002	X	X	X
Coumarin - - / Min. Salts	III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE VI. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 294).

Growth conditions		pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
		0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * **	20° C	6.8	6.9	7.3	7.6	6	66	75		1584		87
	30° C	6.8	7.0	7.3	7.6	23	70	75		1432	292	128
	37° C	6.8	7.4	7.8	8.3	29	90	86		1226	444	105
	45° C	6.8	6.4	6.4	6.3	0	0	0		1563		
Aeration **	20 ml/125 ml flask (shaken)	6.8										
	40 ml/125 ml flask (shaken)	6.8										
	70 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (shaken)	6.8										
	100 ml/125 ml flask (static)	6.8										
Nutrition * **												
CYT medium ∕ 1.00% glucose		6.8	5.2	5.2	5.1	19	28	24		1758	2185	1971
CYT medium ∕ 0.10% glucose		6.8	7.1	7.7	8.3	12	45	48		2066	365	70
CYT medium ∕ 0.01% glucose		6.8	7.0	8.0	8.0	12	38	48		1853	42	67
Coumarin ∕ Tryptone ∕ Y.E. (Difco) ∕ Min. Salts	I	6.8	7.2#	8.4	8.7	15	56	62	3126	2518	76	77
Coumarin ∕ Tryptone ∕ Y.E. (Difco) ∕ Min. Salts	II	7.8	7.5#	8.1	8.8	14	57	58	3026	2423	81	72
Coumarin ∕ Tryptone ∕ Y.E. (BYF) ∕ Min. Salts	I	7.0	7.2#			11			3050	2280		
Coumarin - ∕ Y.E. (Difco) ∕ Min. Salts	I	6.6	7.0#	8.1	8.2	12	39	39	2978	2361	75	86
Coumarin - ∕ Y.E. (BYF) ∕ Min. Salts	I	6.6	6.9#	7.9	7.8	10	54	51	2931	2109	28	24
Coumarin ∕ Tryptone - ∕ Min. Salts	I	6.8	6.4#	7.4	7.6	12	39	41	2978	1772	24	24
Coumarin - - ∕ Min. Salts	I	6.7	6.5#	6.6	6.6	0	0	0	3002			
Coumarin - - ∕ Min. Salts	III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

\# 24 hours.

APPENDIX TABLE VII. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 1395a)

Growth conditions	pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
	0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * ** 20° C	6.8	6.7	7.2	7.2	-	33	48			1127	1100
30° C	6.8	7.1	7.5	7.5	9	60	69		1418	1183	215
37° C	6.8	7.2	7.6	7.7	11	69	69		1349	961	202
45° C	6.8	6.6	6.6	6.5	0	0	0		1351	1363	1539
Aeration ** 20 ml/125 ml flask (shaken)	6.8	7.5	8.9	8.4	32	63	78		1381	89	104
40 ml/125 ml flask (shaken)	6.8	7.4	8.3	8.6	12	60	57		1758	79	108
70 ml/125 ml flask (shaken)	6.8	7.3	7.7	7.7	7	36	44		1853	2043	551
100 ml/125 ml flask (shaken)	6.8	7.2	7.4	7.6	6	26	29		2043	1741	1720
100 ml/125 ml flask (static)	6.8	7.0	7.4	7.2	3	24	30		1551	1473	1473
Nutrition * **											
CYT medium + 1.00% glucose	6.8	5.6	5.4	5.2	17	35	33		1805	1876	1995
CYT medium + 0.10% glucose	6.8	7.2	8.3	8.6	11	69	60		2185	64	70
CYT medium + 0.01% glucose	6.8	7.6	8.5	8.5	13	57	62		2209	180	95
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts I	6.8	7.6#	8.1	8.5	15	42	45	3126	2166	103	72
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts II	7.8	7.8#	8.2	8.6	11	36	44	3026	2228	297	83
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts I	7.0	7.3#	8.0	8.3	5	52	65	3050	2038	122	87
Coumarin - + Y.E. (Difco) + Min. Salts I	6.6	7.3#	8.4	8.1	11	29	33	2978	2123	74	95
Coumarin - + Y.E. (BYF) + Min. Salts I	6.6	7.2#	8.1	7.9	9	45	45	2931	1824	28	24
Coumarin + Tryptone - + Min. Salts I	6.8	7.0#	7.6	7.8	9	29	32	2978	1663	21	24
Coumarin - - + Min. Salts I	6.7	6.5#	6.6	6.6	0	0	0	3002			
Coumarin - - + Min. Salts III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

\# 24 hours.

APPENDIX TABLE VIII. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 295b).

