METABOLISM OF D-TRYPTOPHAN BY A *FLAVOBACTERIUM* SPECIES

By

JERRY ROY MARTIN

Bachelor of Science

Utah State University

Logan, Utah

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METABOLISM OF D-TRYPTOPHAN BY A

FLAVOBACTERIUM SPECIES

Thesis Approved:

Norman D Durham
Thesis Adviser

Eric C. Miller

Lynn P. Hall

Roger E. Koeppe

Jan V Zaloha

J. R. Boggs
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

D-Amino acids were first prepared in 1886 by Schulze and Bosshard [cited in (94) on p. 68] using the asymmetric utilization of racemic amino acids by Penicillium glaucum, but it was several years later that the natural occurrence of D-amino acids was established. D-Amino acids occur in a variety of microorganisms (13, 15, 41, 86) and in natural microbial products (4, 12, 30, 90). Many antibiotics contain D-amino acid moieties, and some are involved in structural elements such as cell walls and capsular materials.

In recent years the structure and mechanism of action of antibiotics have been intensively studied. The peptides in certain antibiotics do not commonly display the regularity in amino acid sequence that has been recognized in proteins. These irregularities encompass structure as well as the steric configuration of the amino acid moieties. It is believed that antibiotic action can be attributed, at least in part, to the presence of D-amino acids (2).

Bacterial cell walls contain the D-isomers of alanine, aspartic and glutamic acids. Capsular material from some species of Bacillus is composed exclusively of both L- and D-glutamic acid, while the material isolated from B. anthracis contains only D-glutamic acid. In both cases the amino acid moieties are γ-linked into a straight chain arrangement.

Several years ago Kogl and Erxleben (50) reported the occurrence

of D-amino acids in tumor proteins and suggested the neoplastic process was related to the presence of D-amino acids. Although most investigators do not share the view of Kogl and Erxleben, Kogl (51) has reported the presence of D-amino acids in animal tissues.

D-Amino acids are no longer considered "unnatural" even though the distribution is limited in nature. Much is known about the properties of D-amino acid enzymes, but little is known about the role of D-amino acids in metabolism. Thus, these compounds have aroused considerable interest.

Many animals can utilize certain D-amino acids (8) and the rate of growth depends principally on the rate of conversion to the L-isomer. In general, the nutritional value of the D-isomers for growth is less than that of the corresponding L-enantiomorphs, although both isomers produce almost equal effects under certain conditions. The metabolic value of D-amino acids presents many questions since it is known that D-amino acids are absorbed much more slowly by the intestional mucosa than are the corresponding L-isomers.

Microorganisms utilize D-amino acids more effectively than do mammals since they adapt to nutritional conditions more readily than higher animals. Bacteria may utilize D-amino acids directly, catalyze inversion by racemization, carry out oxidation and reamination, or use other mechanisms. Rydon (75) listed 26 bacterial species that would use at least one of 13 D-amino acids. The most frequently used D-amino acids were alanine, serine, valine, glutamic acid, aspartic acid, and histidine. The D-isomers of methionine, tryptophan, proline, leucine, tyrosine, phenylalanine, cysteine, and cystine may also be utilized (67).

L-Tryptophan was first isolated from casein in 1902 by Hopkins and

Cole [cited in (26) on p. 2320]. Although D-tryptophan has never been reported to occur naturally, there has been considerable interest in its metabolism. The purpose of this study was to elucidate the pathway of catabolism of D-tryptophan by a species of Flavobacterium and to investigate the influence of tryptophan analogs on the oxidation of D- and L-tryptophan.

CHAPTER II

REVIEW OF LITERATURE

A. Pathways of L-tryptophan metabolism

The metabolism of tryptophan by microorganisms may be accomplished by at least five reactions: (a) oxidation to N-formylkynurenine; (b) conversion to indoleacetic acid; (c) fission to indole, pyruvic acid and ammonia; (d) decarboxylation to tryptamine, and (e) hydroxylation to 5-hydroxytryptophan. Each reaction leads to a separate pathway of metabolism.

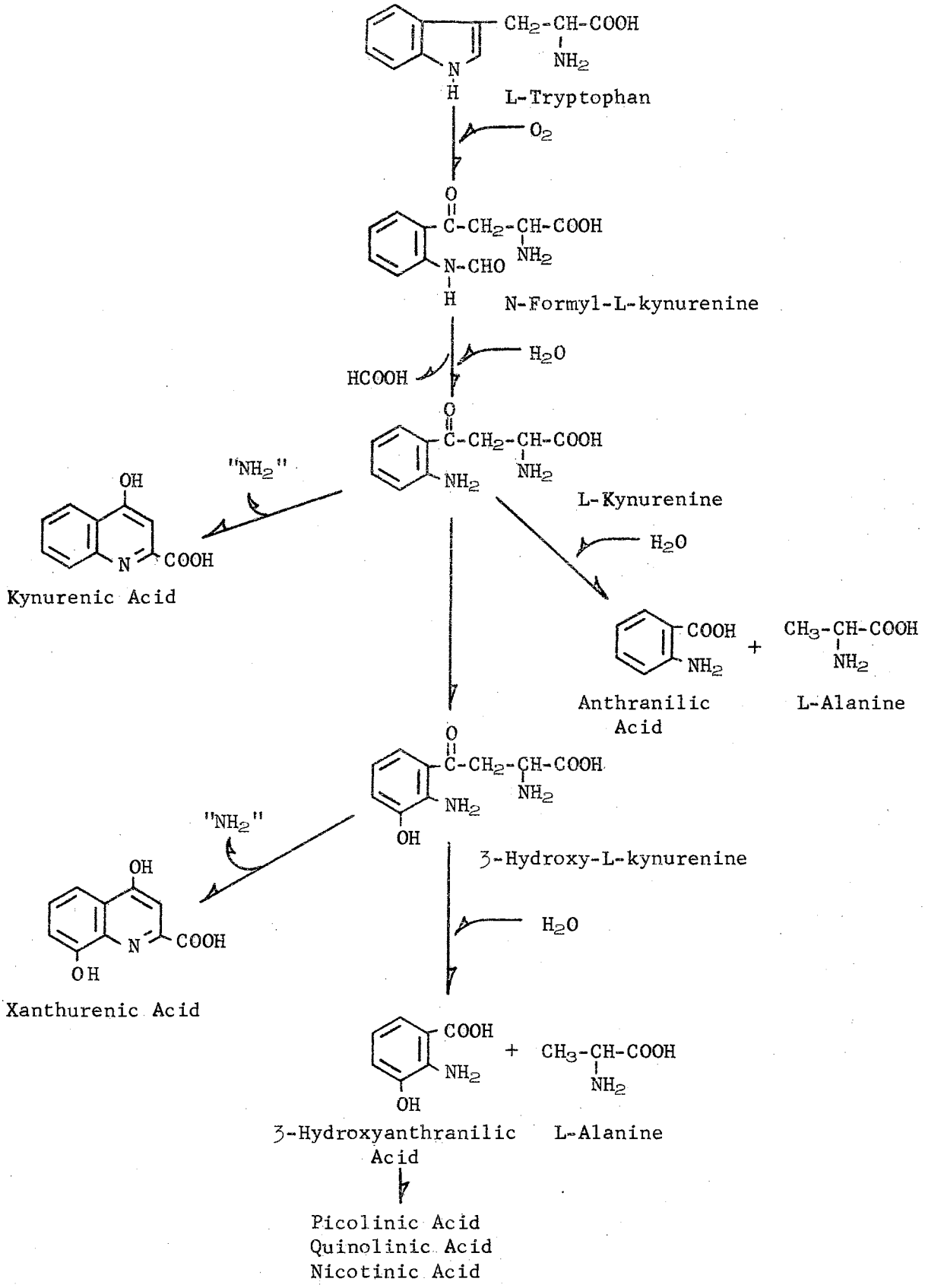
1. The kynurenine-anthranilic acid pathway

This pathway has been formulated mainly from results obtained with Neurospora, Pseudomonas and a number of animal species (Figure 1).

L-Tryptophan is converted to L-kynurenine by addition of O_2 and hydrolysis of N-formylkynurenine to kynurenine and formic acid. Kotake and Masayama named the enzyme system responsible "tryptophan pyrrolase" (54). The cleavage of the indole nucleus is catalyzed by tryptophan peroxidase-oxidase which is inhibited by cyanide, carbon monoxide or catalase (49). Experiments using $^{18}O_2$ and $H_2{}^{18}O$ demonstrated that the oxygen atoms introduced during rupture of the indole nucleous arise from molecular oxygen (34). The formation of N-formylkynurenine with the introduction of 1 mole of oxygen eliminated the need of an intermediate product. 2-Oxindolyalanine (65,77), α,β-dihydroxytryptophan (34), 5- or 7-hydroxytryptophan (21), α-methyltryptophan, or D-tryptophan (92) failed to react in the pyrrolase system.

Figure 1

The kynurenine-anthranilic acid pathway and related reactions.


