THE METABOLISM OF COUMARIN AND O-COUMARIC ACID BY SOIL FUNGI

by

Hang-shan Shieh, M. S_c.

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Department of Agricultural Bacteriology,
McGill University,
Montreal.

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Hang-shan Shieh

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ABSTRACT

Moulds belonging to the Fusarium genus were isolated that are able to utilize coumarin as the sole carbon source in a medium containing inorganic nitrogen and various salts. Factors affecting the growth of the cultures and the rate of coumarin degradation were studied. The intermediate products were identified by paper chromatography and by isolation and purification using both cell suspensions and cell free extracts. The major route for decomposition of coumarin was through dihydrocoumarin to melilotic acid. The dihydrocoumarin hydrolase was isolated and partially purified with very high enzymatic activity but the dehydrogenase preparations had low enzymatic activity.

These Fusarium sp. can utilize also o-coumaric acid as the sole carbon source. The only product isolated from the metabolism of o-coumaric acid was 4-hydroxycoumarin. This latter compound accumulated and was not further metabolized under the test conditions although with added formaldehyde non-enzymatic conversion to dicumarol was found.

These fungi metabolize coumarin and o-coumaric acid differently than reported for bacteria and animals but degrade coumarin by the same route as reported for plants.

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INTRODUCTION

Coumarin is a naturally occuring constituent of sweet clover (Melilotus alba) but is found also in a large variety of other plants. Derivatives of coumarin are used widely as a flavor ingredient in foods, drugs, rodenticides and insecticides. Dicumarol is formed in improperly cured hay and causes a hemorrhagic condition in cattle. Dicumarol (trade name: menadione) is used medically as an anticoagulant. Link et al (163) believed that dicumarol was formed from coumarin because of its common structural relationship.

The metabolism of coumarin and related compounds in higher plants has been extensively studied by Kosuge and Conn (101, 102), and in the animal body by many authors. However, only a few reports on microbial activity on coumarin and related compounds are published. Following studies initiated previously in this laboratory on the degradation of coumarin by bacteria, an attempt is made in this investigation to isolate other microorganisms that utilize coumarin and closely related compounds. A particular interest was the investigation of the biological routes by which coumarin may be transformed into dicumarol.

GENERAL REVIEW OF LITERATURE

The degradation of aromatic compounds by living cells has received increasing interest in recent years. These compounds include natural products, industrial wastes, and synthetic compounds. With modern techniques, the studies, consisting mainly in isolation of intermediates and investigation of induced enzyme patterns, have yielded some fairly definite knowledge about the way in which ring compounds are metabolized.

METABOLISM OF POLYNUCLEAR AROMATIC COMPOUNDS

Metabolism of polynuclear aromatic compounds by bacteria: Fernley and Evans (57) studied the degradation of naphthalene by a soil pseudomonad. They isolated coumarin, and detectable amounts of o-hydroxy-trans-cinnamic acid (o-coumaric acid), o-hydroxyphenylpropionic acid (melilotic acid), and salicylic acid. The naphthalene-grown cells were sequentially induced to oxidize trans-1,2-dihydro-1,2-dihydroxynaphthalene, 1,2-dihydroxy-naphthalene, and salicylic acid; oxygen uptake was observed on coumarin, o-coumaric acid, and melilotic acid, but the rates were not sufficiently high to warrant assumption of their roles as intermediates. Experiments with a crude cell free extract of naphthalene-grown cells demonstrated that during the oxidation of 1,2-dihydroxynaphthalene approximately 3 moles of oxygen were consumed and 1 mole of carbon dioxide was liberated;

coumarin was the major product isolated.The gas exchange data are in agreement with the view that o-hydroxy-cis-cinnamic acid (isolated as coumarin)is produced by oxidative decarboxylation of the postulated precursor o-carboxy-cis-cinnamic acid. Negative results were obtained by resting cells tested on the two o-carboxycinnamic acids which had the trans-configuration. The cells were also inactive on o-carboxyphenyl-propionic acid, phthalide, acetic acid, o-carboxybenzolacetic acid, and phthalic acid. The scheme for degradation of naphthalene by soil bacteria proposed by Fernley and Evans is shown in Fig. 1.

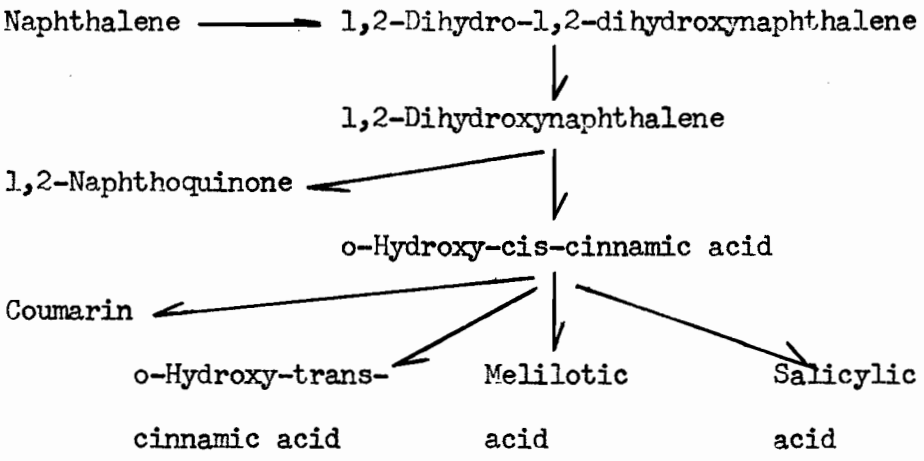


Fig. 1. Degradation of naphthalene by soil bacteria.

Murphy and Stone (115) studied the bacterial dissimilation of naphthalene also. The major pathway found for the oxidation of naphthalene by a Pseudomonas sp. was via salicyclic acid, which is further oxidized through catechol to β-ketoadipic acid. Evidence was found that a second

pathway of naphthalene oxidation produces 1,2-naphthoquinone. The 1,2-naphthoquinone was not further metabolized and was found to be responsible for the characteristic brown to reddish-orange colour of the culture medium.

Rogoff and Wender (131), with a pseudomonad isolated from soil, identified methylsalicylic acids produced from the oxidation of methylnaphthalenes. Sequential induction experiments demonstrated that the methylsalicylic acids and their respective methylcatechols were intermediates in the methylnaphthalene oxidations. A point of interest in these studies is that cells grown on naphthalene, or on either of the methylnaphthalenes, were induced to oxidize salicylic acid, catechol, and their methyl derivatives and suggests that the same enzymes elaborated for oxidation of the parent compounds (salicylic acid, catechol) are involved in the oxidation of the methyl derivatives; the presence of the methyl group on the benzene nucleus apparently does not confer a specificity requirement for the enzymes involved. When the naphthalene nucleus bears a chlorine substituent in place of a methyl group the characteristics of the oxidative pathway are not altered. Walker and Wiltshire (174) studied the degradation of 1-chloronaphthalene by a soil organism and identified 8-chloro-1,2-dihydro-1,2-dihydroxynaphthalene and 3-chlorosalicylic acid as intermediates in the oxidation.

Rogoff and Wender (134) found that a pseudomonad from soil could attack the end ring of phenanthrene. 1-Hydroxy-2-naphthoic acid was isolated from phenanthrene-oxidizing cultures and sequential induction experiments demonstrated that salicylic acid and catechol were also intermediates in this oxidative pathway. Similar experiments with an anthracene-oxidizing strain demonstrated end-ring attack on this compound with the formation of 3-hydroxy-2-naphthoic acid, and sequential induction of anthracene-grown cells to salicylic acid and catechol as shown also by Rogoff and Wender (133). The proposed scheme is shown in Fig. 2.

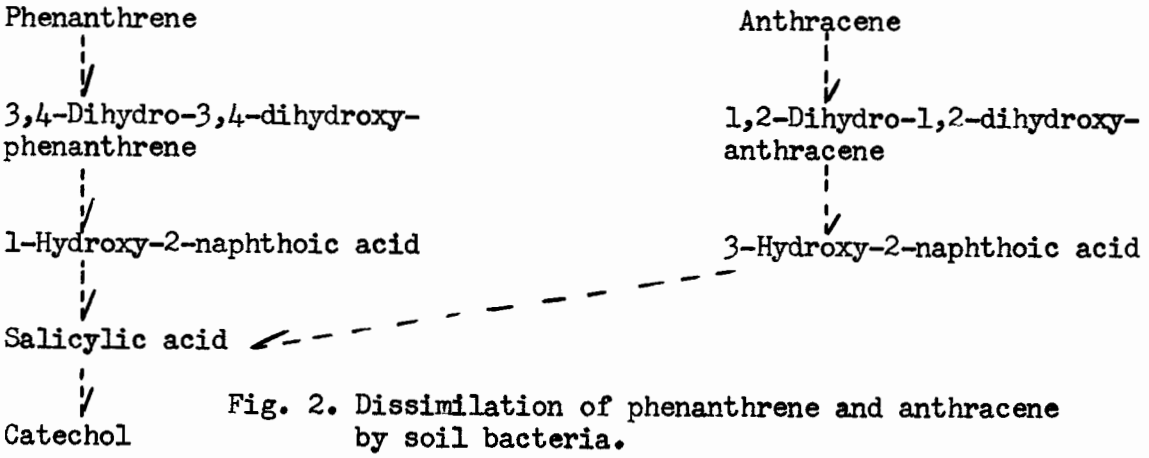


Fig. 2. Dissimilation of phenanthrene and anthracene by soil bacteria.

Metabolism of polynuclear aromatic compounds by fungi: Byrde, Downing and Woodcock (31) studied the metabolism of 2-methoxynaphthalene by Aspergillus niger. 4-Methoxysalicylic acid was isolated as a metabolic product from 2-methoxynaphthalene, and 4-ethoxysalicylic acid was isolated

similarly from 2-ethoxynaphthalene. Although the fungistatic activity of 4-methoxysalicylic acid was less than that of 2-methoxynaphthalene, 4-ethoxysalicylic acid was more active than the corresponding 2-ethoxynaphthalene.

Metabolism of polynuclear aromatic compounds by animals : Booth et al (19, 2o) found that rabbits convert naphthalene into 1-hydroxy-2-naphthyl sulphate, which is also formed when rabbits, rats and guinea pigs are treated with trans-1,2-dihydro-1,2-dihydroxynaphthalene. Slices of livers of male rats convert naphthalene into a compound which is almost certainly S-(1,2-dihydro-2-hydroxy-1-naphthyl) glutathione and which yields S-(1-naphthyl) glutathione when treated with acid at room temperature. Rat-kidney homogenates convert S-(1,2-dihydro-2-hydroxy-1-naphthyl) glutathione into a compound believed to be S-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine and which forms S-(1-naphthyl)-L-cysteine on treatment with acid at room temperature.

METABOLISM OF BENZENOID COMPOUNDS

Metabolism of benzenoid compounds by bacteria: Sleeper and Stanier (149) found that cells of Pseudomonas fluorescens grown on mandelate, or benzoate show complete adaptation to catechol, but not

to protocatechuic acid, whereas cells of P. fluorescens grown on p-hydroxybenzoate show complete adaptation to protocatechuic acid, but not to catechol.

The metabolism of benzoic acid was investigated by Gale (60, 61) in three nonpathogenic species of Mycobacteria. Two of the cultures oxidized benzoate through catechol but later steps were not the same as reported for certain pseudomonads (149). One culture did not oxidize benzoate but did attack catechol.

In studying the enzymatic conversion of mandelic acid to benzoic acid, Gunsalus et al (72, 73) reported that four soluble enzymes were found in cell free extracts prepared from mandelate-grown cells of Pseudomonas fluorescens. These are: mandelic acid racemase, which converts either isomer to a racemic mixture; benzoylformic carboxylase, which catalyzes a decarboxylation of benzoylformic acid to benzaldehyde and requires cocarboxylase for its activity; and two benzaldehyde dehydrogenases, which react respectively with diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide(TPN).

Bacterial oxidation of p-aminobenzoic acid by Pseudomonas fluorescens was studied by Durham (48). Cells of Pseudomonas fluorescens grown on p-aminobenzoic acid showed complete simultaneous adaptation to p-hydroxybenzoic acid and protocatechuic acid and in addition

protocatechuic acid was oxidized further to β-ketoadipic acid. Cells grown on p-hydroxybenzoic acid are adapted to protocatechuic acid but not to p-aminobenzoic acid, while protocatechuic acid grown cells are not adapted to either p-aminobenzoic acid or p-hydroxybenzoic acid. Therefore p-hydroxybenzoic, protocatechuic acid and β-ketoadipic acid are intermediates in the oxidation of p-aminobenzoic acid and the degradation sequence is in this order.

In studying the metabolism of p-nitrobenzoic acid, Durham (49) reported that a strain of Pseudomonas fluorescens is capable of using this compound as a sole source of organic carbon and nitrogen for aerobic growth. Experimental results with living, irradiated and dried cell preparations indicated that p-aminobenzoic acid, p-hydroxybenzoic acid and protocatechuic acid were intermediats in the oxidative scheme and the sequence of appearance was in this order.

Studies involving the metabolism of nitro-phenylcarboxylic acids by microorganisms were investigated by Yang et al (188). Cells grown on o-nitrobenzoic acid showed immediate oxygen uptake on o-nitrobenzoic acid and o-hydroxylamine benzoic acid, indicating that these compounds appear to be intermediates in the metabolism of o-nitrobenzoic acid. Cells previously adapted to o-nitrosobenzoic acid showed simultaneous adaptation to o-hydroxylamine benzoic acid but not

to o-nitrobenzoic acid or anthranilic acid, whereas cells exposed to o-hydroxylamine benzoic acid were not adapted to o-nitrobenzoic acid, o-nitrosobenzoic acid, or anthranilic acid. The conclusion is therefore that o-nitrosobenzoic acid and o-hydroxyamine benzoic acid are intermediates in the metabolism of o-nitrobenzoic acid and the sequence in this order.

Oxidative metabolism of phthalic acid by pseudomonads isolated from soil was studied by Ribbons and Evans (129). Protocatechuic acid was detected and identified in the logarithmic phase of growth in phthalate grown cultures of Pseudomonas sp. The oxidation of protocatechuate by cell free extracts proceeded through cis-cis-β-carboxymuconate and 3-oxoadipate, which were isolated and identified.

Metabolism of benzenoid compounds by fungi: The metabolism of methoxylated aromatic compounds by soil fungi was investigated by Henderson (81). The rates of decomposition of mono-methoxybenzoic acids by Hormodendrum sp. was most rapid in the order para (p), meta (m) and ortho(o). In the initial stage of attack the methoxyl group is replaced by a hydroxyl group. Penicillium sp. also formed p-methoxyphenol from p-methoxybenzoic acid. p-Hydroxybenzoic acid formed from p-methoxybenzoic acid is further metabolized to protocatechuic acid by Hormodendrum sp. and Penicillium sp. When veratric acid (3,4-dimethoxybenzoic acid) is incubated with Hormodendrum sp. and Penicillium sp., the methoxyl group in the p-position is replaced by a hydroxyl group to give vanillic acid.

Metabolism of benzenoid compounds by animals: Methylation of the 3-hydroxy position of benzoid compounds by rat liver and kidney preparations was demonstrated by Pellerin and D'Iorio (121). 3,4-Dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxycinnamic acid were separately incubated with L-methionine-methyl-C^{14} in the presence of rat liver or kidney homogenate. In each case, the radioactive metabolite separated by paper chromatography was found to have migrating properties similar to those of the 3-methoxy-4-hydroxyaromatic acid. This reaction was enchanced by the addition of ATP, Mg, and reduced glutathione. When 3-hydroxybenzoic acid was incubated in this medium no methylated derivative was obtained. Preliminary experiments indicated that the enzymatic activity was contained mostly in the supernatant fraction and that liver homogenate was much more active than kidney homogenate in methylating benzenoid compounds.

METABOLISM OF MISCELLANEOUS AROMATIC COMPOUNDS

Metabolism of miscellaneous aromatic compounds by bacteria: The pathway for the oxidation of nicotinic acid by a strain of Pseudomonas fluorescens was investigated by Behrman and Stanier (6). The first step, catalyzed by a cytochrome-linked particulate enzyme system, is hydroxylation in the 6-position and is followed by an oxidative decarboxylation to yield 2,5-dihydroxypyridine, which in turn is cleaved oxidatively to yield maleamic and formic acids. Maleamic acid undergoes a hydrolytic

deamination to maleic acid, which is isomerized to fumaric acid. Subsequently Behrman and Stanier (7) found that 5-fluoro- and 5-chloro-nicotinic acids were oxidized by Pseudomonas fluorescens after induction in cells grown on nicotinic acid of the enzyme system which oxidize these substituted nicotinic acids. The particulate fraction from induced cells can convert 5-fluoro-nicotinic acid in good yield to 5-fluoro-6-hydroxy-nicotinic acid. The oxidation of 5-fluoro-nicotinic acid by cells results in the accumulation of two acids, chromatographically indistinguishable from acetic and citric acids.

Wada (173) isolated bacteria from soil which can utilize the tobacco alkaloids as a sole source of both nitrogen and carbon. Two microbial types, A and B were separated and Type A bacteria break down nicotine; whereas type B decompose nornicotine and anabasine as well as nicotine. The degradation of nicotine by both types follow the same route:
Nicotine ——→ Pseudooxy-nicotine ——→ γ-Keto-γ-(3-pyridyl)-butyric acid ——→ 3-succinoyl-6-hydroxypyridine ——→ Aliphatic compounds.

An organism capable of breaking down indole-acetic acid has been isolated by Proctor (122) and characterized as a member of the genus Pseudomonas. With standard manometric techniques the adaptation to indoleacetic acid was shown to involve simultaneous adaptation to skatole (3-methylindole), indoxyl (3-hydroxyindole), salicylic acid and catechol

but no adaptation occurred to indole, anthranilic acid, o-aminophenol, o-nitrophenol, o-nitrobenzoic acid or phthalic acid.

A study by Rogoff (132) of the bacterial oxidation of quinic acid showed that strains of Pseudomonas and Achromobacter spp. isolated from soil, utilized quinic acid as a sole source of carbon. Protocatechuic acid was produced as an intermediate in this oxidation but sequential induction experiments indicated that p-hydroxybenzoic acid was not an intermediate in quinic acid oxidation.

Metabolism of cinnamic acid and hydroxycinnamic acid by Lactobacillus pastorianus var. quinicus was shown by Whiting and Carr (181). When this organism was inoculated into a basal medium with the addition of 0.025% cinnamic acid and 0.25% fructose, the cinnamic acid was completely metabolized to dihydrocinnamic acid. In a similar medium containing 0.025% p-coumaric acid (p-hydroxycinnamic acid) and 0.25% fructose, two products, phloretic acid and p-ethylphenol were produced.