Growth conditions	pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
	0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * ** 20° C	6.8	7.0	7.4	7.1	-	45	78			1441	363
30° C	6.8	7.2	7.7	7.7	12	62	90		1587	496	110
37° C	6.8	7.5	7.9	7.5	11	33	74		1610	1266	309
45° C	6.8	6.6	6.7	6.5	0	0	0		1971	2066	2054
Aeration ** 20 ml/125 ml flask (shaken)	6.8	7.7	8.8	8.6	62	60	68		1741	82	95
40 ml/125 ml flask (shaken)	6.8	7.5	8.5	8.6	14	54	54		1786	68	133
70 ml/125 ml flask (shaken)	6.8	7.4	7.7	7.7	7	24	48		2161	2102	710
100 ml/125 ml flask (shaken)	6.8	7.3	7.5	7.5	6	12	30		2114	1964	2019
100 ml/125 ml flask (static)	6.8	7.0	7.4	7.4	4	9	24		1710	1781	1798
Nutrition *											
CYT medium + 1.00% glucose	6.8	5.8	5.4	5.1	30	32	36		2325	2769	2280
CYT medium + 0.10% glucose	6.8	7.6	8.3	8.5	15	54	59		1275	541	58
CYT medium + 0.01% glucose	6.8	7.7	8.3	8.6	15	54	57		1294	65	190
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts I	6.8	7.4	8.3	8.4	17	59	57	3126	2598	95	128
Coumarin + Tryptone + Y.E. (Difco) + Min. Salts II	7.8	7.7	8.3	8.7	17	57	60	3026	2522	108	82
Coumarin + Tryptone + Y.E. (BYF) + Min. Salts I	7.0	7.4	7.8	8.4	14	68	54	3050	2437	51	40
Coumarin - + Y.E. (Difco) + Min. Salts I	6.6	7.2	8.0	8.2	13	42	32	2978	2366	70	68
Coumarin - + Y.E. (BYF) + Min. Salts I	6.6	7.1	7.9	7.8	9	54	42	2931	2157	24	24
Coumarin + Tryptone - + Min. Salts I	6.8	6.4	7.4	7.5	13	39	36	2978	1696	19	43
Coumarin - - + Min. Salts I	6.7	6.5	6.6	6.7	0	0	0	3002			
Coumarin - - + Min. Salts III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE IX. The effect of alterations in temperature, aeration, and nutrition on the utilization of coumarin by a Pseudomonas sp. (Mac 296).

Growth conditions	pH				Turbidity (Coleman nephelos units x 10^2/ml)			Coumarin conc., ugm/ml			
	0 hrs	18 hrs	48 hrs	72 hrs	18 hrs	48 hrs	72 hrs	0 hrs	18 hrs	48 hrs	72 hrs
Temperature * ** 20° C	6.8	6.7	7.3	7.3	2	6	48		1715	1472	1100
30° C	6.8	7.2	7.5	7.5	10	57	67		1596	981	215
37° C	6.8	7.4	7.7	7.7	11	60	69		1777	988	202
45° C	6.8	6.7	6.6	6.5	0	0	0		1596	1876	2138
Aeration ** 20 ml/125 ml flask (shaken)	6.8	7.5	8.9	8.4	81	75	78		1698	70	104
40 ml/125 ml flask (shaken)	6.8	7.5	8.7	8.6	10	57	57		1995	63	108
70 ml/125 ml flask (shaken)	6.8	7.3	7.7	7.7	6	18	44		1793	1351	412
100 ml/125 ml flask (shaken)	6.8	7.2	7.5	7.6	4	12	29		2161	1921	1853
100 ml/125 ml flask (static)	6.8	7.3	7.5	7.2	4	24	30		2066	1980	1480
Nutrition *											
CYT medium ≠ 1.00% glucose	6.8	5.7	5.5	5.2	30	30	33		2185	2256	1995
CYT medium ≠ 0.10% glucose	6.8	7.4	8.4	8.6	14	63	60		2470	68	70
CYT medium ≠ 0.01% glucose	6.8	7.0	8.7	8.5	11	27	62		2233	85	95
Coumarin ≠ Tryptone ≠ Y.E. (Difco) ≠ Min. Salts I	6.8	7.3	8.5	8.4	16	54	51	3126	2399	80	88
Coumarin ≠ Tryptone ≠ Y.E. (Difco) ≠ Min. Salts II	7.8	7.6	8.7	8.5	15	57	63	3026	2516	167	82
Coumarin ≠ Tryptone ≠ Y.E. (BYF) ≠ Min. Salts I	7.0	7.1	8.4	8.3	15	66	60	3050	2470	44	71
Coumarin - ≠ Y.E. (Difco) ≠ Min. Salts I	6.6	7.0	8.2	8.1	13	39	35	2978	2446	85	43
Coumarin - ≠ Y.E. (BYF) ≠ Min. Salts I	6.6	7.1	7.7	7.9	12	48	41	2931	1900	59	24
Coumarin ≠ Tryptone - ≠ Min. Salts I	6.8	6.6	7.1	7.4	14	38	36	2978	1663	42	44
Coumarin - - ≠ Min. Salts I	6.7	6.6	6.4	6.6	0	0	0	3002			
Coumarin - - ≠ Min. Salts III	7.0				0						

* Grown under static conditions.

** 40 ml CYT medium/125 ml flask.

APPENDIX TABLE X. Formation of 'catechol-substance' from coumarin by a Pseudomonas sp. (Mac 291).

Time, hrs	Coumarin conc., ugm/ml	'Catechol-substance', ugm/ml*
0	3000	0
8	-	0
9	-	0
10	1924	15
12	-	14
14	1924	92
16	1948	171
18	1948	181
20	1781	211
22	1306	199
24	684	268
26	855	287
28	352	351
30	109	306
32	66	103
36	18	13

* Color-equivalence of catechol.