L-Tryptophan
O_2
N-Formyl-L-kynurenine
HCOOH
H_2O
"NH_2"
Kynurenic Acid
L-Kynurenine
H_2O
Anthranilic Acid
L-Alanine
"NH_2"
3-Hydroxy-L-kynurenine
H_2O
Xanthurenic Acid
3-Hydroxyanthranilic Acid
L-Alanine
Picolinic Acid
Quinolinic Acid
Nicotinic Acid

Kynurenine formylase catalyzes the hydrolysis of N-formylkynurenine to kynurenine and formic acid in microorganisms (35, 42). When hydrolyzed in the presence of $H_2{}^{18}O$, one atom equivalent of ^{18}O is found in formic acid.

Kynurenine-3-hydroxylase hydroxylates kynurenine at position 3 and is specific for the L-isomer. This enzyme is not widespread in microorganisms but is involved in the major pathway of tryptophan dissimilation in most mammals. Interest was aroused in the relationship between hydroxykynurenine and niacin when Yanofsky and Bonner (107) observed that hydroxykynurenine yielded nicotinic acid in a genetically blocked mutant of Neurospora. The hydroxylation reaction was first observed in vitro by de Castro, Price, and Brown (14) with mitochondrial preparations, and the reaction requires TPNH. Saito, Hayaishi and Rothberg (76) demonstrated that molecular oxygen was required for the hydroxylation by experiments with ^{18}O.

Kynureninase, which requires pyridoxal phosphate, catalyzes cleavage of the side chain from kynurenine to form anthranilic acid or from 3-hydroxykynurenine to form 3-hydroxyanthranilic acid. The second product from both kynurenine and 3-hydroxykynurenine is alanine (68). Anthranilic acid and 3-hydroxyanthranilic acid are key intermediates in divergent pathways. Anthranilic acid is important in the aromatic pathway in bacteria, and 3-hydroxyanthranilic acid is involved in the oxidative scheme in mammals and in niacin synthesis in Neurospora. The enzyme has been studied in Neurospora (43) and in Pseudomonas (31).

3-Hydroxyanthranilic acid oxidase has not been reported in bacteria, but it is prevalent in Neurospora where 3-hydroxyanthranilic acid is an intermediate in nicotinic acid formation from tryptophan (11,

69). The conversion of 3-hydroxyanthranilic acid to nicotinic acid, picolinic acid and quinolinic acid are important steps in the metabolism of tryptophan in vertebrates. Ring cleavage of 3-hydroxyanthranilic acid by 3-hydroxyanthranilic acid oxidase is involved in the complete oxidation of tryptophan in mammals (27). The oxygenase requires oxygen, Fe^{++}, and free SH groups for activity (60). Wiss and Bettendorf (102) studied the spectral properties (absorption at 275 and 360 mμ), and reaction with 2,4-dinitrophenylhydrazine of the unstable aliphatic compound and postulated that the intermediate was 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid.

Quinolinic acid is formed nonenzymically from 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid (60). Picolinic acid is formed enzymically by decarboxylation, but the role of picolinic acid in the metabolism of tryptophan is not clear.

The enzymatic formation of nicotinic acid from 3-hydroxyanthranilic acid has not been established. Recently Nishizuka and Hayaishi (71) reported that 3-hydroxyanthranilic acid is converted to niacin ribonucleotide in the presence of 5-phosphoribosyl-1-pyrophosphate by an enzyme preparation obtained from rat liver, and 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid and quinolinic acid were intermediates in this conversion. Quinolinic acid is utilized by Neurospora mutants and rats (38) as a source of niacin. Wilson and Henderson (100) observed that quinolinic acid is converted to niacin in developing chick embryos.

The terminal route of tryptophan degradation in mammals was proposed by Gholson and co-workers (22, 23, 24). These investigators found that 3-hydroxyanthranilic-1-^{14}C acid yielded acetate labeled in the methyl group. 3-Hydroxyanthranilic acid labeled in both the carboxyl

group and C-1 yielded acetate labeled chiefly in the C-2 position. This leaves either C-2 or C-6 of 3-hydroxyanthranilic acid as the source of the carboxyl carbon of acetate. Carbon-7a of tryptophan formed the carboxyl group of acetate. Gholson, Sanders, and Henderson (25) noted that glutaric acid was involved in the degradation of tryptophan, and Hobbs and Koeppe (39) suggested glutaconic acid, β-hydroxyglutaric acid and β-ketoglutaric acid as intermediates.

2. The quinolinic pathway

This pathway is important in certain bacteria for the complete oxidation of tryptophan, but kynurenic acid is not readily metabolized by most animals. The quinolinic pathway arises by the transamination and cyclization of kynurenine (66).

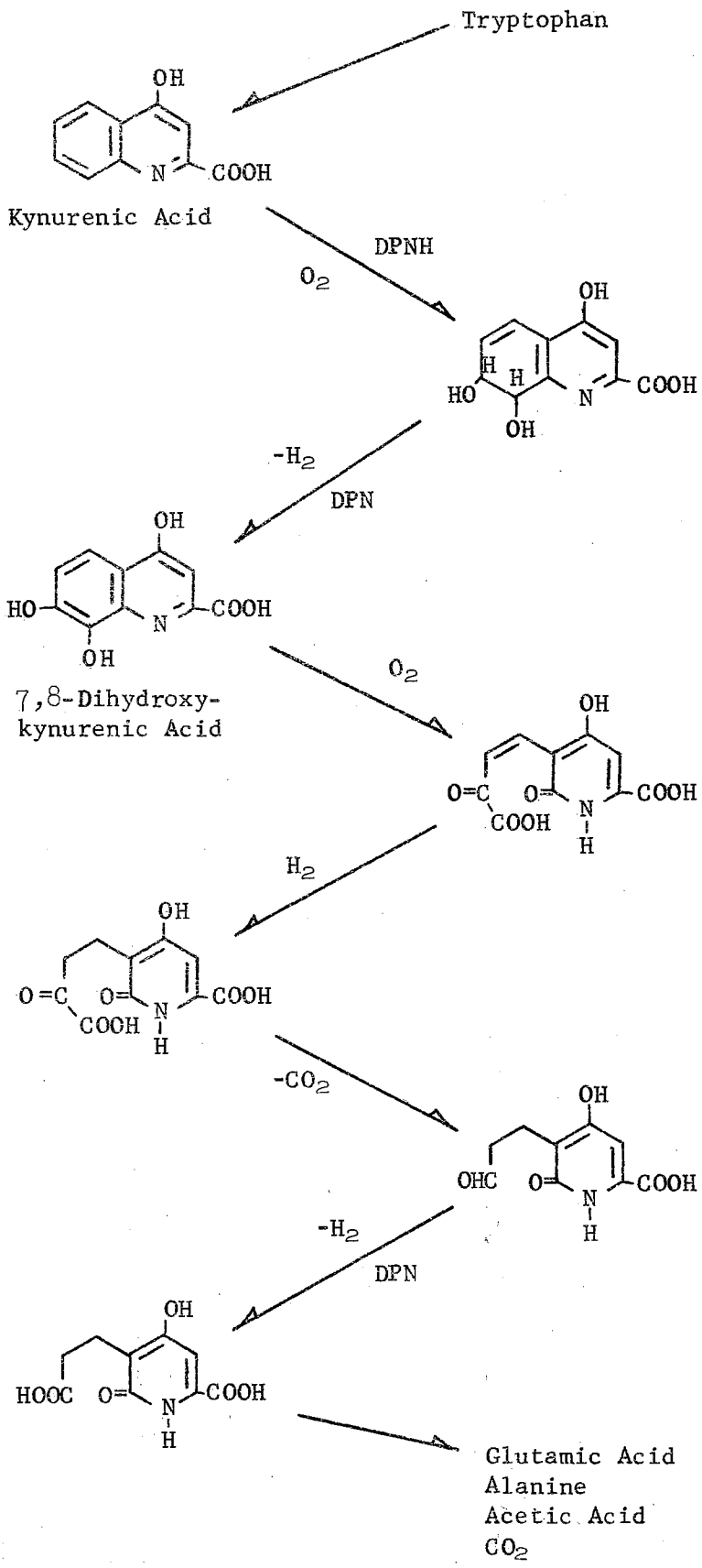
The enzyme responsible for the transamination of both kynurenine and 3-hydroxykynurenine occurs in animal tissue (64, 101), bacteria (106), and fungi (44). It requires pyridoxal phosphate as a coenzyme and a number of α-keto acids will serve as amino group acceptors (44).

Hayaishi and co-workers (33, 36, 37, 40, 93) studied the metabolism of kynurenic acid in Pseudomonas (Figure 2). The degradation proceeds by (a) hydroxylation of carbon atoms 7 and 8, (b) cleavage of the benzene moiety between carbon atoms 8 and 9, (c) reduction of the double bond in the side chain with TPNH, (d) loss of carbon atom 8 as carbon dioxide, and (e) further dissimilation of the resulting pyridone to alanine, acetic acid and carbon dioxide.

Horibata et al. (40) noted that the carbon skeleton of glutamic acid came directly from the benzene moiety of kynurenic acid in Pseudomonas. Carbon atoms 6 and 9 of kynurenic acid were located as sources of carbon atoms 1 and 4 of glutamic acid respectively. Carbon

Figure 2

Pathway of bacterial degradation of kynurenic acid (Quinolinic pathway).