Marr and Stone (109) reported that two organisms, strains of Pseudomonas and of Mycobacterium species were capable of utilizing benzene as sole carbon source. Both organisms oxidized benzene manometrically with sufficient oxygen uptake to account for oxidation to carbon dioxide and water. The metabolic pathway for the breakdown of benzene is via 3,5-cyclo-hexadiene-1,2-diol and catechol. Phenol and o-benzoquinone are not probable intermediates in the oxidation scheme by these organisms.

Metabolism of miscellaneous aromatic compounds by fungi: Henderson et al (83) reported that numbers of fungi isolated from soils under a variety of vegetational types were found to attack p-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. By spectrochemical and paper chromatographic methods vanillin and ferulic acid were shown to be converted to vanillic acid and syringaldehyde converted to syringic acid before the breaking of the benzene ring. Consequently Henderson (85) investigated the influence of trace elements on the metabolism of aromatic compounds by soil fungi. Aspergillus niger, Hormodendrum sp. and Penicillium sp. deficient in various trace elements were studied but only iron had any marked effect on the rates of metabolism and on the accumulation of intermediate products of metabolism.

A number of moulds, obtained from culture collections and by enrichment techniques, were tested by Westlake et al (180) for their ability to degrade rutin. The molds, particularly Aspergillus flavans and A. niger appeared to be most active. The aspergilli when grown on either rutin or quercetin produced extracellular enzymes that degraded both rutin and quercetin but not quercitrin. Rutinose, protocatechuic acid, phloroglucinol carboxylic acid, and a phloroglucinol carboxylic acid-protocatechuic acid ester were identified by paper chromatography as the products.

Metabolism of miscellaneous aromatic compounds by animals: A study has been made of the fate in the rabbit of 1,3,5-tri, penta- and hexa-

chlorobenzene by Parke and Williams (120). 1,3,5-trichlorobenzene is not readily metabolized. The major portion of a dose of 0.5 g/kg. is unchanged in the gut contents and tissues 8 days after dosing. Some of the unchanged chlorohydrocarbon is eliminated in the faeces and expired air. A small proportion is converted into monochlorobenzene, some of which appears in the expired air and as p-chlorophenol in the urine and less than 10% of the dose is excreted as 2,4,6-trichlorophenol.

Pentachlorobenzene is not readily metabolized. Some 10-20% is dechlorinated and eliminated in the expired air as less-chlorinated benzenes, and a small amount of p-chlorophenol was isolated from the urine, thus proving the occurrence of dechlorination. Pentachlorophenol was a very minor urinary metabolite. It is doubtful whether hexachlorobenzene is metabolized at all. Pentachlorobenzene and hexachlorobenzene gave no ethereal sulphates, glucuronides or mercapturic acids.

The fate of 2,4,5-trihydroxybutyrophenone in the rat and dog has been studied by Astill et al (1). At the dose levels studied, 2,4,5-trihydroxy-butyrophenone is almost completely absorbed; about 75% of a single dose of 400 mg./kg. in the rat, and 300 mg./kg. in the dog, is excreted as conjugates, and the path of metabolism is largely by ethereal sulphate and glucuronide conjugation. Ethereal sulphate conjugation at the 5-hydroxyl-group leads to 5-butyryl-2,4-dihydroxy-phenyl hydrogen sulphate, isolated as the potassium salt which was converted then into

5-hydroxy-2,4-dimethoxybutyrophenone. Glucuronic acid conjugation at the 4-hydroxyl group leads to 4-butyryl-2,5-dihydroxy-phenyl glucosiduronic acid, which was not isolated but was converted into 4-hydroxy-2,5-dimethoxy-butyrophenone.

An enzyme system in rabbit liver homogenates which oxidizes p-nitrotoluene to p-nitrobenzoic acid was studied by Gillette (63). p-nitrotoluene is first oxidized to p-nitrobenzyl alcohol by a reduced triphosphopyridine nucleotide-dependent microsomal enzyme system. p-Nitrobenzyl alcohol and p-nitrobenzaldehyde are oxidized by diphospho-pyridine nucleotide-dependent enzymes in the soluble fraction.

In studying the metabolic fate of gallic acid and related compounds, Booth et al (18) showed that o-methylation resulting in the formation of 4-o-methyl gallic acid accounts for the major metabolite in the urine of rats or rabbits ingesting gallic acid, propyl gallate, lauryl gallate, or tannic acid. Decarboxylation accounts for a second metabolite identified as pyrogallol in the urine of rats receiving gallic acid by intraperitoneal injection or rabbits receiving gallic acid in the diet. 3-Methoxy-2,4-dihydroxybenzoic acid was the major urinary metabolite of rats given 2,3,4-trihydroxybenzoic acid. Syringic acid and 3,4-dimethoxy-5-hydroxybenzoic acid were identified as urinary metabolites of rats receiving 3-o-methyl gallic acid.

METABOLISM OF COUMARIN AND RELATED COMPOUNDS

Metabolism of coumarin and related compounds by animals: In studying the metabolism of coumarin and o-coumaric acid in rabbits, Mead, Smith and Williams (112) reported that coumarin is hydroxylated to 3-, 7-, and 8-hydroxycoumarins, which excreted in conjugation with glucose while o-coumaric acid appears to be excreted unchanged.

Booth et al (17) showed that rats and rabbits receiving coumarin orally, produced o-hydroxyphenylacetic acid in the urine which indicate that opening of the lactone ring and decarboxylation occurred. An alternate pathway for the formation of o-hydroxyphenylacetic acid from coumarin involves o-coumaric acid and o-hydroxyphenyllactic acid as intermediates. Some of the o-coumaric acid was converted to o-hydroxyphenylhydracrylic acid and 4-hydroxycoumarin. A species difference was encounted in that rabbits excreted 3-hydroxycoumarin and 7-hydroxycoumarin after ingestion of coumarin whereas rats did not excrete either of these compounds in detectable amounts.

A quantitative study of the metabolism of labelled coumarin in rats and rabbits was reported by Kaighen and Williams (93). When fed to rabbits at a dose level of 50 mg./kg., 80% of the C^{14} of the coumarin was excreted in the urine in 24 hrs. By isotope dilution the metabolites were found to be coumarin (0.5%), an acid-labile coumarin precursor (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%). These metabolites accounted for nearly 95% of the excreted radioactivity. In the rat, about 3% of the dose was excreted in the urine as hydroxycoumarins and 5% as an acid-labile precursor of coumarin while the main urinary metabolite was o-hydroxyphenylacetic acid (20% of the dose).

Metabolism of coumarin and related compounds by higher plants: The metabolism of coumarin in higher plants was studied by Kosuge and Conn (101, 102). Coumarin, when administered to shoots of white sweet clover (Melilotus alba) was rapidly converted to melilotic acid. Subsequent experiments with enzyme extracts of sweet clover showed that this conversion is stimulated by the addition of reduced triphosphopyridine nucleotide or a TPNH-generating system. Apparently coumarin, by hydrogenation, is first converted to dihydrocoumarin and the latter compound, by hydrolysis , is then converted to melilotic acid. When radioactive o-coumaric acid was administered to excised clover shoots, it was converted primarily to o-coumaryl glucoside, and in small amounts to coumarin, melilotic acid, melilotyl glucoside, and other unidentified compounds.

Metabolism of coumarin and related compounds by microorganisms: Metabolism of coumarin and related compounds by cultures of Penicillium

were studied by Bellis (10). Two of the moulds isolated by enrichment techniques were identified as Penicillium jenseni and Penicillium nigricans and were able to utilize coumarin as the sole carbon source. During growth the concentration of coumarin decreased steadily, but it was not possible to isolate any derivatives although in a medium containing sucrose and coumarin, a little umbelliferone was found. When the medium contained o-coumaric acid in place of coumarin as sole carbon source, a large amount of 4-hydroxycoumarin was isolated and in addition to 4-hydroxycoumarin a little dicumarol was detected also.

The effect of temperature, aeration, pH and nutrition on the metabolism of coumarin by nine strains of a Pseudomonas sp. were studied by Halvorson (76). All influence the rate of coumarin breakdown and under optimum conditions, 0.25% coumarin is metabolized in 18 hours by the most active strains. The major route for total decomposition was found to be o-coumaric acid through melilotic acid and 2,3-dihydroxy-phenylpropionic acid with other partially identified or unidentified compounds suggested as further intermediates in the metabolic scheme.

METABOLIC PATHWAY FOR THE DEGRADATION OF AROMATIC RINGS

Metabolic pathway for the degradation of aromatic rings by microorganisms: In general, bacterial enzymes cleave the aromatic ring by the insertion of oxygen between the atoms forming the C-H bonds of two adjacent ring carbons, so that two hydroxyl groups are formed on

the ring in an ortho relationship; the carbon-carbon bond between the hydroxyls is subsequently split.

The key intermediates undergo ring cleavage and further transformation to form aliphatic compounds which can enter the terminal respiratory cycles. The efforts of a number of workers have elucidated the steps involved in these reactions; the pathway by which catechol is degraded is seen in Fig. 3.

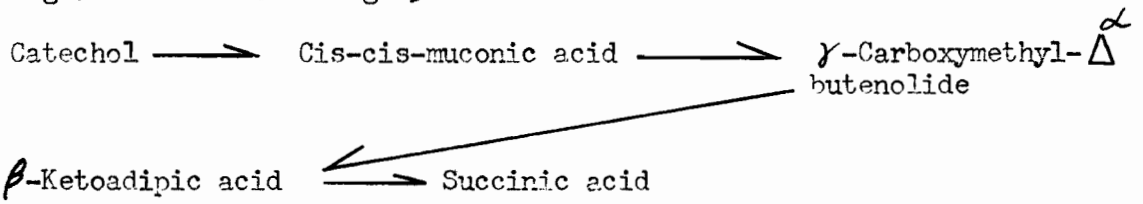


Fig. 3. Oxidative metabolic pathway of catechol by microorganisms.

Protocatechuic acid is also degraded to β-ketoadipic acid; the steps in this degradation are not so well known (Fig. 4) as those of the catechol path.

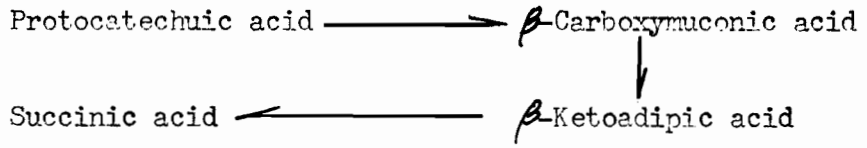


Fig. 4. Oxidative metabolic pathway of protocatechuic acid by microorganisms.

Sleeper and Stanier (149) demonstrated the enzymatic oxidation of catechol and protocatechuic acid to β-ketoadipic acid. Dried cells

prepared from Pseudomonas fluorescens grown on mandelate, benzoate, or phenol are capable of oxidizing catechol to β-ketoadipic acid quantitatively. Dried cells prepared from P. fluorescens grown on p-hydroxybenzoate are capable of oxidizing protocatechuic acid to β-ketoadipic acid quantitatively.

Evans (53) observed that cultures of Vibrio 01 developing with phenol as carbon source gave an intense violet Rothera reaction during the latter stages of growth. The substance responsible for the positive Rothera reaction was not identified but was shown to be an organic acid that was considerably less ether-soluble than aceto-acetic acid. Subsequently Kilby (96) isolated the acid from culture filtrates of Vibrio 01 and identified it as β-ketoadipic acid, a 6-carbon dicarboxylic acid.

The two enzymes involved in the conversion of cis-cis-muconic acid to β-ketoadipic acid were isolated and characterized by Sistrom and Stanier (147). The lactonizing enzyme catalyzes a reversible interconversion of cis-cis-muconic acid and (+)-γ-carboxymethyl-Δ^{α} butenolide, and also a very much slower interconversion of cis-trans-muconic acid and (-)-γ-carboxymethyl-Δ^{α}-butenolide. Mn^{++} is required for full activity. The delactonizing enzyme catalyzes an essentially irreversible conversion of (+)-γ-carboxymethyl-Δ^{α}-butenolide to β-keto-adipic acid. It is stereospecific and no cofactor requirements were found.

A protocatechuic acid oxidase was isolated and purified from Pseudomonas fluorescens by Macdonald, Stanier and Ingraha, (161). The product of the enzyme reaction was identified as one of the geometrical isomers of β-carboxymuconic acid but the configuration was not definitely established, although it is probably the cis-cis-isomer as there is at least one cis double bond.

Metabolic pathway for the degradation of aromatic rings by animals: The above ring cleavage mechanism appears to play little or no part in mammalian metabolism. The rupture of the bond is between the carbon atom bearing a hydroxyl and an adjacent carbon atom carrying a carbon side-chain or carboxyl as shown in Fig. 5.

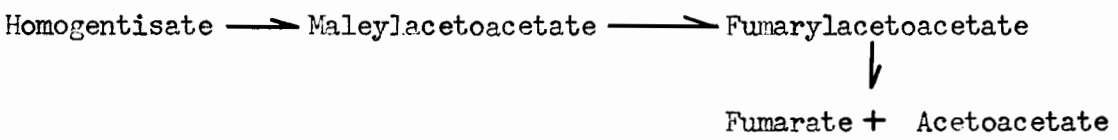


Fig. 5. Oxidative fission of p-dihydroxyphenols by mammals.

Homogentisate oxidase, prepared from liver by alcohol precipitation, formed a diketodicarboxylic acid of the same oxidation level as fumaryl-acetoacetate as shown by Knox and Edwards (98). The participation of iron in this reaction was confirmed. The enzyme possessed essential sulfhydryl groups, and certain preparations were activated non-specifically by ascorbic acid, glutathione, and other thiol compounds except cysteine.

Consequently Knox and Edwards (99) reported that the primary product of homogentisate oxidation by a liver enzyme was identified as an unsaturated β,δ-diketodicarboxylic acid similar to fumarylacetoacetate. It was different from fumarylacetoacetate in its absorption spectra, and it was not hydrolyzed by fumarylacetoacetate hydrolase. The new intermediate spontaneously changed into fumarylacetoacetate in acid but not in alkaline solution. The chromatographic identification of maleic and fumaric acids, respectively, after alkaline hydrolysis of the new intermediate and of fumarylacetoacetate, was consistent with the identification of the new intermediate as 4-maleylacetoacetate, the cis isomer of fumarylacetoacetate. The difference in the absorption spectra of the diketo acids in acid solution was attributed to the existence of fumarylacetoacetic acid in its enol form and of maleylacetoacetic acid in its keto form. Recently Edwards and Knox (51) reported that maleylacetoacetate isomerase, a new type of enzyme which catalyzes a cis-trans isomerization. This enzyme converts the first product of homogentisate oxidation, maleylacetoacetate, to the trans isomer, fumarylacetoacetate. Evidence is provided that maleylacetoacetate and fumarylacetoacetate are intermediates of homogentisate metabolism. Maleylacetoacetate isomerase has been separated from other enzymes of homogentisate metabolism. Glutathione is specifically required as a coenzyme for the reaction.

PART I

METABOLISM OF COUMARIN BY SOIL FUNGI

METHODS

SAMPLING OF SOILS

Soil was collected from the field in the neighbourhood of Macdonald College. The soil was sampled by scraping upwards with a sterilized scoop from a depth of 3-6 inches. The omission of the surface 1/2" was intended to eliminate aerial contaminants not occuring naturally in the soil. The soil was immediately transferred to sterile glass jars and brought to the laboratory. The samples were stored at 5° C until the following day, when dilutions were made.

ISOLATION OF MICROORGANISMS

Microorganisms were isolated from the soil by the enrichment technique with coumarin as the sole carbon source. The medium (medium A) contained:

$(NH_4)_2SO_4$ .....1.0 gm/liter

K_2HPO_4 .....1.0 gm/liter

$MgSO_4 \cdot 7H_2O$ ..... 0.5 gm/liter

NaCl ..... 0.1 gm/liter

Coumarin .....1.0 gm/liter

Isolations were made from the enrichment cultures to an identical medium containing in addition 2% sucrose and 2% agar.

GROWTH OF CULTURES

The cultures were grown on medium A or on medium B throughout the course of this investigation. Medium B had the following composition:

$(NH_4)_2SO_4$ .....1.0 gm/liter

K_2HPO_4..... 1.0 gm/liter

$MgSO_4.7H_2O$ ..... 0.5 gm/liter

NaCl ..... 0.1 gm/liter

$FeSO_4.7H_2O$ ..... 0.01 "

$MnSO_4.H_2O$ ..... 0.005 "

Coumarin ..... 1.0 gm/liter

PREPARATION OF INOCULUM

The inoculum was standardized in the following manner: from a agar slant, a transfer was made into 100 ml medium (medium A) and was incubated at room temperature on a rotary shaker with a speed of 75 r.p.m. for three days; a 5 ml aliquot of this mycelium suspension served as an inoculum in all trials.

IDENTIFICATION OF FUNGI

Growth on Czapek agar medium was examined under the microscope by the following technique: a suspension of spores in melted Czapek agar in a test tube was shaken vigorously then a few loops full of suspension were transferred to a glass slide, covered with a cover slip and the medium allowed to solidfy; the slide was incubated at room temperature and examined under microscope for several days.

EXTRACTION

The mold mycelium was removed by filtration and the filtrate acidified with hydrochloric acid then extracted with ethyl ether. The ethyl ether was removed by evaporation and the residue dissolved in a few drops of ethyl alcohol. These preparations were used for assay by paper chromatography.

SOURCES OF CHEMICALS

Coumarin, salicylic acid, and catechol were obtained from Fisher Scientific Company, Montreal, Canada. Melilotic acid, dihydrocoumarin, and 4-hydroxycoumarin were obtained from K and K Laboratories, Jamaica 33, N. Y. U. S. A. Dicumarol was a gift from Abbott Laboratories Limited, Montreal, Canada. o-Coumaric acid was obtained from Brickman and Company, Montreal, Canada. Cis-cis-muconic acid was kindly furnished by Dr. W. C. Evans, Department of Agricultural Chemistry, School of Agriculture, University College of North Wales, Bangor, England.

PAPER CHROMATOGRAPHY

Chromatography was carried out on strips of Whatman No. 1. filter paper by the asending method with the following solvent systems:

n-Butanol:acetic acid:water:- To 40 parts n-butanol was added 10 parts acetic acid and 50 parts water by volume.

Benzene:acetic acid:water:- To 10 parts benzene was added 10 parts acetic acid and 20 parts water by volume.

The compounds were detected under both acidic and alkaline conditions with the following spray reagents:

(1). Diazotized-p-nitroaniline. p-Nitroaniline, 0.5% in 2 N HCl (5 ml) and $NaNO_2$ (5%) (0.5 ml) were mixed before spraying and sodium acetate solution (20% W/V) (15 ml) added.

This solution gives a purplish-brown to yellow colour with phenols.

(2).Gibbs's reagent. A spray of 0.1% ethanolic 2:6-dibromoquinone chloroimide was followed by a spray of saturated $NaHCO_3$.

This solution gives a pink to blue colour with phenols.

(3). Brentamine fast blue B salt. Papers were sprayed with a 0.01% aqueous solution of this salt. (stabilized, tetrazotized di-o-anisidine).

This spray gives a pink colour with phenols.

(4). Diazotized sulfanilic acid. Sulfanilic acid, 0.3% in 8% (W/V) HCl (25 ml) and $NaNO_2$ (5% W/V) (1.5 ml) mixed immediately before spraying

This solution gives orange to brown colour with phenols.

(5). Ferric chloride. 0.2% in water. This solution gives unstable colour with some phenols.

The R_f values and colours produced between the various solvents and the compounds tested are given in Table 1 and 2.

ABSORPTION SPECTROMETRY

The absorption spectra provided a good means for distinguishing phenolic compounds. Filter paper was illuminated with an ultraviolet lamp to locate the fluorescent spots then qualitative determinations were made by cutting out the areas on the filter paper containing the phenolic compound after extracting with a different solvent. The spectrum was compared to those determined for authentic compounds with a Zeiss spectrophotometer model No. PMQ II.

Table 1

Compound	Solvent* A	Solvent* B	Diazotized p-nitroaniline		Gibbs's reagent	
			Direct spray	First spray with 1 N NaOH	Direct spray	First spray with 1 N NaOH
Coumarin	0.94	0.90	-	Purple	-	Blue
4-Hydroxycoumarin	0.90	0.92	Yellow	Brown	Pink	-
Dicumarol	0.90	0.21	Yellow	Yellow	Pink	-
o-Coumaric acid	0.89	0.82	Yellow	Purple	-	-
Salicylic acid	0.92	0.85	Yellow	Brown	Blue	-
Melilotic acid	0.88	0.84	Yellow	Purple	Pink	Blue
Dihydrocoumarin	0.88	0.84	Yellow	Purple	Pink	Blue
Catechol	0.95	0.80	Brown	Brown	Pink	Brown

* Solvent A is n-butanol-acetic acid-water (4:1:5); Solvent B is benzene-acetic acid-water (1:1:2)

- Colourless.

Table 2

Compound	Brentamine fast blue B salt		Diazotized sulfanilic acid		Ferric chloride	
	Direct spray	First spray with 1 N NaOH	Direct spray	First spray with 1 N NaOH	Direct spray	First sprsy with 1 N NaOH
Coumarin	-	-	-	Brown	-	-
4-Hydroxy-coumarin	Pink	-	-	Yellow	-	-
Dicumarol	Pink	-	-	Yellow	-	-
o-Coumaric acid	-	-	-	-	-	-
Salicylic acid	-	-	-	Yellow	Red	-
Melilotic acid	—	Pink	-	Brown	-	-
Dihydro-coumarin	-	Pink	-	Brown	-	-
Catechol	-	Yellow	Yellow	Brown	Blue	Brown

DETERMINATION OF COUMARIN

Coumarin was estimated by the colorimetric method of Clayton (42). To 1.0 ml aliquot of fermentation liquor was added 2.5 ml of 1.1% sodium carbonate solution and the liquid was heated nearly to boiling on a water bath (90° C) to obtain rapid equilibration between the lactone and acid configuration of the coumarin molecule. The solution was cooled slowly and 5.0 ml of the diazonium solution was added and the volume made up to 50.0 ml with distilled water. The solution were allowed to stand for 30 min. at room temperature before reading in a Coleman colorimeter with a 470 mu filter. The reagents are prepared as follows:

Solution A : p-nitroaniline hydrochloride. p-nitroaniline (3.5g) was dissolved in 45 ml of 37% hydrochloric acid and the solution was diluted to 500 ml with distilled water and filtered. The solution will keep indefinitely if stoppered in a brown bottle at 5° C.

Solution B : sodium nitrite. Sodium nitrite (5.0 g) was dissolved in 100 ml of distilled water. This solution is not as stable as solution A and should be renewed frequently. It is best to keep the solution away from the light and in a dark bottle.

The diazonium solution was prepared after the stock solutions (A and B) were cooled in an ice bath. Then 3.0 ml of solution A

and 3.0 ml solution B were placed in a 100 ml volumetric flask and immersed in an ice bath for five minutes. To this 12.0 ml of solution B was added, the flask was shaken, then returned to the ice bath for an additional five minutes. The flask was then filled to the 100 ml mark with ice cold distilled water and kept on ice until used. A standard curve showing the linear relationship between concentration of coumarin and optical density is given in Fig. 6.

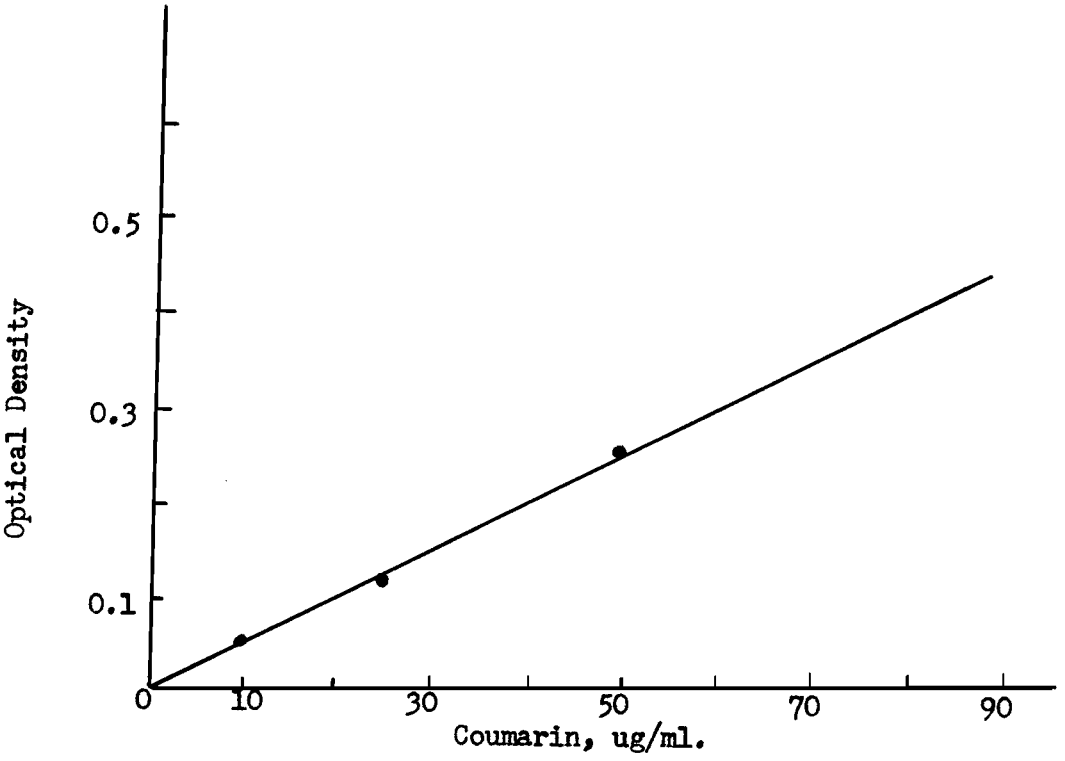


Fig. 6. Standard curve for coumarin.

RESULTS

ISOLATION OF MICROORGANISMS

Isolation of fungi. A total of 36 isolates were obtained from different soil samples.

Isolation of other microorganisms. No actinomycetes or bacteria were isolated in repeated trials.

GROWTH EXPERIMENTS

Tubes (25 ml) containing 10 ml medium A were inoculated with the pure culture under examination and incubated at room temperature for periods of 7 days. The results are shown in Table 3.

Table 3. Comparison of growth of fungi with coumarin as sole carbon source.

Organism No	Growth
31, 32, 33, 34, 35.*	Very good
8 isolates	Poor
23 isolates	Very poor

* These cultures were added to the Macdonald College culture collection and numbered Mac# m31 to m35 respectively.

IDENTIFICATION OF COUMARIN UTILIZING FUNGI

According to a manual of soil fungi (64), all of these active isolates (Mac# m31 to m35) were identified as :

Class ---------- Fungi imperfecti

Order ---------- Moniliales

Family ---------- Tuberculariaceae

Genus ---------- Fusarium

The species was not identified. Typical morphology of the isolated Fusarium sp (Mac# m32) is shown in Fig. 7-10.

Examination of other isolates that demonstrated slower utilization of coumarin, and were described, showed that these fungi belonged to other genera eg. Aspergillus, Penicillium and Mucor.

Fig. 7. Showing spores (X400)

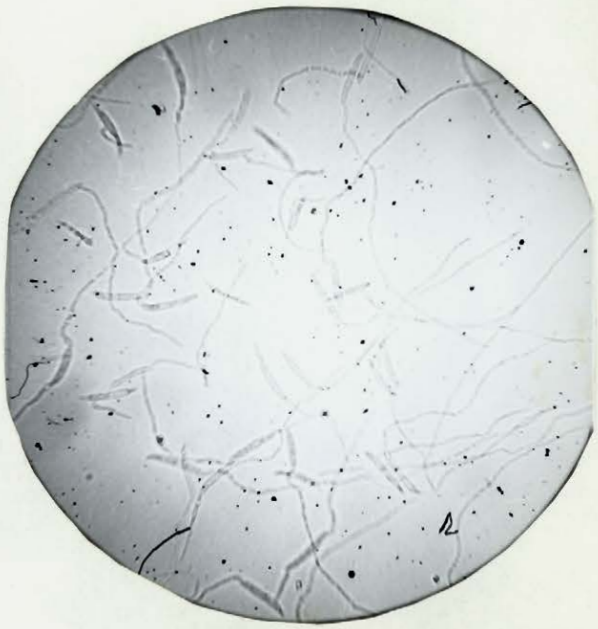


Fig.8. Showing growth by germination(X400)

Fig. 9. Showing spore conjugation (X400)

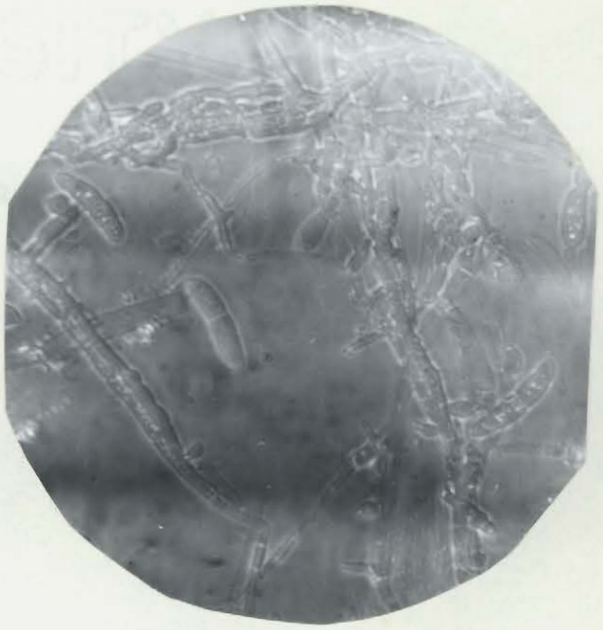


Fig. 10. Showing resultant growth (X1000)

CHANGES IN ACIDITY DURING GROWTH OF THE CULTURES

Flasks(250 ml) containing 100 ml medium A were inoculated with pure cultures Mac# m31, 32, 33, 34, and 35, and incubated at room temperature for periods of 15 days. During growth the solutions become more acid as the results in Table 4 show.

Table 4. Changes in pH during fermentation.

Days	Organism No				
	31	32	33	34	35
	pH				
0	7.1	7.1	7.1	7.1	7.1
3	7.0	6.9	6.9	6.8	7.0
6	6.9	6.8	6.9	6.7	6.9
10	6.7	6.7	6.6	6.7	6.8
15	6.6	6.5	6.5	6.6	6.6

IDENTIFICATION OF MELILOTIC ACID FORMED DURING THE UTILIZATION OF COUMARIN

The isolated moulds are able to utilize coumarin as sole carbon source and the rates of growth are fast. Flasks (250 ml) containing 100 ml medium A were inoculated with cultures Mac# m31, 32, 33, 34 and 35 and incubated at room temperature under static condition for periods of 20 days. During growth the concentration of coumarin falls steadily.

Paper chromatography of the ether soluble materials from these media reveals a compound with identical R_f value and the same colour reaction of melilotic acid after 15-20 days incubation. Chromatographic evidence for the presence of melilotic acid is shown in Fig. 11. Spectroscopic evidence for the presence of melilotic acid is given in Fig. 12.

Fig. 11. Chromatograms of coumarin fermentation liquors of culture Mac# m32 after 15 days incubation.

1. Filtrate of coumarin fermentation after extracted with ethyl ether and sprayed directly with diazotized-p-nitroaniline.
2. Standard melilotic acid sprayed directly with diazotized-p-nitroaniline.
3. coumarin sprayed directly with diazotized-p-nitroaniline.

Solvent: Benzene:Acetic acid: Water (1:1:2).

Origin 1 2 3

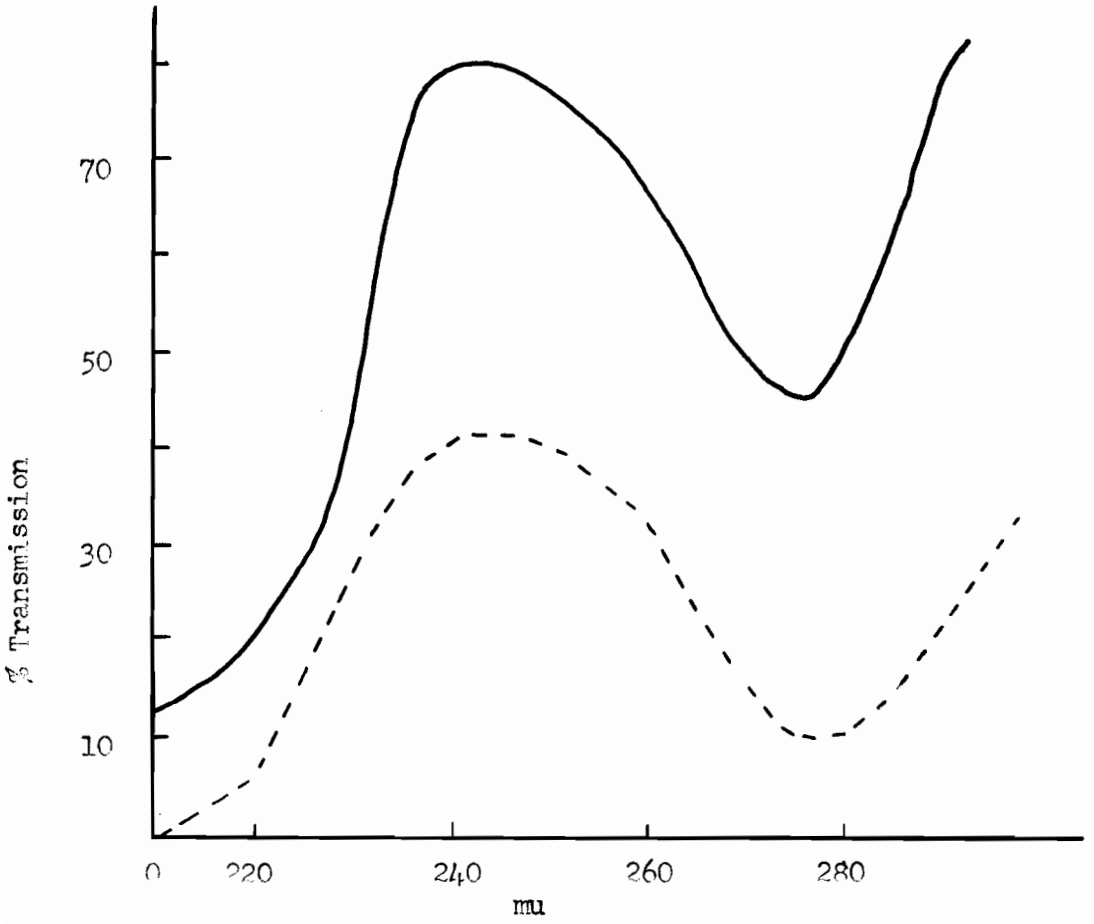


Fig. 12. Ultraviolet absorption spectra of authentic melilotic acid ——— and of isolated melilotic acid - - - - ,eluted from paper chromatograms of culture liquor from Mac# m32 after 15 days incubation.

ISOLATION OF MELILOTIC ACID

Flasks (250 ml) containing 100 ml medium A was inoculated with 5 ml inoculum of culture Mac# m32 and incubated at room temperature at static state. After 9 days, the filtrate was acidified and extracted with ethyl ether. After removal of the solvent from the extract, the residue appeared to contain melilotic acid because of its characteristic honey-like odor. After dissolving in a small amount of water, it was recrystallized from benzene and dried in vacuo. The melting point of the isolated melilotic acid was 85-87° C (uncorrected) authentic 87° C (27) ; mixed melting point 86° C. A comparison of the absorption spectra of the isolated melilotic acid with the authentic sample is shown in Fig. 13. On the basis of these tests the isolated product was definitely identified as melilotic acid.