Tryptophan
Kynurenic Acid
DPNH
O_2
$-H_2$
DPN
7,8-Dihydroxy-
kynurenic Acid
O_2
H_2
$-CO_2$
$-H_2$
DPN
Glutamic Acid
Alanine
Acetic Acid
CO_2

atoms 2, 3, 4 and 5 of glutamic acid were derived from carbon atoms 2 and 3 of kynurenic acid. The carbon atoms of the pyridine ring of kynurenic acid gave rise to alanine and acetic acid. The pyridine moiety of kynurenic acid is degraded and glutamic acid synthesized from small fragments.

3. The aromatic pathway

Oxidation by this pathway commences with the degradation of anthranilic acid. This pathway is the major metabolic route for tryptophan dissimilation in some bacteria, but anthranilic acid appears to be metabolically inert in mammals. Stanier, Hayashi and co-workers (88, 91) reported that anthranilic acid was degraded via catechol, cis, cis-muconic acid, muconolactone and β-ketoadipic acid in pseudomonads (Figure 3). The intermediates leading to catechol remain obscure. Catechol is also an intermediate in the degradation of many other aromatic compounds, e.g., benzoic acid and phenol (85).

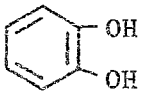
Pyrocatechase purified from P. fluorescens (32) catalyzes the oxidative splitting of catechol to form cis, cis-muconic acid. Experiments with ^{18}O and $H_2{}^{18}O$ indicate that molecular oxygen is introduced into the product (32). The purified enzyme is specific for catechol (32) and contains two atoms of ferrous iron per molecule.

The enzymes which convert cis, cis-muconic acid to β-ketoadipic acid were partially purified from mandilic acid induced Pseudomonas cells by Sistrom and Stanier (83). The laconizing enzyme requires Mn^{++} and converts cis, cis-muconic acid to muconolactone. The delactonizing enzyme hydrolyzes the γ-lactone.

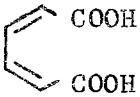
Katagira and Hayaishi (48) reported that cell extracts of tryptophan induced Pseudomonas converted β-ketoadipic acid to succinic

Tryptophan

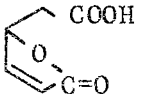
Anthranilic Acid



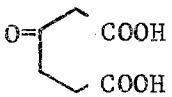
Catechol



cis, cis-Muconic Acid



Muconolactone



β-Ketoadipic Acid

Acetyl Coenzyme A + Succinic Acid

acid and acetylcoenzyme A in the presence of coenzyme A and succinyl coenzyme A. These products readily enter the Kreb's cycle for complete oxidation.

4. The indoleacetic acid pathway

Experiments with animals (98), bacteria (47, 63, 98), and plants (20, 57) suggest that indoleacetic acid is formed from tryptophan (Figure 4). The pathways involve the transamination or oxidative deamination to indolepyruvic acid followed by decarboxylation (47, 99); decarboxylation to tryptamine followed by oxidative deamination to give indoleacetaldehyde which is oxidized to indoleacetic acid (98); hydrolysis of indoleacetamide to indoleacetic acid (63); and the sequence--tryptophan, indoleacetonitrile, indoleacetamide and indoleacetic acid (46).

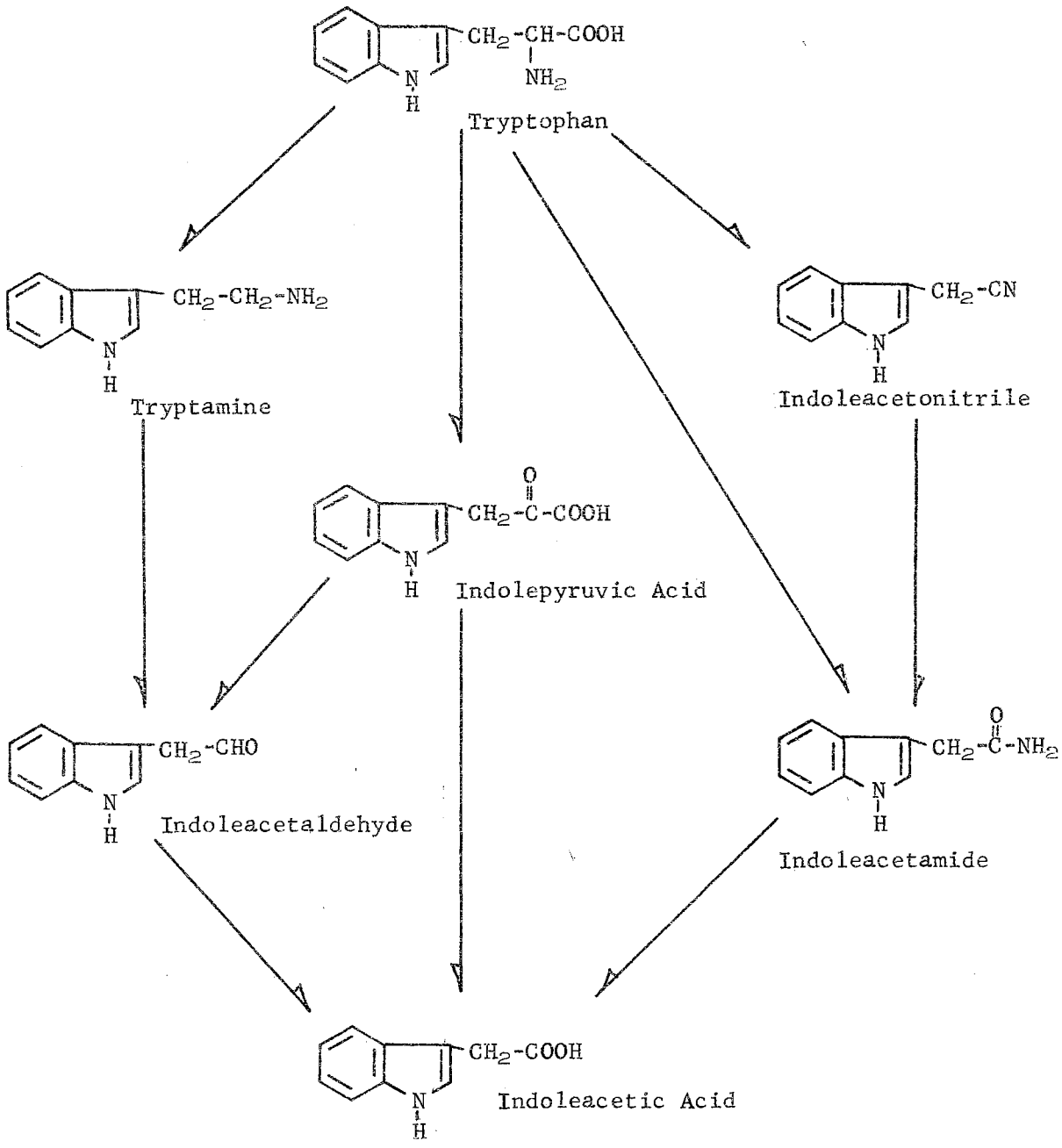
Indoleacetic acid, a normal constitute of urine, was once thought to be due to intestional flora (19). Lin, Civen, and Knox (58) found two independent tryptophan transaminases in rat liver that produce indolepyruvic acid which is converted to indoleacetic acid. Weissbach et al. (98) found that liver and kidney, as well as fecal bacteria, were able to decarboxylate tryptophan to tryptamine. Monoamine oxidase from mammalian tissue converts tryptamine to indoleacetic acid and a microsomal preparation hydroxylates tryptamine in the presence of TPNH to yield 6-hydroxytryptamine (45). Lin et al. (59) reported an enzyme that catalyzed the enol-keto tautomerization of indolepyruvic acid.

5. The 5-hydroxyindole pathway

The formation of serotonin from tryptophan by tryptophan 5-hydroxylase requires hydroxylation at carbon 5. 5-Hydroxytryptophan, the hydroxylated product, has been observed in bacteria (10), toads (80), and in the urine of certain carcinoid patients (84). The 5-hydroxyindole

Figure 4

Possible reaction sequences of the indoleacetic acid pathway.


CH2-CH-COOH
NH2
Tryptophan
CH2-CH2-NH2
Tryptamine
CH2-CN
Indoleacetonitrile
O
CH2-C-COOH
Indolepyruvic Acid
CH2-CHO
Indoleacetaldehyde
O
CH2-C-NH2
Indoleacetamide
CH2-COOH
Indoleacetic Acid

pathway of tryptophan is shown in Figure 5.

Chromobacterium violaceum (70, 96) produced L-5-hydroxytryptophan, 5-hydroxyindolepyruvic acid and 5-hydroxyindoleacetic acid when incubated with L-tryptophan. Serotonin is not formed since this bacterium lacks the decarboxylation enzyme. Most of the 5-hydroxytryptophan is used for the formation of the purple pigment violacein.