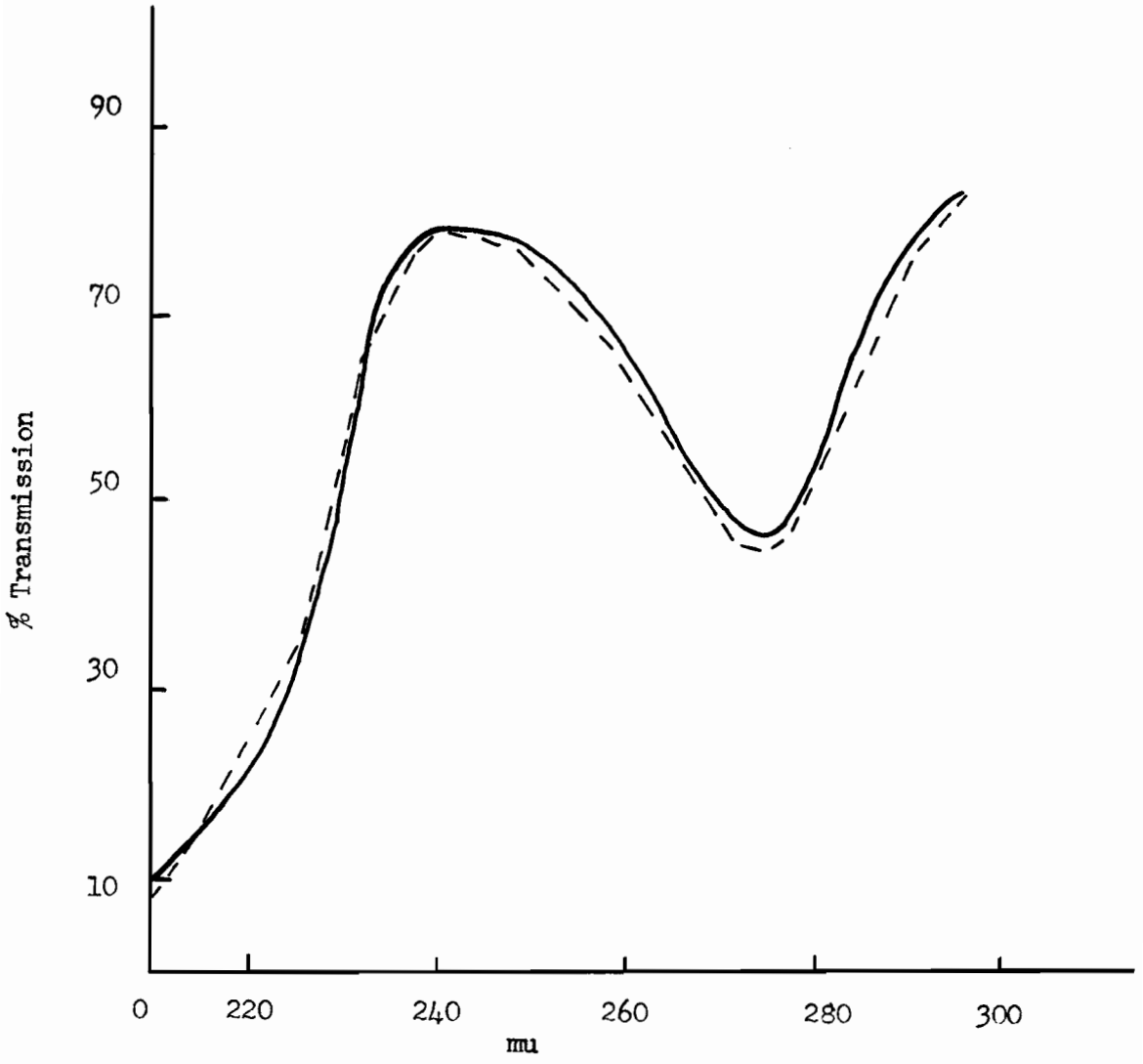


Fig. 13. Absorption spectra: ——— Melilotic acid 3.0 mg in 100 ml 95% alcohol. ---- Isolated melilotic acid 3.0 mg in 100 ml 95% alcohol.

CONVERSION OF DIHYDROCOUMARIN TO MELILOTIC ACID

A medium containing 0.05 g of dihydrocoumarin, 100 ml of phosphate buffer, pH 6.8 and 0.04 g (dry basis) of intact cells of Mac# m32 was incubated at room temperature for 12 hours on a shaker at a speed of 75 r.p.m. The mycelium was removed by filtration and the cleared liquor was acidified and extracted with ethyl ether. The ethereal solution containing the product was evaporated to dryness and a material was crystallized from benzene that had the properties of melilotic acid (m.p. 86° C) and a similar absorption spectrum. High yields of melilotic acid were obtained eg. from 50 mg of dihydrocoumarin 46.0 mg of melilotic acid was crystallized (83% yield based on theory).

THE UTILIZATION OF COUMARIN IN THE PRESENCE OF AN OTHER SOURCE OF CARBON (SUCROSE)

Medium A 100 ml to which sucrose 0.1g had been added as a substrate was inoculated with culture Mac# m32 and incubated at room temperature at static state for periods up to 45 days. The rate of growth is slow. During growth the pH drops as acid is produced as shown in Table 5.

Table 5. Variations in pH.

Days	0	5	10	15	20	25	30
pH	7.5	7.3	6.7	6.0	5.5	5.2	5.0

Paper chromatography of the ether soluble material in the medium revealed after 30 days incubation that a compound with identical R_f value and the same colour reaction as melilotic acid was present.

EFFECT OF AERATION ON THE UTILIZATION OF COUMARIN

Flasks (500ml) containing 100 ml medium A was inoculated with culture Mac# m32 and incubated at 25° C for 10 days in a shaker with a speed of 75 r.p.m. Paper chromatography of the ether soluble material in the medium did not reveal the presence of melilotic acid after 7 days.

EFFECT OF TRACE ELEMENTS ON THE UTILIZATION OF COUMARIN

Flasks (500 ml) containing 100 ml medium A and 100 ml medium B were inoculated with 5 ml inoculum of culture Mac# m32 and incubated at room temperature for five days on a shaker with a speed of 75 r.p.m. The mycelia were removed by filtration. The filtrate was diluted with distilled water to a suitable concentration and the amount of coumarin remaining in the medium determined. Table 6 gives the results of the typical experiment.

Table 6. Effect of trace elements (Fe^{++} and Mn^{++}) on the utilization of coumarin.

Time of incubation days	Residual coumarin mg/100 ml		% Utilization	
	Medium A	Medium B*	Medium A	Medium B
0	50.00	50.00	0	0
1	42.10	34.50	16	31
2	35.26	28.75	30	42
3	24.60	15.00	49	70
4	16.51	0	67	100
5	0		100	

* Medium B contains Fe^{++} and Mn^{++} which medium A does not.

COMPARISON OF UTILIZATION OF MELILOTIC ACID AND CATECHOL

Medium B in which melilotic acid or catechol replaced coumarin as substrate was inoculated with culture Mac# m32 and incubated at 25° C on a rotary shaker at a speed of 75 r.p.m. Paper chromatography of the ether soluble material in the medium to which melilotic acid was added as a carbon source showed that after 2 days no melilotic acid remained.

When catechol was added as a carbon source the medium still contained catechol after 4 days incubation as demonstrated by paper chromatography.

CIS - CIS MUCONIC ACID FORMED DURING THE UTILIZATION OF COUMARIN

Flasks (500 ml) containing 200 ml medium B was inoculated with culture Mac# m32 and incubated at 25° C on a rotary shaker with a speed of 75 r.p.m. After ten days incubation the ether soluble material from the fermentation liquid contained a compound with a similar absorption spectrum to cis-cis-muconic acid (Fig 14). Crystallization of cis-cis-muconic acid from similar fermentation liquor was not successful in repeated trials.

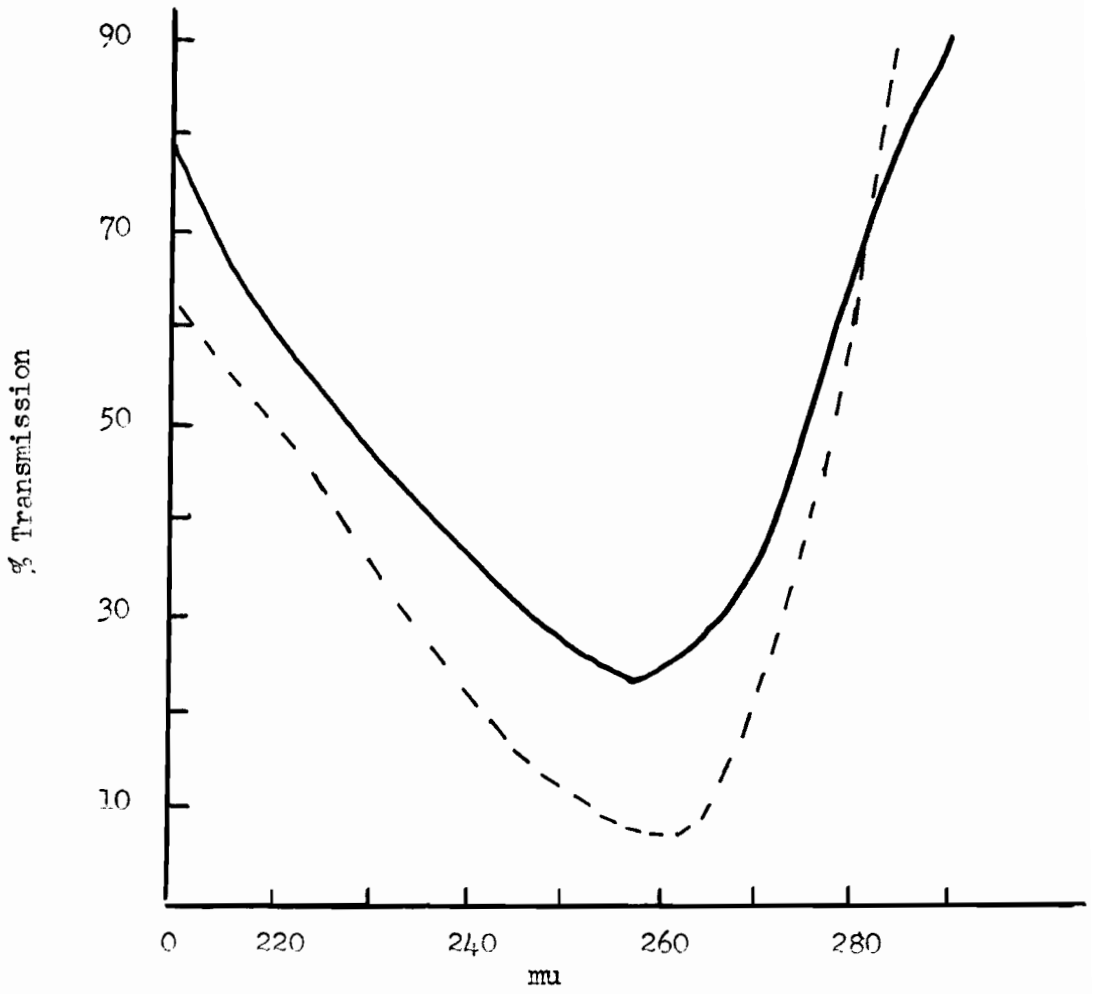


Fig. 14. Ultraviolet absorption spectra of authentic cis-cis-muconic acid ——— and of isolated cis-cis-muconic acid – – – , extracted from coumarin fermentation liquid of culture Mac# m32 after 10 days incubation.

DISCUSSION

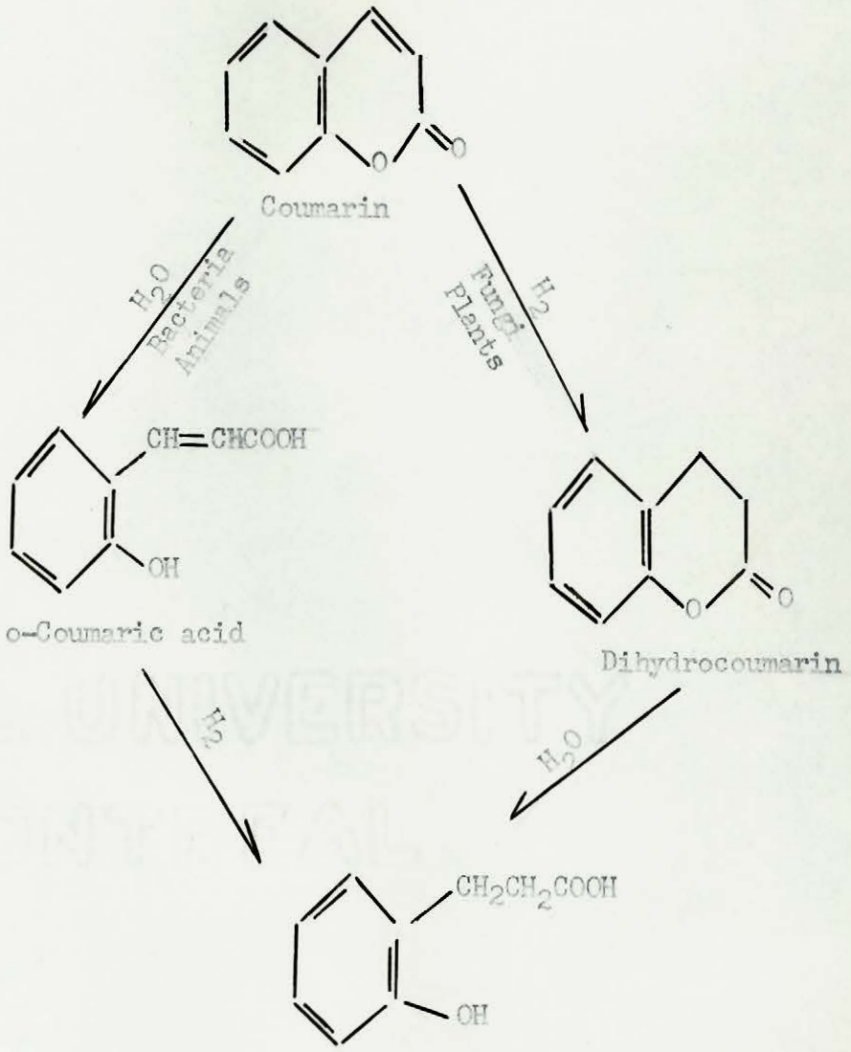
Studies by Bellis (10) on the metabolic fate of coumarin in moulds showed that coumarin was utilized as sole carbon source in a modified Czapek medium by two moulds identified as Penicillium jenseni and Penicillium nigricans. The rate of growth was slow and at no time was it possible to isolate any intermediates. In a similar medium containing sucrose and coumarin, a little umbelliferone was formed. In this study, the five moulds isolated from soil, the Fusarium sp, are able to utilize coumarin as sole carbon source in a simple salts medium, although the rate of growth is slow. During growth the concentration of coumarin falls steadily and the melilotic acid is formed very easily. In a similar medium containing sucrose and coumarin, growth is again slow but melilotic acid is still detectable. The isolated melilotic acid has been identified by paper chromatography, by its melting point and by its ultraviolet spectrum. These results were demonstrated for the isolated fungus, Fusarium sp ., where melilotic acid was found to be the intermediate in the coumarin metabolism. This metabolic pattern is absolutely different from Penicillium sp. as reported by Bellis (10).

The biological oxidation of coumarin in bacteria has been studied by Halvorson (76) who demonstrated that with tritiated coumarin as a

substrate that melilotic acid and 2,3-dihydroxyphenylpropionic acid were radioactive. The degradative mechanism from coumarin to melilotic acid was suggested through o-coumaric acid. This confirmed the evidence of Booth et al (17) who studied the urinary metabolism of coumarin by rats or rabbits. Booth et al (17) showed that o-coumaric acid was the first intermediate found and resulted from the opening of the heterocyclic ring of coumarin. In this study never was it possible to detect or identify o-coumaric acid as an intermediate and on the other hand, the isolated fungus, Fusarium sp., utilized o-coumaric acid as a sole carbon source and produced high yields of 4-hydroxycoumarin.

From phosphate buffered dihydrocoumarin medium melilotic acid was almost quantitatively recovered with resting intact fungus cells. Based on these data the conclusion is that the metabolism of coumarin in Fusarium sp. is similar to that found in higher plants by Kosuge and Conn (101, 102). The mechanism then is that coumarin, by hydrogenation, is first converted to dihydrocoumarin and the latter compound by hydrolysis is then converted to melilotic acid.

In summarizing the results, there appear to be two major pathways for the biological degradation of coumarin: (1), from coumarin through o-coumaric acid to melilotic acid;(2), from coumarin through dihydrocoumarin to melilotic acid. These pathways are demonstrated in the accompanying diagram:



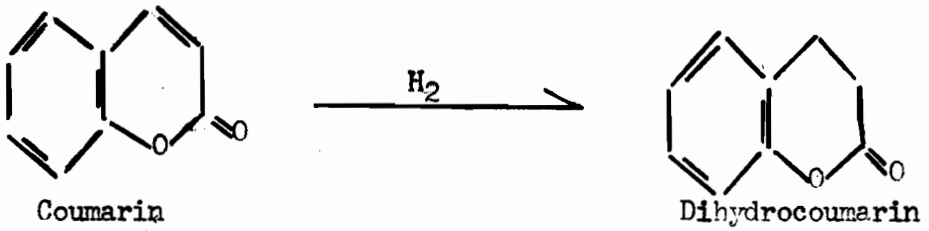
Melilotic acid

Studies by Henderson (85) on the influence of trace elements on the metabolism of aromatic compounds by soil fungi showed that iron had marked effect on the rates of metabolism of the ring compounds. This seems to be true for the metabolism of coumarin by the isolated fungus , Fusarium sp. Aw advantage in using basal medium without

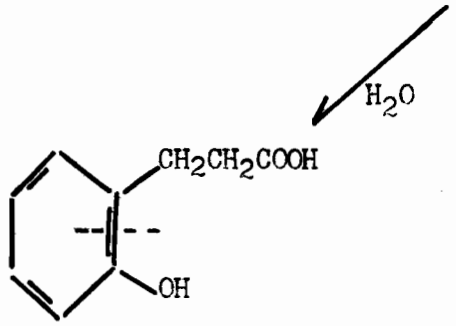
trace elements is that the intermediate , melilotic acid, accumulates in fermentation liquor for a long time and is therefore very easily to identify and isolate.

To cleave the aromatic nucleus bacteria, in general, break the aromatic ring by the insertion of oxygen between the atoms forming the C-H bonds of two adjacent ring carbons, so that two hydroxyl groups are present on the ring in an ortho relationship and then the carbon-carbon bond between the hydroxyls is subsequently split. Benzenoid compounds containing various substituent groups undergo enzymatic manipulations to reach this orthohydroxylated state, eg. phenol is hydroxylated to catechol, which is cleaved. However, all biological systems do not follow the same route eg. homogentisic acid is ruptured at the bond between the carbon atom bearing a hydroxyl and an adjacent carbon carrying a carbon side-chain by mammals (98, 99). This second pathway could be the route of metabolism of coumarin by the Fusarium sp. as the data indicates that a mineral salts medium with either melilotic acid or catechol added as substrate behaved differently after incubation. The melilotic acid was completely metabolized after 2 days, but the catechol was not utilized even after 4 days incubation. Therefore, catechol is not likely a intermediate in the metabolism of coumarin by the isolated fungus, Fusarium sp. Moreover, besides melilotic acid, no other ring compound was detected although evidence was found for the presence of cis-cis-muconic acid. These results suggest that the carbon -carbon bond may be broken between the carbon atom bearing a hydroxyl and an adjacent carbon

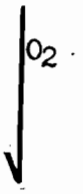
atom carrying a carbon side chain eg. melilotic acid. Based on this theory , a mechanism of coumarin metabolism by the soil fungus Fusarium sp. is presented.

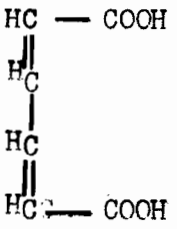


Coumarin Dihydrocoumarin



Melilotic acid





cis-cis-Muconic acid

PART II

COUMARIN METABOLISM BY CELL FREE EXTRACTS

METHODS

It is well understood that whole cells of many microorganisms are impermeable, or nearly so, to certain intermediates in the metabolism of some compounds. The use of cell free extracts helps to overcome the problem of permeability and results obtained with them often are different than those acquired with intact cells. The purpose of the present work is to compare the metabolic patterns of intact cells and cell free extracts.