The decarboxylation of 5-hydroxytryptophan has not been demonstrated in bacteria, but is found in mammalian kidney, liver, intestine, lung and gastric mucosa (16). The enzyme is highly specific, attacking L- but not D-5-hydroxytryptophan.

A soluble bacterial monoamine oxidase attacks both serotonin and tryptamine (52). Mammalian amine oxidase occurs both in the mitochondria and in the soluble fraction of the cell. 5-Hydroxyindoleacetaldehyde is oxidized to 5-hydroxyindoleacetic acid by an aldehyde dehydrogenase. Serotonin may also be methylated to yield N-methylserotonin (96).

6. The indole pathway

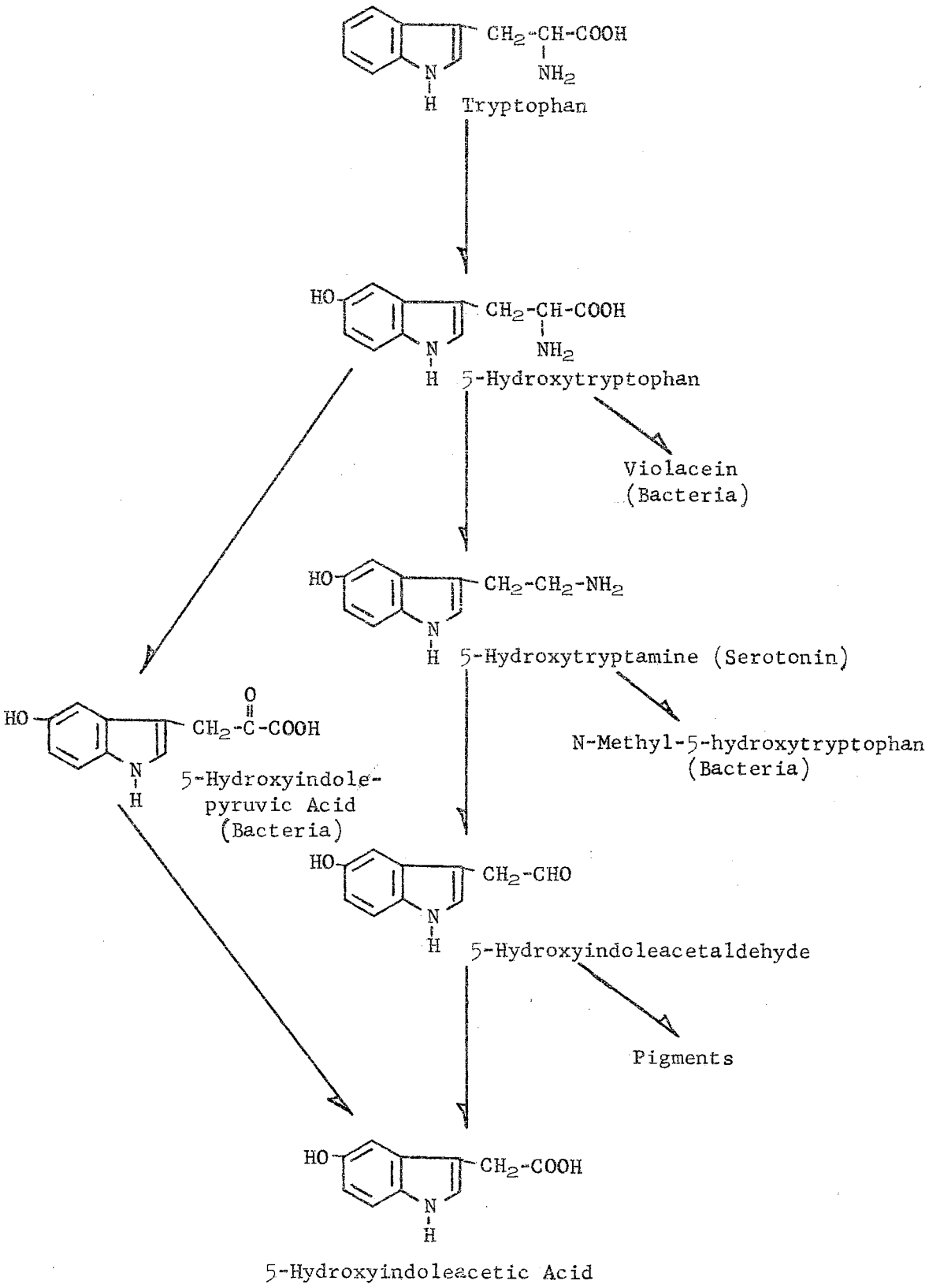
Various bacteria, particularly Escherichia coli, cleave the side chain of tryptophan to yield indole, ammonia and pyruvic acid (29, 103, 104, 105). This reaction appears to be of little consequence in living forms. Indole is further catabolized by certain bacteria via isatin, formylanthranilic acid, anthranilic acid, salicylic acid and catechol (95).

B. D-Tryptophan metabolism by vertebrates

Since animal tissues do not contain D-amino acids, a dietary D-amino acid must be converted to the L-isomer before it can be used in the synthesis of new tissue protein or in replacement of old. The

Figure 5

The 5-hydroxyindole pathway of tryptophan metabolism.


Tryptophan
5-Hydroxytryptophan
Violacein
(Bacteria)
5-Hydroxytryptamine (Serotonin)
N-Methyl-5-hydroxytryptophan
(Bacteria)
5-Hydroxyindole-
pyruvic Acid
(Bacteria)
5-Hydroxyindoleacetaldehyde
Pigments
5-Hydroxyindoleacetic Acid

mechanism of inversion involves deamination of the D-amino acids, with loss of asymmetry, followed by reamination (9). Kidney and liver tissue of all vertebrates tested contain an enzyme which oxidatively deaminates D-amino acids (55). Mammalian D-amino acid oxidases show an absolute stereospecificity and oxidize different D-amino acids at different rates. All D-amino acids that undergo inversion in animals (histidine, tryptophan, methionine, phenylalanine, valine, leucine and arginine) are first converted by D-amino acid oxidase to the corresponding α-keto acid.

The α-keto acid is converted to the L-amino acid, and the α-keto acid frequently substitutes for the corresponding L-amino acid for protein synthesis. Sakurai (78, 79) prepared L-tryptophan and L-phenylalanine from the corresponding α-keto acids using pig heart muscle transaminase and L-aspartic and L-glutamic acids as amino donors.

Reductive amination represents an alternate pathway for conversion of the α-keto acid to an amino acid. Radhakrishnan and Meister (73) observed that L-amino acid oxidase from snake venom and D-amino acid oxidase from sheep kidney will catalyze production of L- and D-amino acid isomers. L-Amino acids were not active in the D-amino acid oxidase system and vice versa.

Conrad and Berg (18) proved inversion of the D-isomer of an essential amino acid. They found that the increment of L-histidine in the tissues of young rats, fed histidine-deficient diets supplemented with D-histidine, exceeded the amount present in the tissue when the feeding began.

Kotake and Goto (53) using kidney and liver tissue from several animal species proved the "stereonaturalization" of D-tryptophan. The production of indole by a strain of E. coli, which attacked only L-

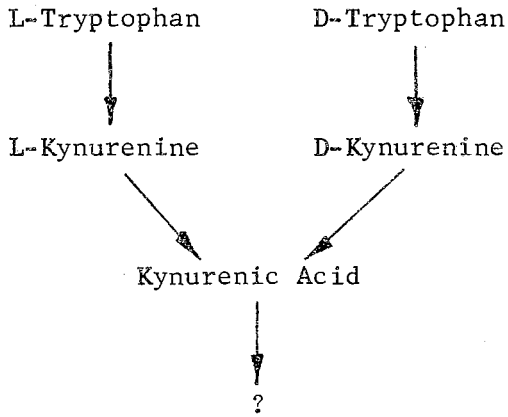
tryptophan, indicated an inversion. In the presence of the animal tissue, but not in its absence, E. coli produced indole from indolepyruvic acid suggesting conversion of the latter by the tissue to L-tryptophan.

Addition of a racemic mixture or of D-amino acids to mammals can result in the urinary excretion of the D-isomers. Larger amounts of tryptophan are excreted by man following administration of DL-tryptophan than when the L-isomer is used (3, 81). Baldwin and Berg (3) and Langner and Berg (56) reported that D-tryptophan is excreted more readily by animals than is L-tryptophan.

C. D-Tryptophan metabolism by microorganisms

Microorganisms utilize D-amino acids more effectively than higher animals as evidenced by the frequent occurrence of D-amino acids in cell structures.

Stanier and Tsuchida (89) reported an unidentified Pseudomonas attacked both isomers of tryptophan. Tryptophan-induced cells were induced to the corresponding isomers of kynurenine and to kynurenic acid but not to a wide variety of other compounds including anthranilic acid. The adaptive patterns implied distinct mechanisms of attack for the stereoisomers of tryptophan and kynurenine, and hence the following steps were postulated:



L-Tryptophan-grown Pseudomonas showed a two-step oxidation with an intervening inductive lag for a racemic mixture of tryptophan. The initial oxidation of DL-tryptophan proceeded at a rate characteristic of the L-isomer but ceased abruptly when the total oxygen uptake was exactly half that of an equal quantity of the L-isomer. Following reversion to the endogenous rate, there was a slow and continuous increase in the rate of oxygen uptake until the total amount was twice that at the initial break, when a return to the endogenous rate occurred. The secondary rise in oxygen consumption reflected a much slower oxidation of the D-isomer by means of a second, initially uninduced, enzyme system. Since the final total oxygen uptake per mole of DL-tryptophan was identical for the L-isomer, it was concluded that the two enzyme systems oxidized tryptophan to the same extent.

Behrman and Cullen (5) reported that a Pseudomonas grown on either D- or L-tryptophan oxidized both isomers of tryptophan, N-formylkynurenine, kynurenine, and alanine at the same rate and to the same extent. Anaerobic incubation of D- or L-tryptophan with cell extracts produced a racemic mixture of tryptophan indicating a direct racemization had occurred.

Stanier and Hayaishi (88) tested 27 species of Pseudomonas and found that 20 followed the aromatic pathway and 5 followed the quinoline pathway. Every strain using the quinoline pathway oxidized D-tryptophan; whereas strains using the aromatic pathway either did not degrade D-tryptophan or oxidized it at a rate significantly lower than the rate of L-tryptophan oxidation. Two strains displayed inductive patterns that were incompatible with either the aromatic or the quinoline pathway. One of these strains had an early metabolic block and the other quantita-

tively converted tryptophan to indole.

Schwarze and Frandsen (82) showed that Pen. notatum degraded D-tryptophan to kynurenine (probably D-kynurenine) and degraded L-tryptophan via kynurenine so that nitrogen was readily available for growth.

Luckner (62) reported that D-tryptophan was metabolized to D-kynurenine, α-N-acetyl-D-tryptophan and α-N-acetyl-D-kynurenine by Pen. viridicatum while L-kynurenine, anthranilic acid, and 3-hydroxyanthranilic were produced from L-tryptophan. L-Kynurenine was rapidly degraded but D-kynurenine was acetylated to yield the N-acetyl derivative. α-N-Acetyl-D-tryptophan was not degraded, but α-N-acetyl-L-tryptophan was completely metabolized.

CHAPTER III

EXPERIMENTAL PROCEDURE

Growth experiments

Test organism and maintenance: The organism used in this study was isolated from soil using an enrichment technique and possessed the characteristics of the genus Flavobacterium as described in the seventh edition of Bergey's Manual of Determinative Bacteriology. Stock cultures were maintained on synthetic salts-DL-tryptophan-agar slants. Cultures of the organism were grown at 37 C and stored at 4 C. Subcultures were made at intervals of 1 to 2 weeks.

Synthetic medium: The synthetic medium used for growth studies had the following composition: NaCl, 0.2 g; NH_4Cl, 0.2 g; KH_2PO_4, 0.32 g; and K_2HPO_4, 0.42 g in 100 ml of distilled water. The pH was adjusted to 6.8-7.0 and the medium sterilized by autoclaving for 15 minutes at 121 C. Each 100 ml of the synthetic medium was supplemented with 0.1 ml of a mineral salts solution that was autoclaved separately and added aseptically just prior to use. The mineral salts contained: $MgSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4$, 0.1 g; $FeCl_3$, 1.0 g; and $CaCl_2$, 0.5 g in 100 ml of distilled water. Two percent agar was added when a solid medium was needed.

Growth studies: Growth studies were conducted in 15 x 150 mm cotton stoppered Pyrex culture tubes. To each tube was added aseptically 4.0 ml of sterile basal medium and the desired carbon source which was dissolved in distilled water, adjusted to pH 6.8-7.0, and sterilized by filtration.

All tubes were brought to a 5.0 ml volume with sterile distilled water.

Inoculum: Inoculum cells were grown on 0.07 percent DL-tryptophan agar slants for 18 to 24 hr, and the growth from 2 slants was harvested, washed and suspended in sterile physiological saline solution to an absorbancy of 0.7 at 540 mμ. One-tenth ml of this suspension was used to inoculate each tube.

Measurement of growth: The tubes were incubated on a reciprocating shaker at 37 C. Growth was determined by measuring absorbancy at 540 mμ with a Bausch and Lomb "Spectronic 20" colorimeter. An uninoculated tube of medium was used as a standard. In some experiments growth was followed by measuring protein (mg/ml) as described under protein determination.

Respirometric experiments

Preparation of cell suspensions: Synthetic medium agar slants, containing the desired carbon source, were inoculated from a stock culture and incubated for 20 to 24 hr at 37 C. The resulting growth was suspended in sterile 0.01 M potassium phosphate buffer (pH 7.0) and 5 or 6 drops of the suspension placed on synthetic agar plates containing the desired carbon source. The inoculum was spread over the agar surface with a sterile glass rod and the plates incubated for 20 to 24 hr at 37 C. The cells were harvested with 0.01 M potassium phosphate buffer (pH 7.0), washed twice by centrifugation and suspended in buffer.

Gas exchange measurements: All respirometric experiments were performed in double-sidearm Warburg apparatus at 37 C with air as the gas phase (97). To determine oxygen uptake, 0.2 ml of 20 percent potassium hydroxide (w/v) was placed in the center well containing a piece of fluted filter paper. The cell suspension or cell extract was placed in the main reaction chamber while substrates, inhibitors, or other additives

were placed in the sidearms.

The direct method was used to measure carbon dioxide (97).

Spectrophotometric studies

The oxidase activity of the cell extract was determined by measuring the reduction of 2,6-dichlorophenolindophenol at 600 mμ in the Bausch and Lomb "505" spectrophotometer at room temperature. The following components were added to quartz cuvettes: 0.1 ml crude extract (containing the indicated protein concentration); 0.12 μmole 2,6-dichlorophenolindophenol; 1.0 μmole sodium cyanide; 200 μmoles Tris [tris(hydroxymethyl)aminomethane] buffer (pH 7.4); substrate and/or inhibitor as indicated; and water to a total volume of 3.0 ml. The enzymatic reaction was started by addition of the substrate with an adder-mixer which permitted mixing immediately upon addition of the enzyme.

Chemical and preparative procedures

Preparation of cell extract: Cells were grown 16 to 18 hr at 37 C on synthetic medium agar plates containing 0.07 percent of the appropriate carbon source, harvested and washed twice with cold 0.01 M Tris buffer (pH 7.4). The sedimented organisms were frozen in small pellets and ruptured by 2 passages through an X-press at dry ice temperatures. The preparation was thawed, diluted with 0.01 M Tris buffer (pH 7.4) and cell debris removed by centrifugation at 20,000 x g for 30 minutes at 4 C. The supernatant solution was carefully decanted and used in experiments requiring cell extracts.

Protein determination: Protein was determined by a modification of the procedure of Lowry et al. (61). To 1.0 ml of sample (10 to 300 μg protein) was added 5.0 ml of a dilute alkaline copper solution (prepared by adding 1.0 ml of 0.025 percent $CuSO_4 \cdot 5H_2O$ to 50 ml of a solution

containing 3.0 percent Na_2CO_3 and 0.5 percent potassium sodium tartrate). The mixture was incubated at room temperature for 15 minutes and 0.5 ml of phenol reagent (Folin-Ciocalteau) diluted 1:2 with water was added. The tubes were mixed, permitted to stand at room temperature for 30 minutes and the absorbency measured at 540 mμ in a Bausch and Lomb "Spectronic 20" colorimeter. A standard curve using bovine serum albumin was prepared concurrently.

Cell fractionation for protein determination: Whole cells were washed 2 times and suspended in cold physiological saline. Two ml of the cell suspension were incubated at 70 C for 30 minutes with 0.1 ml 70 percent $HClO_4$. The solid material was sedimented and washed once by centrifugation. The sediment was dissolved in 0.1 N NaOH and the protein content determined as described above.

Paper chromatography techniques: The samples were spotted on Whatman No. 1 paper and developed by ascending chromatography in the appropriate solvent. Two-dimensional chromatograms were developed in the solvent systems of Redfield (74). The first solvent system consisted of methanol:water:pyridine (80:20:4 v/v) while the second system consisted of tert-butanol:methyl ethyl ketone:water:diethylamine (40:40:20:4 v/v). One dimensional chromatograms were developed using either isopropanol:ammonium hydroxide(sp. gr. 0.896):water (10:1:1 v/v) (Solvent System A) or isopropanol:glacial acetic acid:water (4:1:1 v/v)(Solvent System B). After development, each chromatogram was dried in a stream of warm air and the amino acids located by spraying with an ethanol solution of 1.0 percent ninhydrin. Indole derivatives were developed with either Salkowski reagent (50 ml of 50 percent $HClO_4$ added to 1.0 ml of 0.05 M $FeCl_3$) or Ehrlich reagent (660 mg of p-dimethylaminobenzaldehyde

dissolved in a mixture of 36 ml of ethanol and 8 ml conc. HCl). Anthranilic acid was located by scanning with ultraviolet light (Wood Lamp).