GROWTH OF CELLS

Flasks (500 ml) containing 200 ml medium (composition as medium B in Part I but with 0.1g coumarin) were inoculated with 5 ml inoculum (prepared as described in Part I) of culture Mac# m32 and incubated at 25^{0} C for 7 days on a rotary shaker with a speed of 75 r.p.m.

PREPARATION OF CELL FREE EXTRACTS

The mycelia obtained of culture Mac# m32 were harvested by filtering through a funnel, then were washed with cold 0.1 M phosphate

buffer, pH 6.8. The crude enzyme extract was prepared by grinding the wet mycelia with an equal weight of sand prewashed with 0.1 M phosphate buffer at pH 6.8 . The cells were ground in a chilled mortar for 10-15 min., then 4 to 5 volumes of 0.1 M phosphate buffer, pH 6.8, was added and the cellular debris and the sand were removed by filtering through Whatman No. 2 filter paper,.The supernatant was used as enzyme preparation for the study of the enzymatic degradation of coumarin.

PREPARATION OF AMMONIUM SULFATE PRECIPITATES

Fifty millilitres of cell free extract (protein, 1.4 mg/ml) was stirred continuously and 7.6 g of finely powdered $(NH_4)_2SO_4$ was added to give 0.2 saturation; this was allowed to stand for 15 min. The precipitate was separated by centrifugation at 8000 r.p.m. for 15 min., then dissolved in 20 ml of 0.1 M phosphate buffer at pH 6.8. The supernatant was treated again in the same manner with an additional 11.4 g of $(NH_4)_2SO_4$ to obtain 0.5 saturation and again after a similar treatment with an additional 7.6 g of $(NH_4)_2SO_4$ to obtain 0.7 saturation. The precipitates were recovered and dissolved in 0.1 M phosphate buffer, pH 6.8. All the solutions were dialyzed against 1 liter of 0.1 M phosphate buffer, pH 6.8, for 12 hours and then analysed for enzyme activity.

ENZYMATIC ANALYSES

The enzymatic degradation of coumarin and hydrolysis of dihydrocoumarin was estimated by measuring the formation of melilotic acid. Since dihydrocoumarin is difficultly soluble in water, it was first dissolved in a small amount of ethanol then diluted with distilled water. Freshly prepared dihydrocoumarin solution is relatively stable for about three hours if kept cold.

CHEMICAL ANALYSES

Determination of protein : The protein content of the cell free extracts was estimated by turbidity measurements of trichloroacetic acid precipitates (152). The cell free extract, containing 0.1 to 5.0 mg of protein, was made up with water to a volume of 2.0 ml, and 3.0 ml of 5.0 % trichloroacetic acid solution were added. The resulting suspension was allowed to stand for 30 seconds and the turbidity was measured in a Coleman colorimeter with a 525 mu filter. Crystalline egg-albumin was used as a standard and a linear function over the range of protein concentration from 0.1 to 3.0 mg was found as shown in Fig 15.

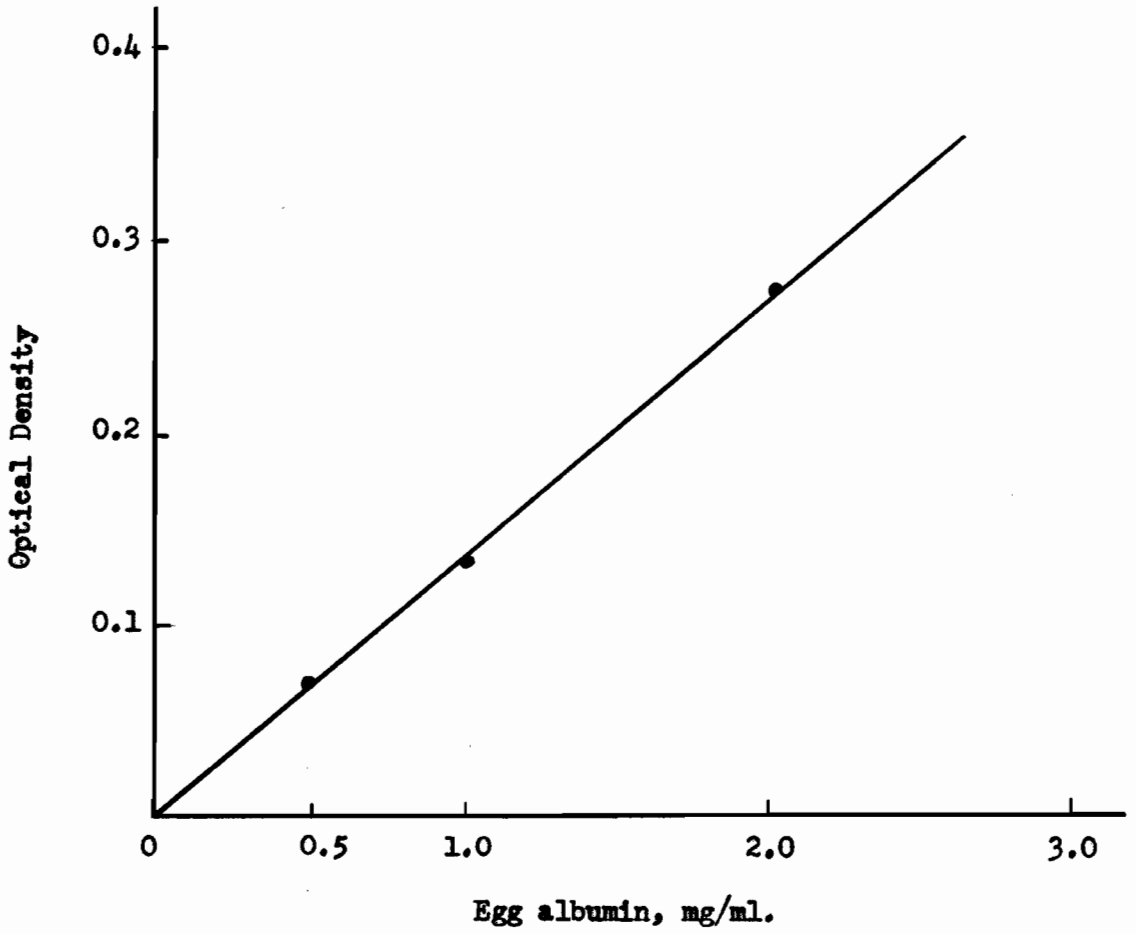


Fig. 15. Standard curve of protein solution.

Determination of coumarin: See above on page 30 for a description of the method.

Determination of melilotic acid: Melilotic acid reacts with diazonium solution in an alkaline condition to give a reddish colour, as does coumarin. Thus melilotic acid was estimated by the same method used in the determination of coumarin and a standard curve is given in Fig. 16.

Assay of a mixture of melilotic acid and coumarin: The principle of the estimation of mixture of coumarin and melilotic acid is based on the finding that both compounds react, as shown, with diazonium solution at an alkaline pH but that at a neutral or acid pH, melilotic acid gives a yellow color and coumarin no color.

In the assay procedure to 1.0 ml of a sample at neutral pH was added 5.0 ml of water, followed by 1.0 ml of diazonium solution (freshly prepared), then after shaking for 30 seconds, the mixture was allowed to stand for 30 minutes and the absorption at 470 mu was determined and compared with that of a standard curve. A typical standard curve is given in Fig. 17.

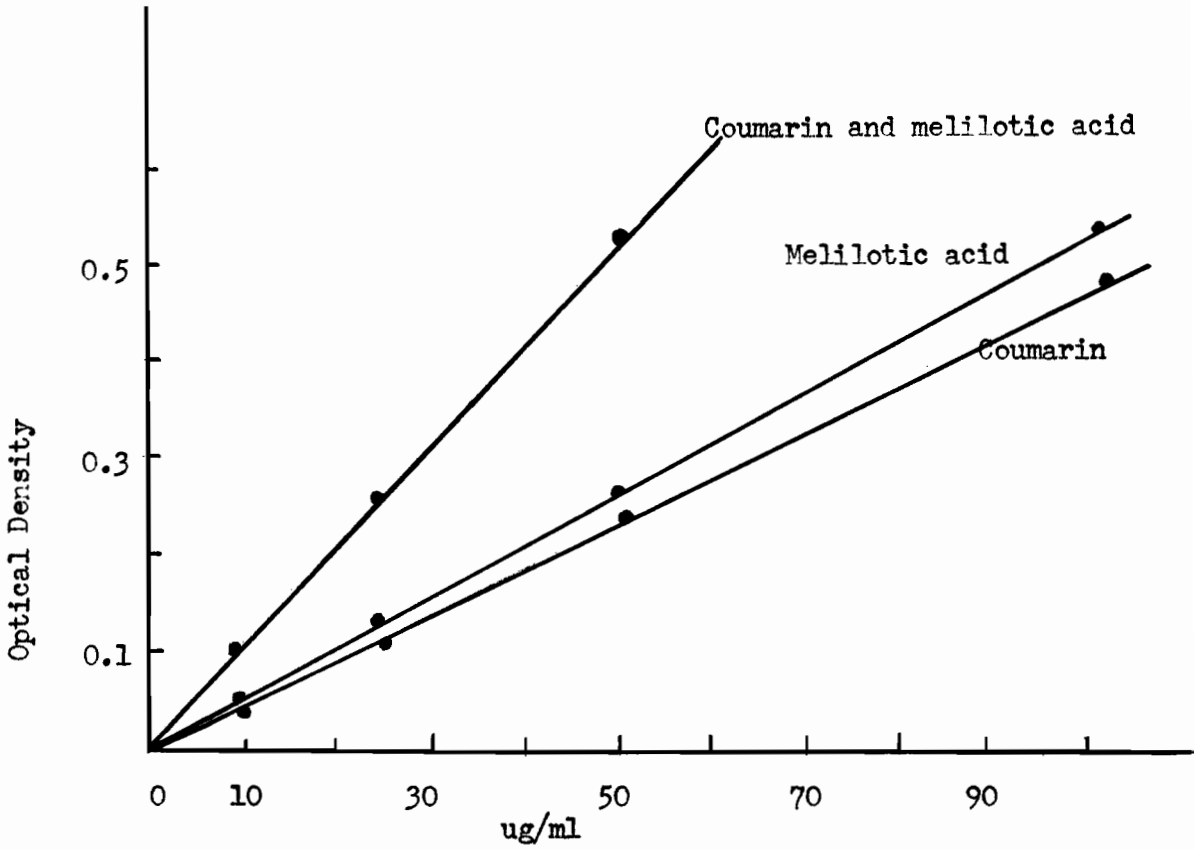


Fig. 16. Standard curve of coumarin, melilotic acid , and a mixture of coumarin and melilotic acid in an alkaline condition.

A mixture of coumarin and melilotic acid is 1:1 ratio.

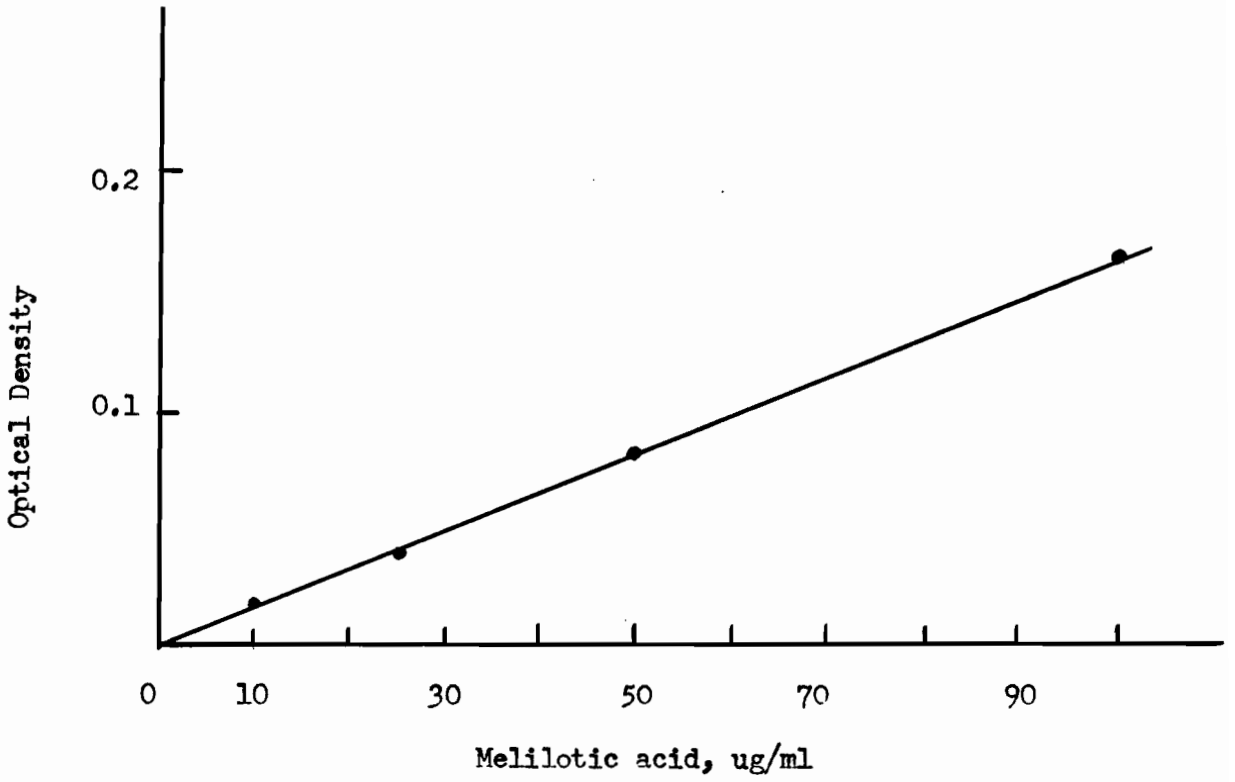


Fig. 17. Standard curve of melilotic acid in neutral condition.

The reason for selecting 470 mu is based on the following data(Table 7) showing the relatively linear relationship between concentration of melilotic acid and optical density at 470 mu.

Table 7. The relationship between wave length and optical density for melilotic acid assay at neutral pH.

Wave length	Melilotic acid, ug/ml			
	50	100	150	200
	Optical Density			
390	0.39	0.60	0.70	0.75
430	0.27	0.44	0.54	0.59
470	0.08	0.15	0.22	0.28
525	0.02	0.03	0.04	0.04
590	0.009	0.01	0.012	0.014

To determine the concentration of coumarin, the total amount of coumarin and melilotic acid is estimated at an alkaline pH (see Fig 17 for a standard curve) and the melilotic acid concentration is subtracted giving the amount of coumarin in the sample.

Dihydrocoumarin (below 100 ug/ml) and cis-cis-muconic acid do not give a colour reaction at neutral pH.

Examples of the assay are given in Table 8.

Table 8. Assay of a mixture of melilotic acid and coumarin.

Coumarin ug	Compounds added		pH		Products recovered			
	Melilotic acid,ug	cis-cis-Muconic acid,ug	Alkaline Optical Density	Neutral Optical Density	Melilotic acid ug	%	Coumarin ug	%
25	25	25	0.26	0.04	24	96	24.4	97
50	50	50	0.50	0.079	48.6	97	49	98

RESULTS

CONVERSION OF COUMARIN TO MELILOTIC ACID BY CELL FREE EXTRACTS

Qualitative demonstration of enzymatic activity: Enzymatic activity was indicated by paper chromatographic assay. A 500 ml Erlenmeyer flask containing 20 ml of cell free extract (protein, 2.0 mg/ml), 80 ml 0.1 M phosphate buffer at pH 6.8, and 0.05 g coumarin was incubated at 25° C for 24 hours on a rotary shaker. The solution was extracted with ethyl ether, the ether was removed by evaporation, and the residue was dissolved in a few drops of water. Spots were applied to Whatman No. 1 filter paper with a capillary pipette. The papers were developed for a few hours at room temperature by the ascending technique with a solvent consisting of benzene-acetic acid-water (1:1:2). The papers were air dried then sprayed with diazotized-p-nitroaniline. A compound with a identical color reaction and an R_f value similar to melilotic acid was found.

Effect of pH on enzyme activity: The effect of pH on enzyme activity was studied by incubating 1.0 ml of cell free extract (protein, 1.4 mg/ml) with 2.0 ml of buffers at pH 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9, and 10, prepared as described in the Appendix Tables 3 and 4 following the method of Clark and Lubs (165); to this was added 2.0 ml of coumarin

solution (500 ug/ml), and these were incubated at 30° C for 3 hours. After incubation the resulting mixture was made to pH 7.0 and suitable aliquot was removed and analysed for melilotic acid and coumarin.

The results are shown in Fig 18. and are calculated as activity units where one unit of enzyme is defined as the amount required to degrade 0.1 mg of coumarin in 3 hours at 30° C.

The optimum pH for degradation of coumarin by the cell free extracts of culture Mac# m32 is approximately pH 6.5.

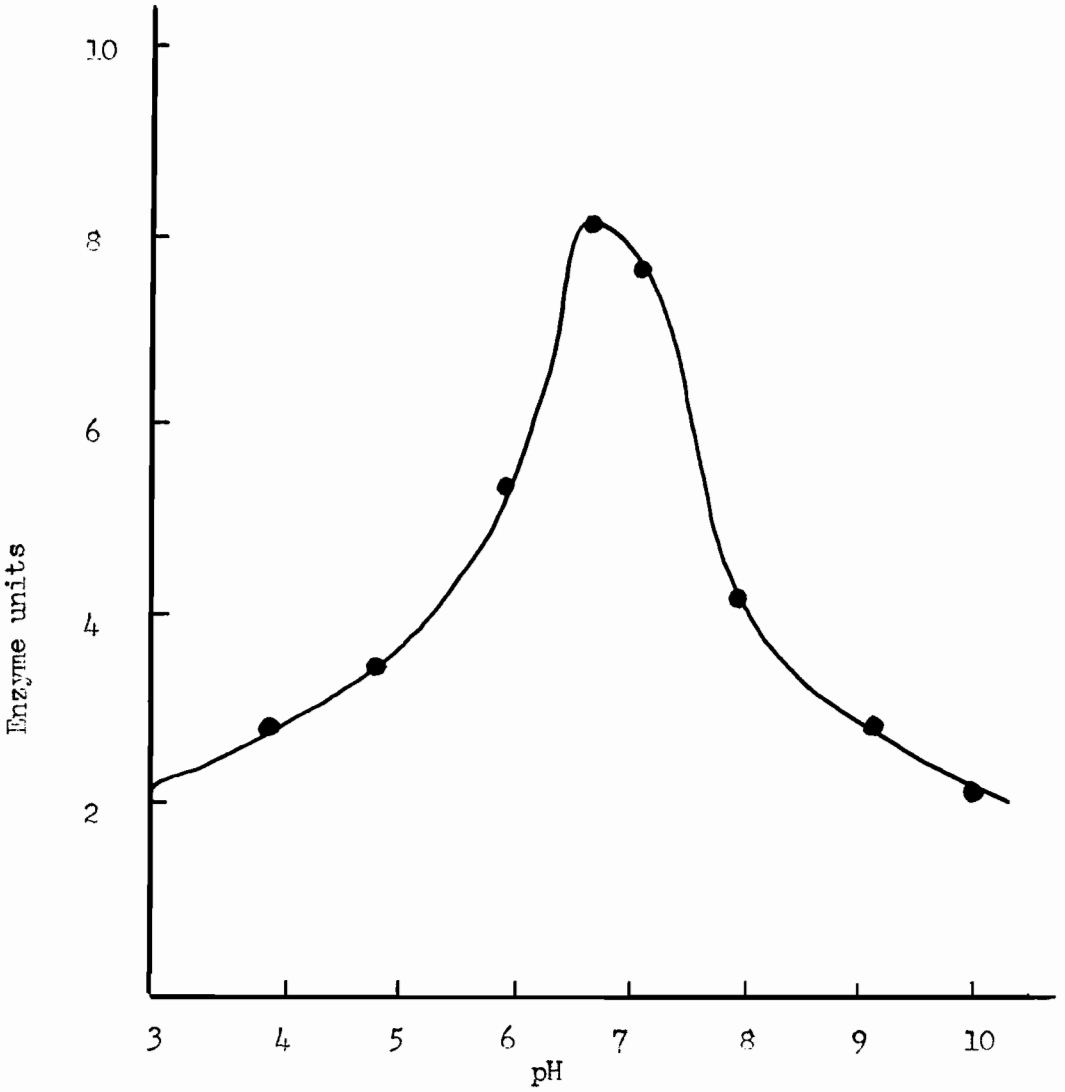


Fig. 18. The effect of pH on the reaction rate.

Effect of temperature on enzyme activity: The effect of temperature on enzyme activity was studied by incubating 1.0 ml of cell free extract (protein, 0.7 mg/ml) with 2.0 ml of buffer (pH6.5) and 2.0 ml of coumarin (500 ug/ml) for 6 hrs at temperature of 5, 10, 20, 30, 35, and 40° C. Suitable aliquots were analysed and the results are shown in Fig. 19, calculated on the basis of enzyme activity units.

The optimum temperature for degradation of coumarin by the cell free extracts of culture Mac# m32 is approximately 35° C.

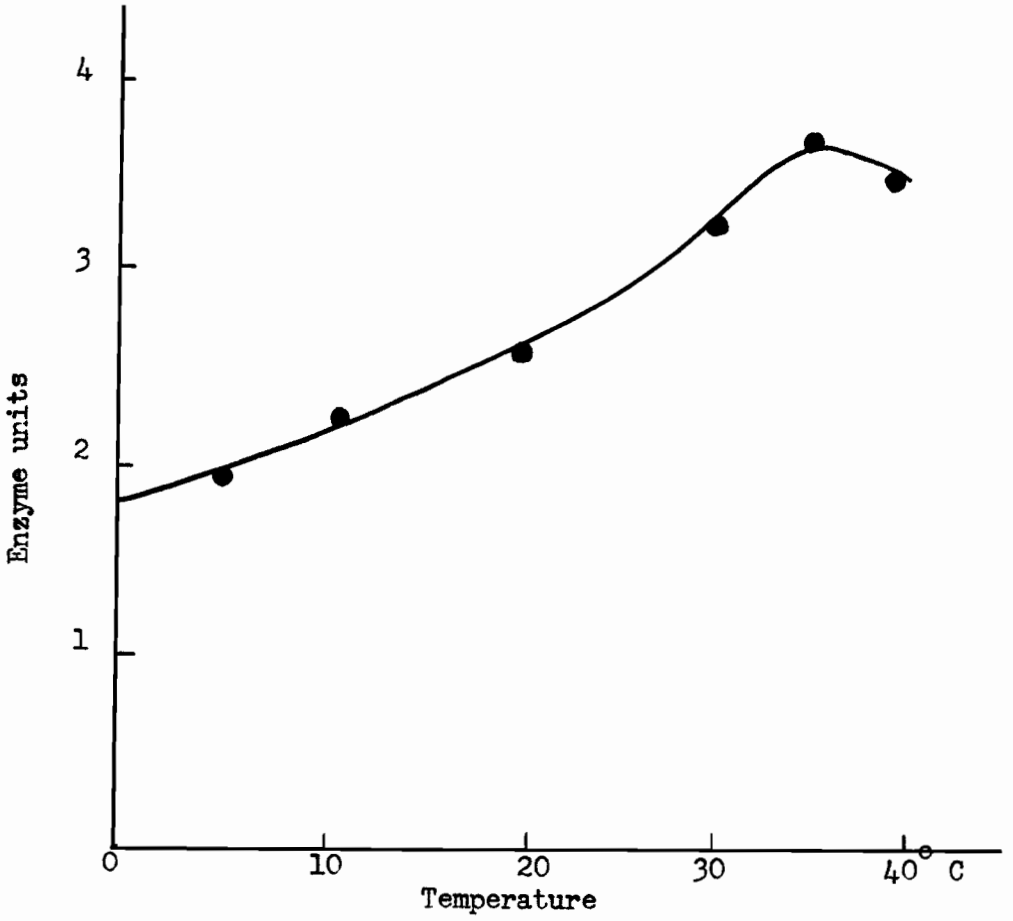


Fig. 19. Effect of temperature on enzyme activity.

Effect of mineral elements on enzyme activity: The effect of mineral elements on enzyme activity was estimated by incubating 1.0 ml of a cell free extract (protein, 0.7 mg/ml) with 2.0 ml of buffer (pH 6.5), 2.0 ml of coumarin (500 ug/ml) and 1.0 ml of mineral elements (final concentration, 10^{-3} M) in a test tube for 6 hours at 37° C. A suitable aliquot was removed from the resulting mixture and analysed and the results are shown in Table 9.

Table 9. Effect of mineral elements on enzyme activity.

Compound added	Melilotic acid produced ug/ml	Relative activity compared to control
None	50	100
KCN	48	96
$CuSO_4$	50	100
$ZnSO_4 \cdot 7H_2O$	55	110
$MgSO_4 \cdot 7H_2O$	60	120

The enzyme poison, cyanide, inhibited slightly the activity, but the enzyme was stimulated slightly by magnesium and zinc.

Effect of reduced pyridine nucleotides on enzyme activity: The effect of reduced pyridine nucleotides on enzyme activity was estimated by incubating 0.2 ml of enzyme solution, prepared from 0.2 $(NH_4)_2SO_4$

saturation (protein, 0.3 mg/ml), with 7.8 ml of 0.1 M phosphate buffer pH 6.8, and 2.0 ml of coumarin (500 ug/ml). To this was added 0.3 mg of DPNH or TPNH and these were incubated at 35° C for 30 mins. Suitable aliquots were analysed for melilotic acid and the results are shown in Table 10.

Table 10. Effect of reduced pyridine nucleotides on enzyme activity.

Compound added	Relative activity compared to control
None	100
DPNH	200
TPNH	280

The conversion of coumarin to melilotic acid is stimulated by the addition of reduced diphosphopyridine nucleotide (DPNH) or reduced triphosphopyridine nucleotide (TPNH).

CONVERSION OF DIHYDROCOUMARIN TO MELILOTIC ACID BY CELL FREE EXTRACTS

Partial purification of dihydrocoumarin hydrolase was obtained by the ammonium sulfate precipitation technique. (see above to page 53 for a description of the method). The results are given in Table 11. calculated as enzyme activity units where one unit of dihydrocoumarin hydrolase activity is defined as that amount of enzyme which forms 10 ug of melilotic acid in 1.0 min. The assay tube contains 1000 ug dihydrocoumarin, 0.9 ml of 0.1 M phosphate buffer at pH 6.8 and 1.0 ml of enzyme preparation. This was incubated at 30° C.

Table 11. Partial purification of dihydrocoumarin hydrolase.

Fraction	Total volume (ml)	Units/ml	Total units	Protein mg/ml	Total protein (mg)	Specific activity units/mg
Crude extract	50	5.1	2550	1.4	70	36
0.2 $(NH_4)_2SO_4$ saturation	20	0.4	80	0.4	8	10
0.2-0.5 $(NH_4)_2SO_4$ saturation	20	4.5	900	0.7	14	64
0.5-0.7 $(NH_4)_2SO_4$ saturation	20	3.8	760	0.8	16	47

Most of the enzyme activity of dihydrocoumarin hydrolase was found in the fraction of 0.2-0.5 $(NH_4)_2SO_4$ saturation and this preparation was purified 2-fold compared to the crude extract with a 35% recovery of the enzyme activity.

Effect of temperature on dihydrocoumarin hydrolase was studied by incubating 1.0 ml of enzyme solution (protein, 0.3 mg/ml) with 8.9 ml of 0.1 M phosphate buffer at pH 6.8, and 0.1 ml of dihydrocoumarin (100 ug) for 10 min. at temperatures of 5, 10, 20, 30, 35, and 40° C. A suitable aliquot was taken and analysed for melilotic acid and the results are shown in Fig. 20.

The optimum temperature for hydrolysis of dihydrocoumarin by the partial purified dihydrocoumarin hydrolase of culture Mac# m32 is approximately 35° C.

Effect of pH on dihydrocoumarin hydrolase was studied by incubating 1.0 ml of enzyme solution (protein, 0.75 mg/ml) with 3.9 ml of various buffers prepared as shown in the Appendix Tables 3 and 4 following the method of Clark and Lubs (165) (pH 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9, and 10) and 0.1 ml of dihydrocoumarin(100 ug) for 2 min. at 35° C. After incubation the resulting mixture was immediately made to a neutral pH then heated to stop the enzyme action and a suitable aliquot was analysed for melilotic acid. The results are shown in Fig. 21.

The optimum pH for hydrolysis of dihydrocoumarin by the partially purified dihydrocoumarin hydrolase of culture Mac# m32 is approximately pH 7.5.

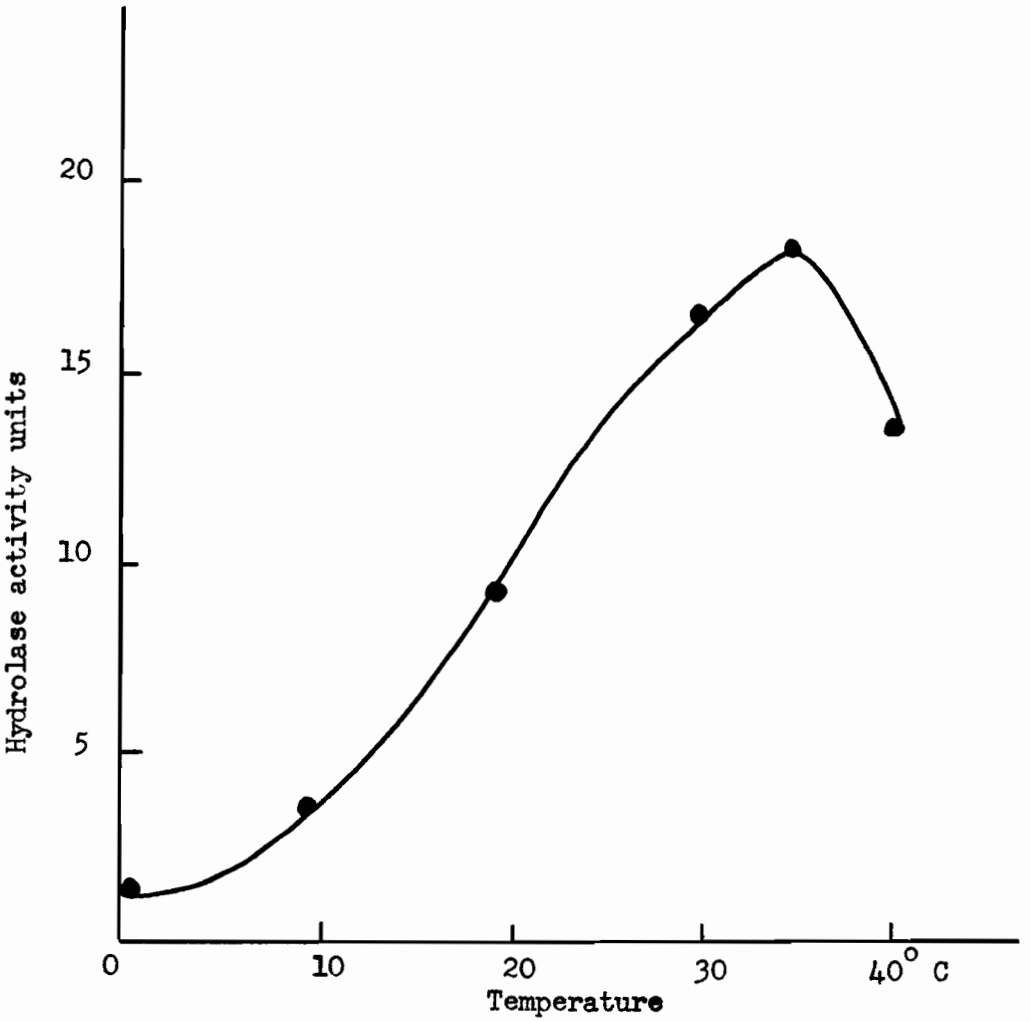


Fig. 20. Effect of temperature on dihydrocoumarin hydrolase.

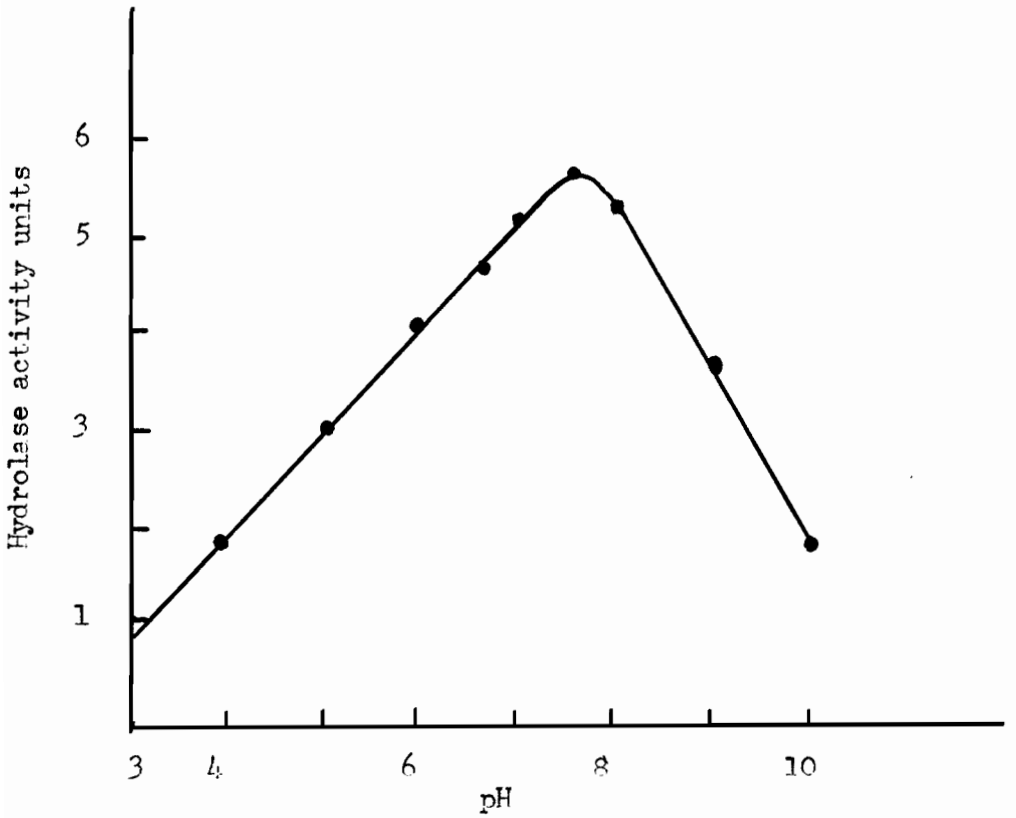


Fig 21. Effect of pH on dihydrocoumarin hydrolase

Effect of mineral elements on dihydrocoumarin hydrolase was estimated by incubating 1.0 ml of enzyme solution (protein, 0.3 mg/ml) with 7.9 ml of buffer at pH 7.0 (prepared as shown in the Appendix), 0.1 ml of dihydrocoumarin (100 ug) and 1.0 ml of mineral elements solution (final concentration 3.0×10^{-3}M) for 15 min. at 35° C. A suitable aliquot was taken from the resulting mixture and analysed for melilotic acid. The results are shown in Table 12.

Table 12. Effect of mineral elements on dihydrocoumarin hydrolase.

Compound added	Relative activity compared to control
None	100
NaI	63
NaF	78
LiCl	84
$CdSO_4$	112
$ZnCl_2$	126

The enzyme was stimulated by cadmium and zinc, but iodide, fluoride, and lithium salt inhibited slightly the enzyme activity.

DISCUSSION

The conversion of coumarin or dihydrocoumarin to melilotic acid by the isolated fungus, Fusarium sp., was demonstrated in intact cells. These experiments with cell free extracts show that the step for conversion of coumarin to melilotic acid is slow, but the conversion of dihydrocoumarin to melilotic acid by the cell free extracts of Fusarium sp., is rapid. Such results suggest that dihydrocoumarin hydrolase activity could prevent the accumulation of dihydrocoumarin in the fermentation liquor.

Studies by Kosuge and Conn (101, 102) on the metabolic fate of coumarin in high plants showed that coumarin, when administered to shoots of white sweet clover, Melilotus alba, is rapidly converted to melilotic acid. Subsequent experiments with cell free extracts of sweet clover have shown that this conversion is stimulated by the addition of reduced triphosphopyridine nucleotide or a TPNH-generating system. In this investigation the step for the conversion of coumarin to melilotic acid is stimulated both by the addition of reduced diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH) to the cell free extracts of Fusarium sp. This reaction was demonstrated by the

colourmetrical method (refer to page 66), but cannot be shown clearly with absorption spectra where the optical density at 340 mu is determined; the reason for this is that these ring compounds interfere seriously. The conversion of coumarin via dihydrocoumarin to melilotic acid is not a single step, moreover the reversible reaction probably occurs. Therefore, it is difficult to assay enzyme activity by conventional assay techniques.

From these results it appears that the initial steps in coumarin metabolism by cell free extracts of Fusarium sp. is first the conversion of coumarin to dihydrocoumarin by hydrogenation. The latter compound, by hydrolysis, is then converted to melilotic acid. These were in confirmation of what have been demonstrated by the intact cells.

PART III

METABOLISM OF O-COUMARIC ACID BY SOIL FUNGI

METHODS

Experiments with isolated fungus, Fusarium sp., have shown that both coumarin and dihydrocoumarin are rapidly converted to melilotic acid. Since o-coumaric acid is a related compound, it is therefore some interest to know the metabolic pattern of o-coumaric acid by the isolated fungi.