Bioautographic procedures: The material to be assayed was spotted on Whatman No. 1 paper and chromatographed in the appropriate solvent system. The developed chromatogram was dried in a stream of warm air, and strips, 1/2 inch wide, containing the migration areas were cut out. The strips were overlaid on a large pyrex plate containing tryptophan assay medium (Difco) plus 2.0 percent agar seeded with Lactobacillus plantarum 17-5. The plates were incubated 24 hr at 37 C. The presence or absence of L-tryptophan was determined by observing for growth in the area corresponding to the R_f value of the compound. Since tryptophan is water soluble, it diffuses from the chromatostrip into the agar, producing easily distinguishable zones of growth.

Quantitative anthranilic acid procedure: The procedure is a modification of that used by Snell and Snell (87) for p-aminobenzoic acid determination. To a 5.0 ml sample was added 1.0 ml of 7.5 percent trichloroacetic acid solution, and after 5 minutes incubation, 1.0 ml of 0.025 percent solution of $NaNO_2$ was added. The mixture was allowed to stand at room temperature 20 minutes, then 1.0 ml of 0.5 percent sodium ammonium sulphamate solution and 1.0 ml of 0.065 percent N-(1-naphthyl)-ethylenediamine dihydrochloride solution were added and the color permitted to develop for 30 minutes. Values were calculated from a standard curve prepared concurrently. Controls indicated that tryptophan and indolepyruvic acid, in the concentrations used in this investigation, did not influence the test. However, high concentrations of indolepyruvic acid produced a red precipitate which gave erroneous anthranilic acid values.

CHAPTER IV

RESULTS AND DISCUSSION

Growth on tryptophan

The Flavobacterium species used in this study could utilize D-, L- or DL-tryptophan as the sole carbon, nitrogen and energy source. Typical growth curves (Figure 6), obtained when using DL-tryptophan-induced organisms, showed that growth on L- or DL-tryptophan commenced approximately 2 to 3 hours after inoculation. However, growth on the D-isomer of tryptophan was not evident until 9 to 10 hours after inoculation and was considerably slower than growth in L-tryptophan. The total maximum growth on L-tryptophan was greater than the growth obtained with equimolar concentrations of D- or DL-tryptophan. Oesterling and Rose (72) reported that rats utilize D-tryptophan for growth, but the amount of growth per unit of D-tryptophan was less than with a like quantity of L-tryptophan.

Experiments conducted to substantiate the turbidity studies showed that the quantity of protein synthesized per μmole of L-, D- and DL-tryptophan was 61, 50 and 55 μg respectively (Table I).

After maximum growth was obtained, free tryptophan could not be demonstrated in the growth medium by paper chromatography or by absorption at 270 mμ in a Beckman DU spectrophotometer. Since more growth was obtained from L-tryptophan, the L-isomer must be metabolized more efficiently than the D-isomer, i.e., either less net adenosine triphosphate is pro-

Figure 6

Growth of a Flavobacterium species in equimolar concentrations of D-, L- and DL-tryptophan as the carbon, nitrogen, and energy source. △, 1.2×10^{-3} M L-tryptophan; ○, 1.2×10^{-3} M D-tryptophan; ●, 1.2×10^{-3} M DL-tryptophan.

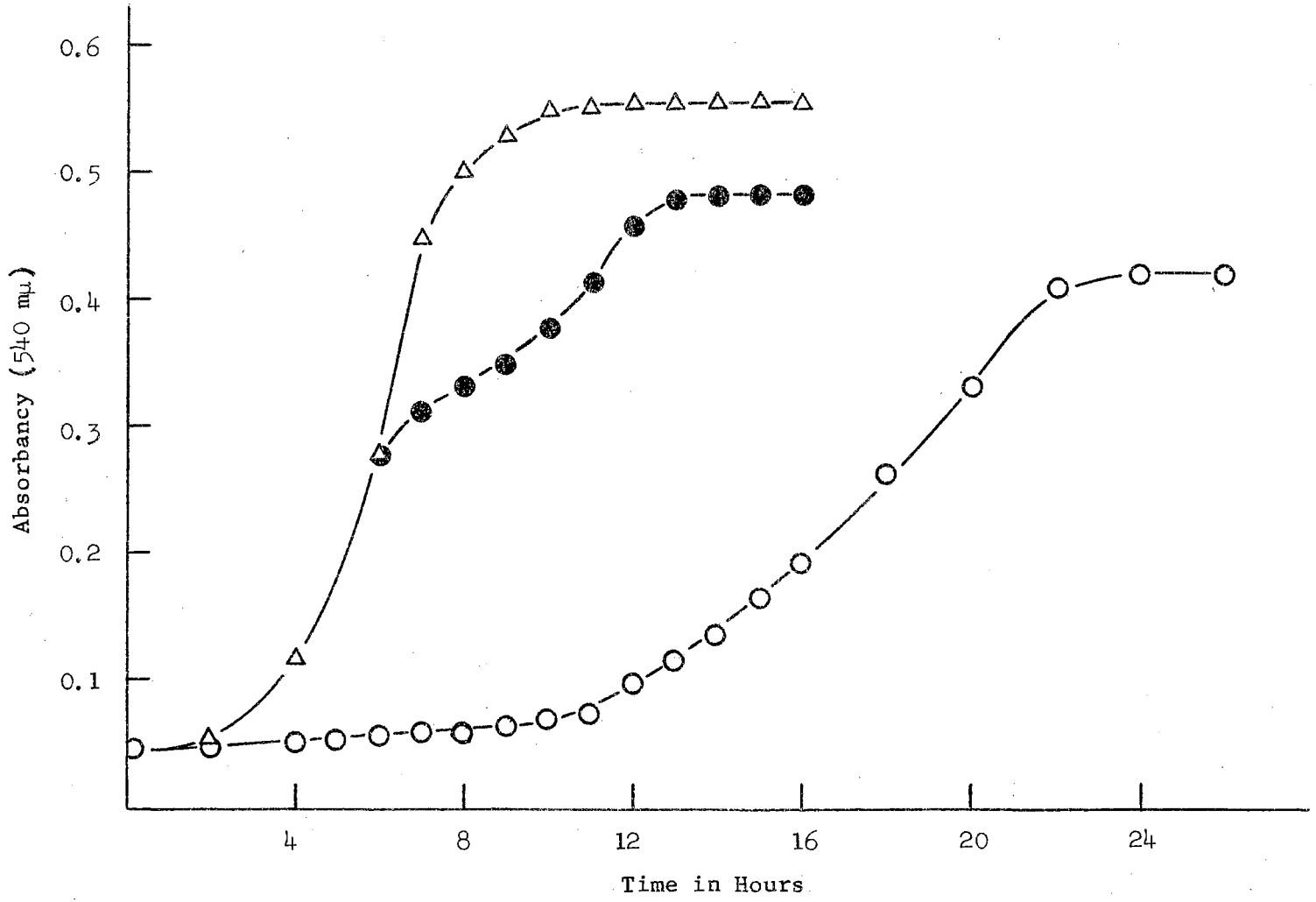

0.6
0.5
0.4
0.3
0.2
0.1
Absorbancy (540 mμ)
4
8
12
16
20
24
Time in Hours

TABLE I

QUANTITY OF PROTEIN SYNTHESIZED BY FLAVOBACTERIUM PER MICROMOLE D-, L-, AND DL-TRYPTOPHAN

Substrate	Total Substrate (μmoles)	Total Protein Synthesized (μgrams)	Protein Synthesized per μmole Substrate (μgrams)
L-Tryptophan	10	600	61
	20	1250	
D-Tryptophan	10	510	50
	20	980	
DL-Tryptophan	10	540	55
	20	1120	

duced during metabolism of D-tryptophan than during metabolism of the L-isomer, or more energy must be expended for the metabolism of D-tryptophan. The significance of this is discussed in forthcoming sections.

The utilization of the D-isomer from the medium containing DL-tryptophan was much faster than when D-tryptophan served as the sole carbon source in the medium (Figure 6), suggesting that the presence of a readily oxidizable energy source, such as L-tryptophan, accelerated the utilization of D-tryptophan.

Experiments, using other readily oxidizable substrates, showed that addition of succinic acid to a synthetic medium containing D-tryptophan "sparked" the utilization of D-tryptophan as a source of carbon and energy for growth (Figure 7). Growth on L-tryptophan as the carbon and energy source continued uninterrupted, whereas growth on only D-tryptophan was not evident for several hours. Similar results were obtained using L-alanine or pyruvic acid in combination with D-tryptophan. Thus, any readily oxidizable substrate will enhance utilization of D-tryptophan. L-Tryptophan was readily utilized for growth although supplementation with succinic acid slightly increased the rate of growth.

Induction studies using washed cell suspensions

Sequential induction studies were conducted to determine the metabolic degradation pathway of D-tryptophan. The adaptive response of noninduced asparagine-grown cells to D-tryptophan, L-tryptophan, L-kynurenine, DL-kynurenine, kynurenic acid, and anthranilic acid, as indicated by the marked lag in oxygen uptake, is shown in Figure 8.

Cells induced to L-tryptophan, by growth on this substrate, were sequentially induced to L-tryptophan, L-kynurenine, and anthranilic acid (Figure 9), indicating these compounds may serve as intermediates in the

Figure 7

Influence of succinic acid on growth of Flavobacterium species on D-tryptophan. ● , 1.2 x 10^{-3} M L-tryptophan plus 2 x 10^{-3} M succinic acid; △ , 1.2 x 10^{-3} M D-tryptophan plus 2 x 10^{-3} M succinic acid; ▽ , 1.2 x 10^{-3} M L-tryptophan; ◇ , 1.2 x 10^{-3} M D-tryptophan; ○ , 2 x 10^{-3} M succinic acid.

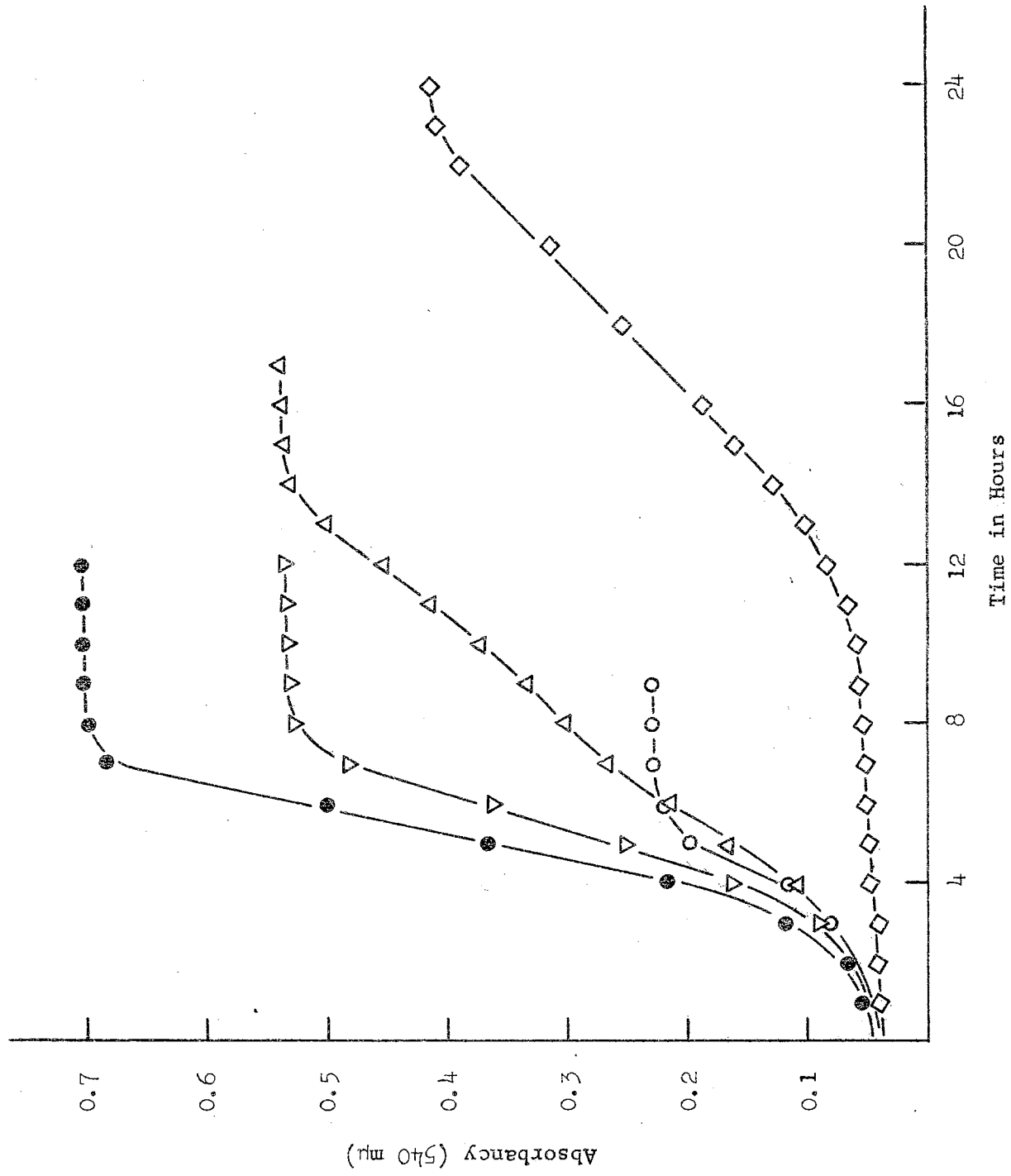

Absorbancy (540 mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
4
8
12
16
20
24
Time in Hours

Figure 8

The oxidation of D- and L-tryptophan, D- and L-kynurenine, anthranilic acid and kynurenic acid by washed cells of the Flavobacterium species grown on asparagine. ◆, 2 μmoles D-tryptophan; ○, 2 μmoles L-tryptophan; ◑, 2 μmoles D-kynurenine; ▽, 2 μmoles L-kynurenine; □, 2 μmoles anthranilic acid; ●, 2 μmoles kynurenic acid; ▼, endogenous.

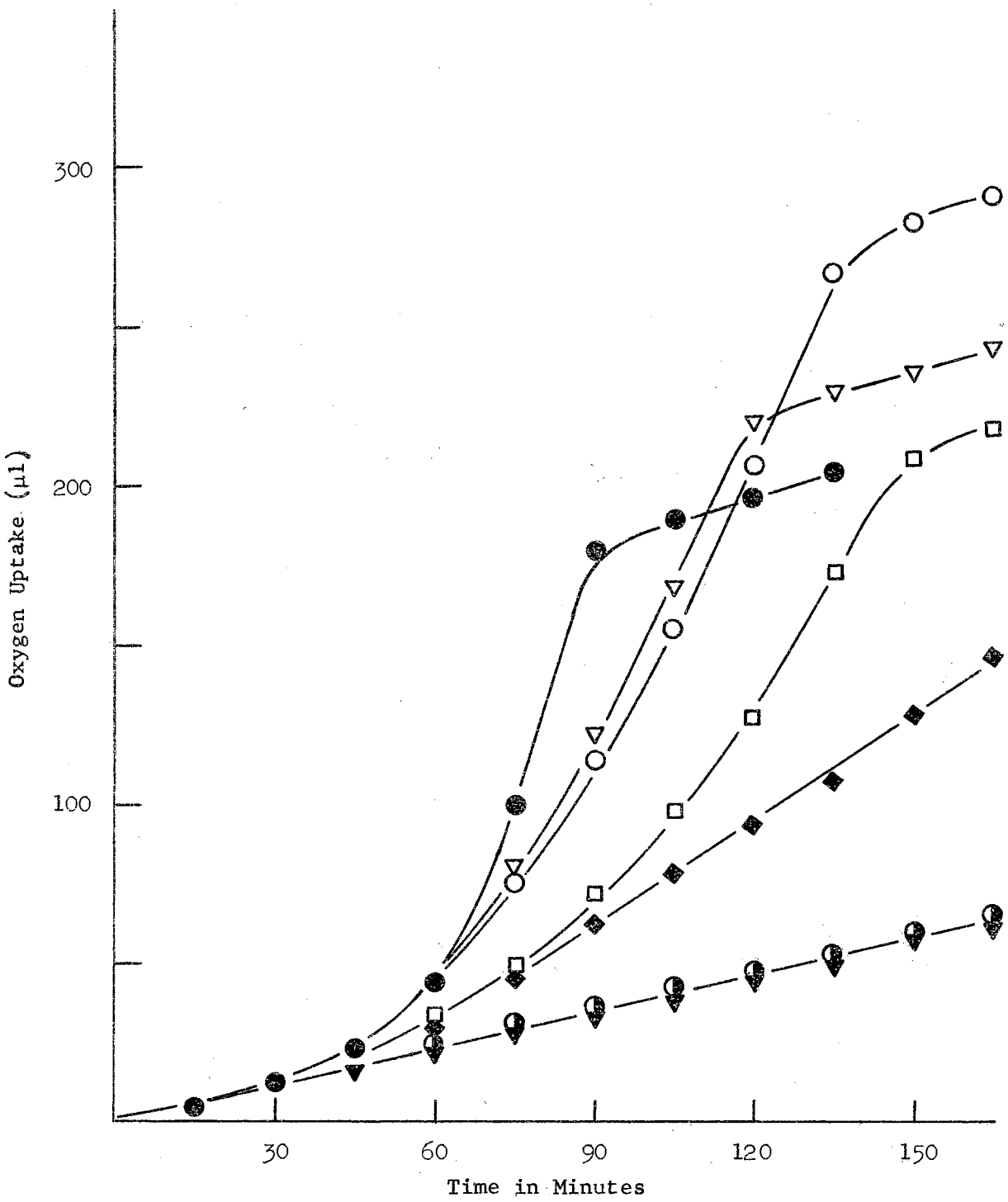
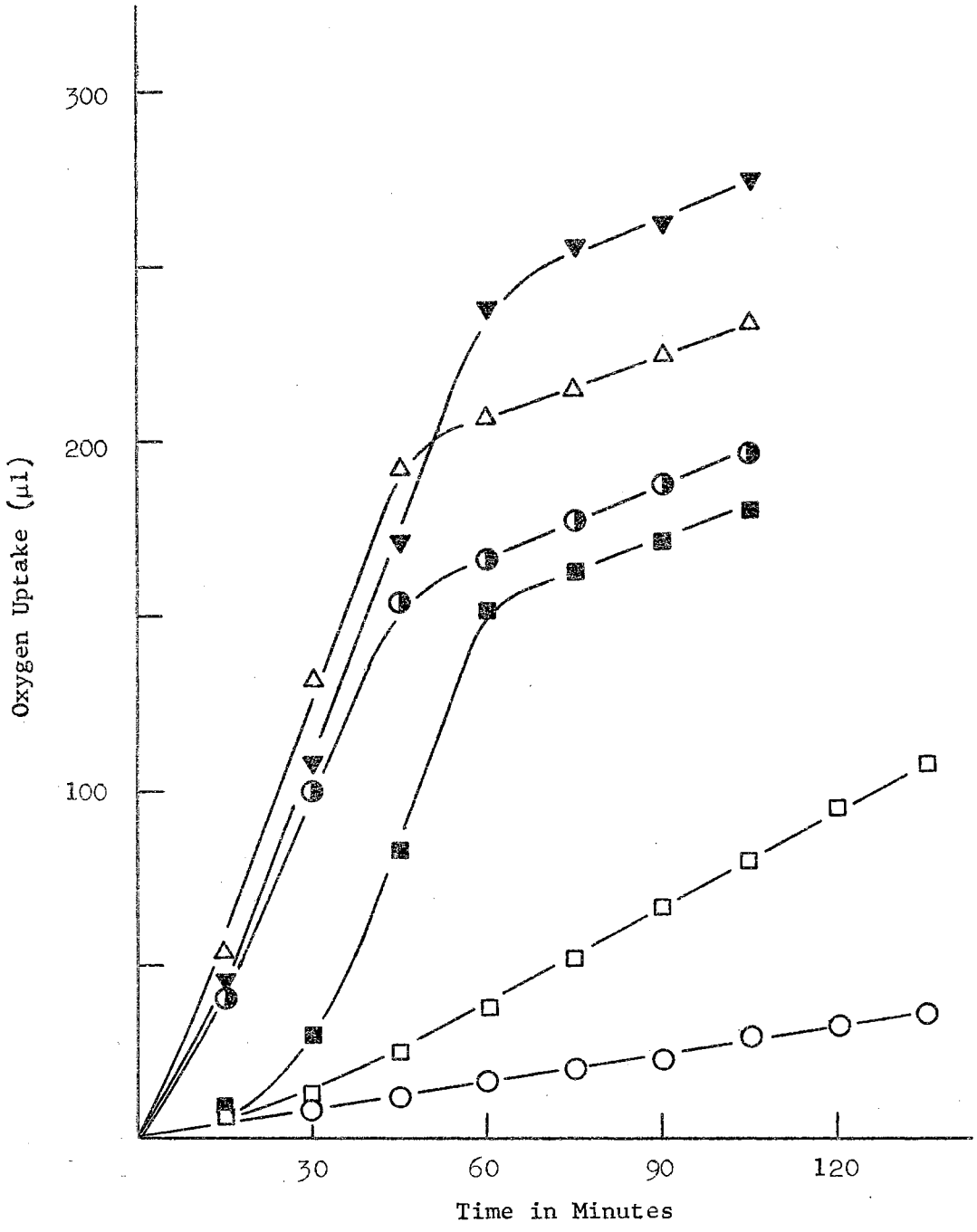