MICROORGANISMS

The isolated fungi, Mac# m 31, 32, 33, 34, and 35 were used for this investigation.

MEDIA

The basic medium (Medium C) contained:

$(NH_4)_2SO_4$ .....1.0 gm/liter

K_2HPO_4.....1.0 gm/liter

$MgSO_4$.....0.5 gm/liter

NaCl ..... 0.1 gm/liter

o-Coumaric acid .....0.5 gm/liter

In addition to medium C two other media were used:

Medium D-- 1 gram of sucrose was added to medium C.

Medium E-- 0.01 gram of $FeSO_4.7H_2O$ and 0.005 gram of $MnSO_4$ were added to medium D.

INOCULUM

Cultures for inoculum were grown as slants for four days at room temperature on o-coumaric acid and sucrose agar (composition as medium E). They were stored at 5° C and transfers to fresh medium were made every four weeks. During the four week period all necessary inocula were taken from these slants.

GROWTH CONDITIONS

All cultures were grown at room temperature in either a static condition or more commonly on a rotary shaker running at a speed at 75 r.p.m.

ENZYMATIC ANALYSIS

The enzymatic products were demonstrated with paper chromatographic techniques(see above to page 26)

CHEMICAL ANALYSIS

Determination of o-coumaric acid: o-Coumaric acid was estimated by the colorimetric method used in the determination of coumarin. 4-Hydroxycoumarin does not interfere with the colour reaction. A standard curve is given in Fig. 22.

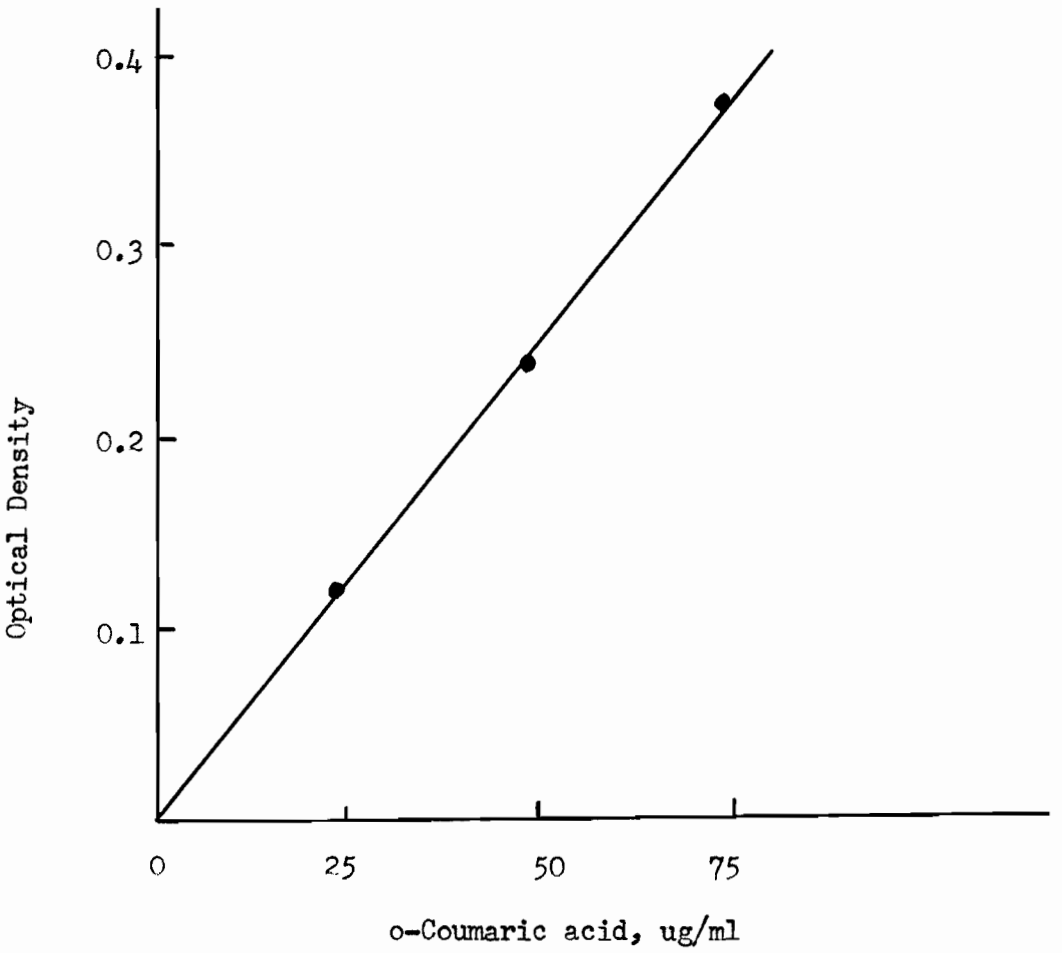


Fig. 22. Standard curve of o-coumaric acid.

RESULTS

GROWTH EXPERIMENTS

Tubes (25 ml) containing 10 ml of medium C were inoculated with cultures Mac# m31, 32, 33, 34 and 35 and incubated at 25° C for periods of 10 days. The results are shown in Table 13.

Table 13. Growth of fungi with o-coumaric acid as sole carbon source.

Organism No.	Growth
Mac# m31	Very good
Mac# m32, 33, 34 and 35.	Good

CHANGES IN ACIDITY DURING GROWTH OF THE CULTURE

Flask (250 ml) containing 100 ml of medium C was inoculated with culture Mac# m31 and incubated at room temperature for a period of 15 days. During growth the solution become slightly acid in a medium containing o-coumaric acid as the sole carbon source as the results in Table 14 indicate.

Table 14. Changes in pH during fermentation of o-coumaric acid.

Days	0	3	10	15
pH	6.9	6.9	6.8	6.7

IDENTIFICATION OF 4-HYDROXYCOUMARIN FORMED DURING THE UTILIZATION OF O-COUMARIC ACID

The moulds isolated from coumarin enriched medium are able to utilize o-coumaric acid as sole carbon source although the rates of growth are very slow. Flasks (250 ml) containing 100 ml of medium C were inoculated with culture Mac# m31, 32, 33, 34 and 35 and incubated at 25° C under a static condition for periods of 30 days. During growth the concentration of o-coumaric acid fell steadily. Paper chromatography of the ether soluble materials from these media after 15-30 days incubation revealed a compound with identical R_f value and the same colour reaction as 4-hydroxycoumarin. Spectroscopic evidence for the formation of 4-hydroxycoumarin is given in Fig. 23. Chromatographic evidence for the formation of 4-hydroxycoumarin is shown in Fig. 24.

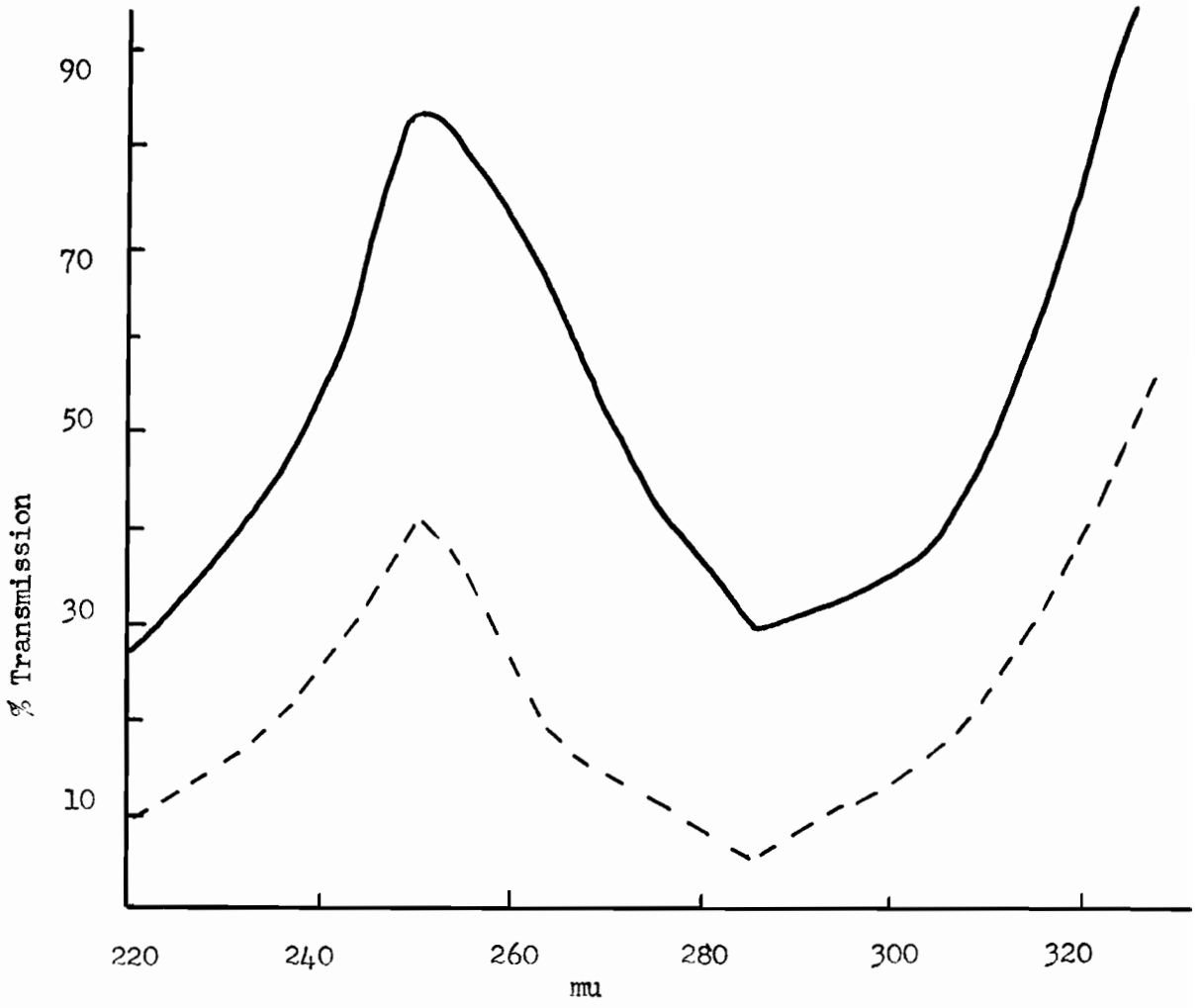


Fig 23. Ultraviolet absorption spectra of authentic 4-hydroxycoumarin ——— and of isolated 4-hydroxycoumarin– – – eluted from paper chromatograms of culture liquor from Mac# m31 after 30 days incubation.

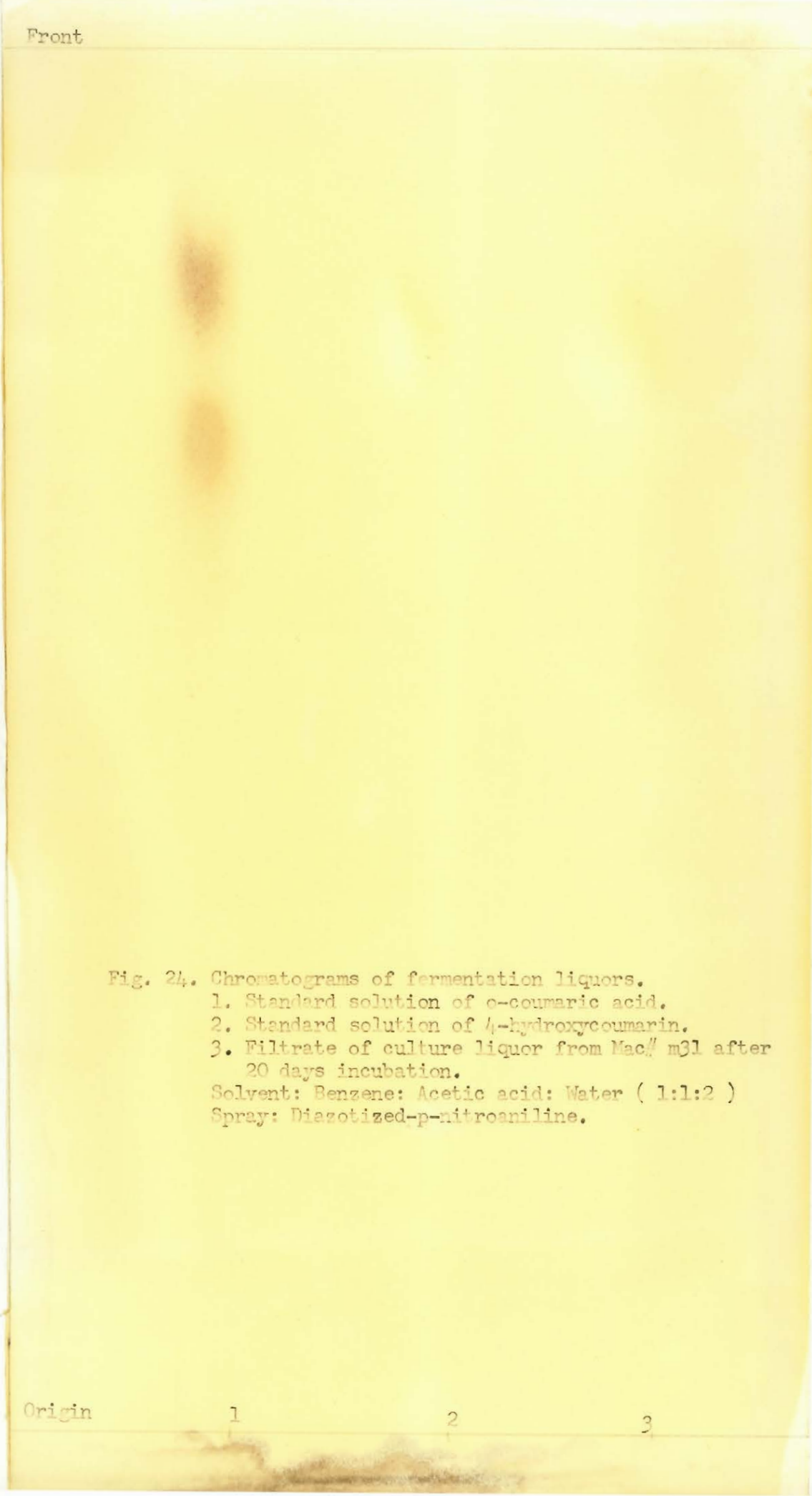


Fig. 24. Chromatograms of fermentation liquors.
1. Standard solution of o-coumaric acid.
2. Standard solution of 4-hydroxycoumarin.
3. Filtrate of culture liquor from Mac. m31 after 20 days incubation.
Solvent: Benzene: Acetic acid: Water (1:1:2)
Spray: Diazotized-p-nitroaniline.

IDENTIFICATION OF THE FERMENTATION PRODUCT: 4-HYDROXYCOUMARIN

Flasks (250 ml) containing 100 ml medium C were inoculated with culture Mac# m31 and incubated at 25° C in a static state. After 30 days, the filtrate was acidified and extracted with ethyl ether. After removal of the solvent from the extract, the residue was recrystallized from hydrochloric acid following by isolation of the crystalline material by filtration. After drying in 100° C oven, the melting point was found to be 210° C (uncorrected); authentic 210° C (163); mixed melting point 210° C. A comparison of the absorption spectra of the isolated 4-hydroxycoumarin with the authentic sample is shown in Fig 26. On the bases of these tests the isolated product was definitely identified as 4-hydroxycoumarin.

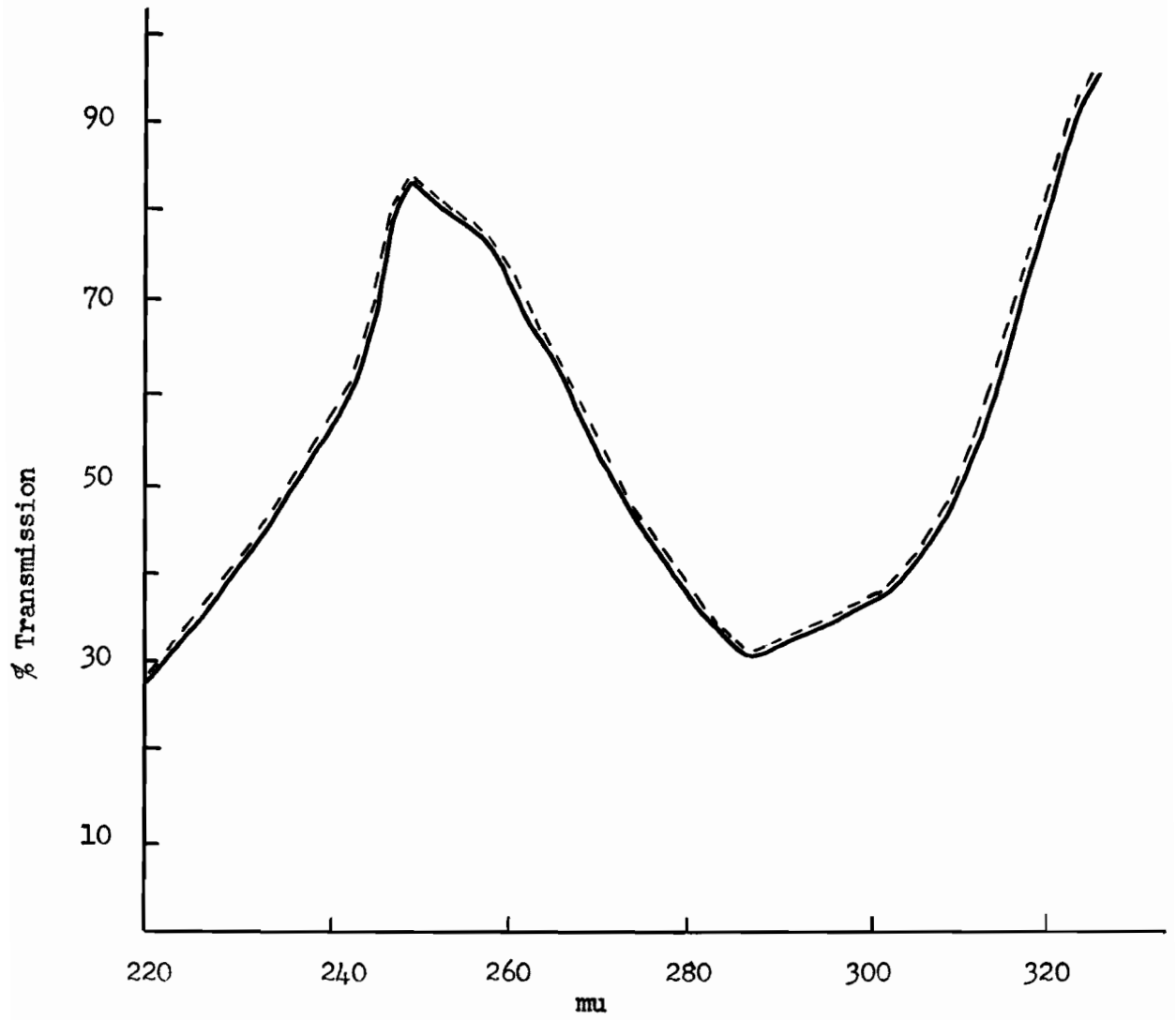


Fig. 26. Absorption spectra: ——— 4-hydroxycoumarin 0.001g in 100 ml 1% NaOH. - - - - isolated 4-hydroxycoumarin 0.001g in 100 ml 1% NaOH.

THE FORMATION OF 4-HYDROXYCOUMARIN IN THE PRESENCE OF AN OTHER SOURCE OF CARBON (SUCROSE)

Flasks (250 ml) containing 100 ml of medium D were inoculated with culture Mac # m31 and incubated at 25° C under static state for periods of 15 days. The rate of growth is more vigorus with sucrose added than previously found with o-coumaric acid as the sole carbon source. During growth the pH drops as more acid is produced as shown in Table 15.

Table 15. Variations in pH with sucrose and o-coumaric acid as carbon sources.

Days	0	3	10	15
pH	6.7	6.5	6.0	5.7

Paper chromatography of the ether soluble material in the medium after 20 days incubation revealed that a compound with identical R_f value and the same colour reactions as 4-hydroxycoumarin was present.

The formation of 4-hydroxycoumarin with sucrose as the carbon source in addition to o-coumaric acid is more rapid than where o-coumaric acid is the only source of carbon.

EFFECT OF AERATION ON THE FORMATION OF 4-HYDROXYCOUMARIN

Flasks (500 ml) containing 100 ml medium D were inoculated with culture# m31 and incubated at 25° C for 10 days on a shaker rotating at

75 r.p.m. Paper chromatography of the ether soluble material in the medium after 10 days reveal the presence of 4-hydroxycoumarin only. Aeration was shown to be essential to the rapid utilization of o-coumaric acid and all tests were incubated with aeration in subsequent trials.

EFFECT OF TRACE ELEMENTS ON THE FORMATION OF 4-HYDROXYCOUMARIN

The effect of trace elements on the formation of 4-hydroxycoumarin was estimated by measuring the disappearance of o-coumaric acid.

Flasks (500 ml) containing 100 ml of medium D or 100 ml of medium E were inoculated with culture Mac# m31 and incubated at 25° C for 7 days in a shaker with a speed of 75 r.p.m. In a medium containing $FeSO_4$ and $MnSO_4$ the rate of disappearance of o-coumaric acid is considerably faster as shown in Table 16.

Table 16. The amount of o-coumaric acid in the medium during the different periods.

Days	o-Coumaric acid, ug/ml	
	Medium D (without $FeSO_4$ and $MnSO_4$)	Medium E (with $FeSO_4$ and $MnSO_4$)
0	500	500
2	370	230
4	116	64
5	62	12
6	24	0
7	0	

PRODUCTION OF 4-HYDROXYCOUMARIN

Flasks (500ml) containing 100 ml of medium E were inoculated with Mac# m31 and incubated at room temperature on a shaker with a speed of 75 r.p.m. for 9 days. The mycelia were removed by filtration and the filtrate was acidified then extracted with ethyl ether. The ether was removed by evaporation and 4-hydroxycoumarin was crystallized twice from hydrochloric acid and dried in an oven at 100° C , high yield of crystalline 4-hydroxycoumarin (52%) was obtained as shown in Table 17.

Table 17. Production of 4-hydroxycoumarin.

Experiment No	o-Coumaric acid supplied grams/3 flasks	4-Hydroxycoumarin recovered grams/3 flasks	% Yield
1	0.15	0.081	53.3
2	0.15	0.079	52.2

CONVERSION OF 4-HYDROXYCOUMARIN TO DICUMAROL

Flasks (250ml) containing 0.05 gram of 4-hydroxycoumarin, (dissolved in 100 ml hot water), and 1 ml of 40% HCHO was left at room temperature in a static state. After 12 hours a large amount white crystalline

material was formed. After recovery by filtration, and drying, 0.04 g was recovered (93% of theory). This material was identified as dicumarol by behavior on paper chromatograms and by its solubility in various solvents. This experiment proves (as suggested by Bellis (10)) that in the presence of formaldehyde, 4-hydroxycoumarin is spontaneously converted to dicumarol. Any experiments to prove this conversion biologically must be carried out under conditions that rigidly exclude contamination through the air with formaldehyde.

DISCUSSION

Studies by Bellis (10) on the metabolic fate of o-coumaric acid in the moulds, Penicillium jenseni and Penicillium nigricans showed that o-coumaric acid was converted to 4-hydroxycoumarin. The presence of sucrose in the medium in addition to o-coumaric acid did not interfere with this hydroxylation. In this investigation, the moulds isolated from coumarin enriched medium(Fusarium sp.) are able to utilize o-coumaric acid as sole carbon source in a simple salts medium, although the rate of growth is very slow. In a similar medium containing sucrose and o-coumaric acid, growth is vigorous. Paper chromatography of the ether soluble matter in both media revealed a compound with identical R_f value and the same colour reactions as 4-hydroxycoumarin. Isolated 4-hydroxycoumarin was identified by its melting point and by its ultraviolet spectrum in sodium hydroxide solution. These results confirm the observations of Bellis (10). With these Fusarium sp., aeration and trace elements increase the rate of formation of 4-hydroxycoumarin. No other products were detected.

Metabolism of o-coumaric acid in rats and rabbits was investigated by Booth et al (17). These workers suggest that o-coumaric acid is converted first to o-hydroxyphenylhydracrylic acid and then to 4-hydroxy-coumarin. In this study no evidence was found that o-hydroxyphenylhydra-

crylic acid is an intermediate and mechanism of o-coumaric acid to 4-hydroxycoumarin is still obscure.

As for the origin of dicumarol, Stahman, Huebner, and Link (163) identified dicumarol as the substance in improperly cured hay which causes a hemorrhagic condition in cattle. They suggested that dicumarol was formed from coumarin because of the similar structure. Growth of species of Penicillium in a medium containing coumarin as the sole carbon source gave no intermediates (10). Growth of Fusarium sp.(this investigation), or Pseudomonas sp (76) in a medium containing coumarin as the sole carbon source resulted in no detectable amounts of dicumarol or the precursor, 4-hydroxycoumarin. On the other hand, growth of either species of Penicillium in a medium containing 4-hydroxycoumarin and sucrose provided some evidence for the presence of a small amount of dicumarol (10), but no evidence was found in this investigation that 4-hydroxycoumarin accumulated in the medium can be further converted to dicumarol. The possibility that 4-hydroxycoumarin is converted to dicumarol by absorption of formaldehyde from the atmosphere or formaldehyde produced by microorganisms cannot be ruled out since dicumarol was formed readily in a mixture of 4-hydroxycoumarin and formaldehyde. Based on these data dicumarol is probably formed from o-coumaric acid through 4-hydroxycoumarin to dicumarol.

GENERAL DISCUSSION

Stanier's 'sequential induction' technique has been used as a useful tool for study of oxidative metabolism of ring compounds, but in some instances this technique has failed to demonstrate a sequence of reaction in the compounds most readily oxidized. For example, a study by Rogoff (132) of the bacterial oxidation of quinic acid showed that strains of Pseudomonas and Achromobacter spp, isolated from soil, utilized quinic acid as a sole source of carbon, protocatechuic acid was produced as an intermediate in this oxidation. Protocatechuic acid was also produced in the course of oxidation of p-hydroxybenzoic acid by these organisms. Sequential induction experiments indicate that p-hydroxybenzoic acid is not an intermediate in quinic acid oxidation by the organisms used. Mead et al (112) 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 (17) showed that the enzyme reducing o-coumaric acid to melilotic acid is reversible. Thus no sequence of intermediates could be established by the 'sequential induction' technique. Halvorson (76) reported that the 'sequential induction' technique

of Stanier was applied to the metabolism of Pseudomonas sp grown on coumarin and found that of 23 compounds tested only o-coumaric acid, melilotic acid, and 2,3-dihydroxyphenylpropionic acid behaved as intermediates of coumarin degradation but the technique failed to reveal a reaction sequence for these compounds. For these reasons, Stanier's 'sequential induction' technique was not used in this study.

Bellis reported that coumarin was utilized as sole carbon source by two moulds, Penicillium jenseni and Penicillium nigricans (10). The concentration of coumarin fell steadily but no intermediates were found. In this investigation, the moulds isolated by enrichment techniques were Fusarium sp, and these were able to utilize coumarin as the sole carbon source. The intermediate product, melilotic acid, was formed in large amounts and was identified. Metabolism of coumarin in bacteria was studied by Halvorson (76) who demonstrated that from tritiated coumarin as a substrate, melilotic acid was produced that was radioactive. The mechanism for the conversion of coumarin to melilotic acid in the Pseudomonas sp. was suggested by Halvorson to go through o-coumaric acid since melilotic acid was identified also in fermentation liquor containing o-coumaric acid as the sole carbon substrate. This confirms the data of

Booth et al (17) studying coumarin metabolism in rats or rabbits.

The mechanism of conversion of coumarin to melilotic acid in the fungus, Fusarium sp., is certainly not the same scheme since the Fusarium sp.utilize o-coumaric acid as sole carbon source but produce a high yield of 4-hydroxycoumarin while with coumarin as the carbon source melilotic acid is produced. Moreover, a cell preparation of this fungus was shown to convert dihydrocoumarin to melilotic acid is almost quantitative. Thus the mechanism of degradation for these Fusarium sp. is that coumarin, by hydrogenation, is first converted to dihydrocoumarin and the latter compound by hydrolysis is then converted to melilotic acid. With cell free extracts of Fusarium sp., the evidence for this route of metabolism was very good. The data indicates that the conversion of coumarin to melilotic acid is slow but the hydrolase converting dihydrocoumarin to melilotic acid is very active. This suggests that the active dihydrocoumarin hydrolase prevents the accumulation of dihydrocoumarin in the fermentation liquor. The conversion of coumarin to melilotic acid is stimulated both by the addition of reduced diphosphopyridine nucleotide (DPNH) and triphosphopyridine nucleotide(TPNH) (this reaction is difficult to demonstrate by the usual assay technique as the phenolic compounds have an absorption spectrum in the the ultraviolet that interferes with these measurements). These findings confirm the observations of Kosuge and Conn (101,102) in higher plants.

The conversion of coumarin to 2,3-dihydroxyphenylpropionic acid was demonstrated conclusively by Halvorson (76) for Pseudomonas sp. but previously a mechanism of this nature was not encountered and this is the most fruitful path way to pursue in future degradation studies.

The side chain of melilotic acid was originally thought to be the site of biological attack, such that each carbon atom of the side chain was removed successively with the resultant formation of catechol. No evidence was obtained in Pseudomonas (76) or Fusarium (this investigation) to prove this meehanism of degradation.

Studies by Bellis (10) on the metabolic fate of o-coumaric acid in moulds, Penicillium jenseni and Penicillium nigricans showed that o-coumaric acid was converted to 4-hydroxycoumarin. In this study, the moulds belonging to the Fusarium sp. also converted o-coumaric acid to 4-hydroxycoumarin. Booth et al (17) reported that o-coumaric acid was first converted to o-hydroxyphenylhydracrylic acid then to 4-hydroxycoumarin in animals. In this investigation, no evidence was shown to favour this mechanism.

Concerning the origin of dicumarol in spoiling sweet clover, Halvorson (76) reported that with Pseudomonas sp. neither 4-hydroxycoumarin, nor dicumarol, nor o-coumaric acid were detected in the

fermentation liquor with coumarin as the substrate nor was 4-hydroxy-coumarin utilized by these Pseudomonas sp. under manometric conditions, i.e. no evidence was obtained to suggest any of the Pseudomonas cultures play a role in the formation of dicumarol in spoilt sweet clover hay. Growth of either species of Penicillium in a medium containing 4-hydroxy-coumarin and sucrose provided evidence for the presence of a small amount of dicumarol (10), but with Fusarium sp. growing on o-coumaric acid , 4-hydroxycoumarin accumulates in the medium and no evidence was found to indicate any further conversion to dicumarol. The possibility that 4-hydroxycoumarin is converted to dicumarol by non-biological reactions by absorption of formaldehyde from the atmosphere is certainly the most logical explanation based on the evidence.

SUMMARY

Part I. Metabolism of coumarin by soil fungi.

Moulds belonging to the Fusarium genus, isolated by an enrichment technique, are able to utilize coumarin as the sole carbon source in a medium containing inorganic nitrogen and various salts. The rate of growth of these fungi is slow but the concentration of coumarin falls steadily and melilotic acid is formed. In a similar medium containing sucrose and coumarin as sources of carbon, growth is still slow and melilotic acid is formed at a slower rate but is still detectable. Trials indicated that coumarin metabolism is more rapid in well-aerated medium on a rotary shaker but under these conditions melilotic acid does not accumulate. The rate of metabolism of coumarin was enhanced by the addition of iron and manganese in trace amounts to the usual salts medium.

With resting cells in a phosphate buffer containing coumarin, production of melilotic acid is slow but is measurable. However, with dihydrocoumarin as the substrate, melilotic acid is recovered almost quantitatively.

Melilotic acid was isolated from various culture liquors and was identified by its melting point and by its ultra violet spectrum.

A chemical method for determining the relative concentrations of coumarin and melilotic acid in mixtures was developed.

Part II. Coumarin metabolism by cell free extracts.

Experiments with cell free extracts indicated that the conversion of coumarin to melilotic acid is slow, but that the conversion of dihydrocoumarin to melilotic acid is rapid. The latter enzyme, dihydrocoumarin hydrolase, was partially purified and concentrated and its properties investigated. Maximum activity was obtained at pH 7.5 and a temperature optimum at 35° C. Zinc and cadmium were found to activate the enzyme while iodine, fluorine and lithium were inhibitors.

Part III. Metabolism of o-coumaric acid by soil fungi.

Fusarium sp. isolated from coumarin enriched medium can utilize o-coumaric acid as the sole carbon source, but the rate of growth is very slow. In a similar medium containing sucrose and o-coumaric acid, growth is vigorous and the rate of utilization of o-coumaric acid is rapid. Both aeration and the addition of trace elements increased the rate of substrate utilization and product formation.

Paper chromatography of the ether soluble material from the medium revealed a compound with identical R_f value and colour reaction of 4-hydroxycoumarin. 4-Hydroxycoumarin accumulates in the medium (50% of the theoretical yield was obtained as a crystalline product) and was identified by its melting point and by its ultraviolet spectrum.

No other products were found from o-coumaric acid utilization. No evidence for the biological conversion of 4-hydroxycoumarin to dicumarol was found although experiments showed that in the presence of added formaldehyde, dicumarol was formed in high yields from 4-hydroxycoumarin by a non-enzymatic reaction.

CLAIM OF CONTRIBUTION TO KNOWLEDGE

The following information, as revealed in these studies, is claimed to be a contribution to knowledge.

(1). Fungi belonging to the Fusarium genus, isolated by enrichment technique, are able to utilize coumarin as the sole carbon source in a medium containing various salts and inorganic nitrogen. Most other fungi are inhibited by coumarin.

(2). Factors affecting growth of these cultures and those affecting the rate of coumarin utilization were studied.

(3). By testing growing cultures, resting cells, and cell free extracts, the route of coumarin degradation was found to proceed first by reduction to dihydrocoumarin and then by hydration to melilotic acid. Active enzyme preparations were isolated for both conversions and dihydrocoumarin hydrolase was partially purified to give a very active preparation.

(4). A chemical method for determining the relative concentrations of coumarin and melilotic acid in mixtures was developed.

(5). These same Fusarium sp. utilize o-coumaric acid as the sole carbon source. The route of metabolism is different than from coumarin and the only product found was 4-hydroxycoumarin. The 4-hydroxycoumarin accumulated in the medium and was not further metabolized.

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APPENDIX

APPENDIX TABLE 1

Absorption spectra.

mu	Melilotic acid 3.0 mg/100 ml, 95% alcohol. %T	Isolated melilotic acid 3.0 mg/100ml, 95% alcohol %T
210	14.7	10.6
215	15.2	13
220	18.5	19.4
225	28.3	32.55
230	49.7	52.55
235	73.3	72.2
240	79	78.9
245	78.5	77.8
250	76.8	74.1
255	72	66.5
260	65.2	57.2
265	56.9	50
270	49.5	45
275	45.5	44
280	48.6	47
285	67.4	52.5
290	77.7	74
295	82	80

APPENDIX TABLE 2

Absorption spectra.

mu	4-Hydroxycoumarin 1.0 mg/100 ml, 1% NaOH. %T	Isolated 4-hydroxycoumarin 1.0 mg/100 ml, 1% NaOH. %T
220	25.8	29.4
225	33	36.2
230	39.5	42
235	46.2	48.4
240	55.6	57.2
245	71	71.55
250	83.6	84.1
255	80.2	80.4
260	72.4	73.05
265	62.6	64.2
270	52.55	54.55
275	42.6	45.05
280	37.55	40.2
285	30.75	33.4
290	32.4	35
295	34	36.7
300	35.2	37.9
305	40.2	43
310	48.49	51
315	61.2	63.4
320	79.2	80.5
325	91.1	91.5
330	95.5	95.3

APPENDIX TABLE 3

Standard buffer solution.

pH*	50 ml, 0.2M KH-ortho-phthalate and X ml 0.2M HCl X
2.2	46.70
2.4	39.60
2.6	32.95
2.8	26.42
3.0	20.32
3.2	14.70
3.4	9.90
3.6	5.97
3.8	2.63

pH*	50 ml, 0.2M KH-ortho-phthalate and X ml 0.2M NaOH X
4.0	0.40
4.2	3.70
4.4	7.50
4.6	12.15
4.8	17.70
5.0	23.85
5.2	29.95
5.4	35.45
5.6	39.85
5.8	43.00
6.0	45.45
6.2	47.00

* To obtain the required pH, mix the solutions in the proportions indicated, and dilute to 200 ml.

APPENDIX TABLE 4

Standard buffer solution.

pH	ml A (0.2 M disodium phosphate)	ml B (0.1M citric acid)
6.4	13.85	6.45
6.6	14.55	5.45
6.8	15.45	4.55
7.0	16.47	3.53
7.2	17.39	2.61
7.4	18.17	1.83
7.6	18.73	1.27

pH*	50 ml, 0.2M KCl and 50 ml boric acid containing 12.405 g of H_3BO_3 per liter and X ml 0.2 M NaOH
	X
7.8	2.61
8.0	3.97
8.2	5.90
8.4	8.50
8.6	12.00
8.8	16.30
9.0	21.30
9.2	26.70
9.4	32.00
9.6	36.85
9.8	40.80
10.0	43.90

* To obtain the required pH, mix the solutions in the proportions indicated, and dilute to 200 ml.