300
200
100
Oxygen Uptake (µl)
30
60
90
120
150
Time in Minutes

Figure 9

The oxidation of D- and L-tryptophan, L-kynurenine, kynurenic acid, and anthranilic acid by L-tryptophan-grown cells. □, 2 μmoles D-tryptophan; ▼, 2 μmoles L-tryptophan; △, 2 μmoles L-kynurenine; ▩, 2 μmoles kynurenic acid; ◑, 2 μmoles anthranilic acid; ○, endogenous.


300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

oxidative degradation of L-tryptophan. Failure of the L-tryptophan-induced cells to immediately oxidize D-tryptophan, D-kynurenine, DL-3-hydroxykynurenine, DL-5-hydroxykynurenine, kynurenic acid, DL-oxindole-alanine, tryptamine, tryptophol, xanthurenic acid, or indole suggests that these substrates are not intermediates in L-tryptophan metabolism. Both D-tryptophan and kynurenic acid were oxidized following a lag period (Figure 9). Cells induced to kynurenic acid were able to oxidize this substrate very rapidly, indicating that permeability is probably not a factor in the failure of L-tryptophan-grown cells to oxidize this compound. Cells induced to D-tryptophan immediately oxidized both isomers of tryptophan, L-kynurenine and anthranilic acid, suggesting these compounds serve as intermediates in the oxidative degradation of D-tryptophan. Table II shows the oxidation pattern of DL-tryptophan-induced cells to other possible tryptophan intermediates.

Quantitative oxidation studies indicated that D-tryptophan-induced cells utilized 90 μliters of oxygen during the metabolism of 1 μmole L-kynurenine and 46 μliters of oxygen when 1 μmole of DL-kynurenine was used as substrate. The quantity of oxygen consumed during the oxidation of 1 μmole of a racemic mixture of kynurenine was equivalent to that observed when 1/2 μmole of L-kynurenine is oxidized. D-Kynurenine was not oxidized by cells induced to D-tryptophan. These results indicate that only the L-isomer of kynurenine was oxidized and that the D-isomer was not attacked. Similar results were obtained using L-tryptophan-induced cells. Thus, cells induced to D- or L-tryptophan only oxidize the L-isomer of kynurenine which establishes that D-tryptophan is converted to L-tryptophan and is not oxidized through D-kynurenine.

The addition of chloramphenicol or streptomycin (60 μg./ml) did

TABLE II

OXIDATION OF POSSIBLE INTERMEDIATES OF DL-TRYPTOPHAN METABOLISM BY A FLAVOBACTERIUM SPECIES AFTER GROWTH ON DL-TRYPTOPHAN*

Compound	Cells Adapted after a Lag Period(+)	Cells Did Not Adapt(-)
Isatin	+	
Indole	±**	
D-Kynurenine		-
Kynurenic Acid	+	
Xanthurenic Acid	+	
Quinaldic Acid		-
3-Hydroxyanthranilic Acid		-
DL-3-Hydroxykynurenine		-
DL-5-Hydroxykynurenine		-
Indolelactic Acid		-
Indoleacetic Acid		-
Indolepropionic Acid		-
DL-Oxindolealanine		-

* The oxidation studies were conducted for a maximum of 4 hours.

**Shows a slight but consistant increase in oxygen uptake over endogenous respiration.

not influence the oxidation of D- or L-tryptophan by D-tryptophan-grown cells (Table III). The antibiotics did not affect the oxidation of L-tryptophan by L-tryptophan-grown cells but did inhibit oxidation of D-tryptophan. These results substantiate the previous findings that D-tryptophan-induced cells are sequentially induced to L-tryptophan, but cells grown on the L-isomer are not induced to D-tryptophan.

The oxidation of D-tryptophan by DL-tryptophan-grown cells proceeded at a low rate -- 35 to 50 percent of the rate at which L-tryptophan was oxidized (Figure 10). Stanier and Hayaishi (88) reported that pseudomonad strains, using the quinoline pathway, oxidized D-tryptophan at a high rate, whereas strains using the aromatic pathway either did not attack D-tryptophan or oxidized D-tryptophan much slower than the L-isomer. However, Behrman and Cullen (5) isolated a pseudomonad with inducible patterns typical of an aromatic pathway organism, and this bacterium oxidized D- and L-tryptophan at the same rate and to the same extent.

Since anthranilic acid was one of the intermediates of D-tryptophan oxidation, experiments were conducted to determine the intermediates of anthranilic acid degradation. Table IV shows the oxidation pattern of anthranilic acid-induced cells on various aromatic compounds. Salicylic acid and gentisic acid were oxidized immediately by anthranilic acid-induced cells, and the oxidation was not inhibited by addition of streptomycin or chloramphenicol (60 μg./ml) or by exposing the cell suspension to ultraviolet radiation for three minutes. These results suggest that salicylic acid and gentisic acid are intermediates in anthranilic acid degradation.

Catechol gave a slight but consistent increase in oxygen uptake over

TABLE III

OXIDATION OF SUBSTRATES IN THE PRESENCE OF CHLORAMPHENICOL BY CELLS GROWN ON D- OR L-TRYPTOPHAN*

Substrate	Cells Grown On	
	D-Tryptophan	L-Tryptophan
D-Tryptophan	+	-
L-Tryptophan	+	+
L-Kynurenine	+	+
Anthranilic Acid	+	+

* +, substrate oxidized; -, substrate not oxidized.

Figure 10

The oxidation of D- and L-tryptophan by cells of a Flavobacterium species grown on DL-tryptophan. ○, 2 μmoles L-tryptophan; ▲, 2 μmoles D-tryptophan; □, endogenous.

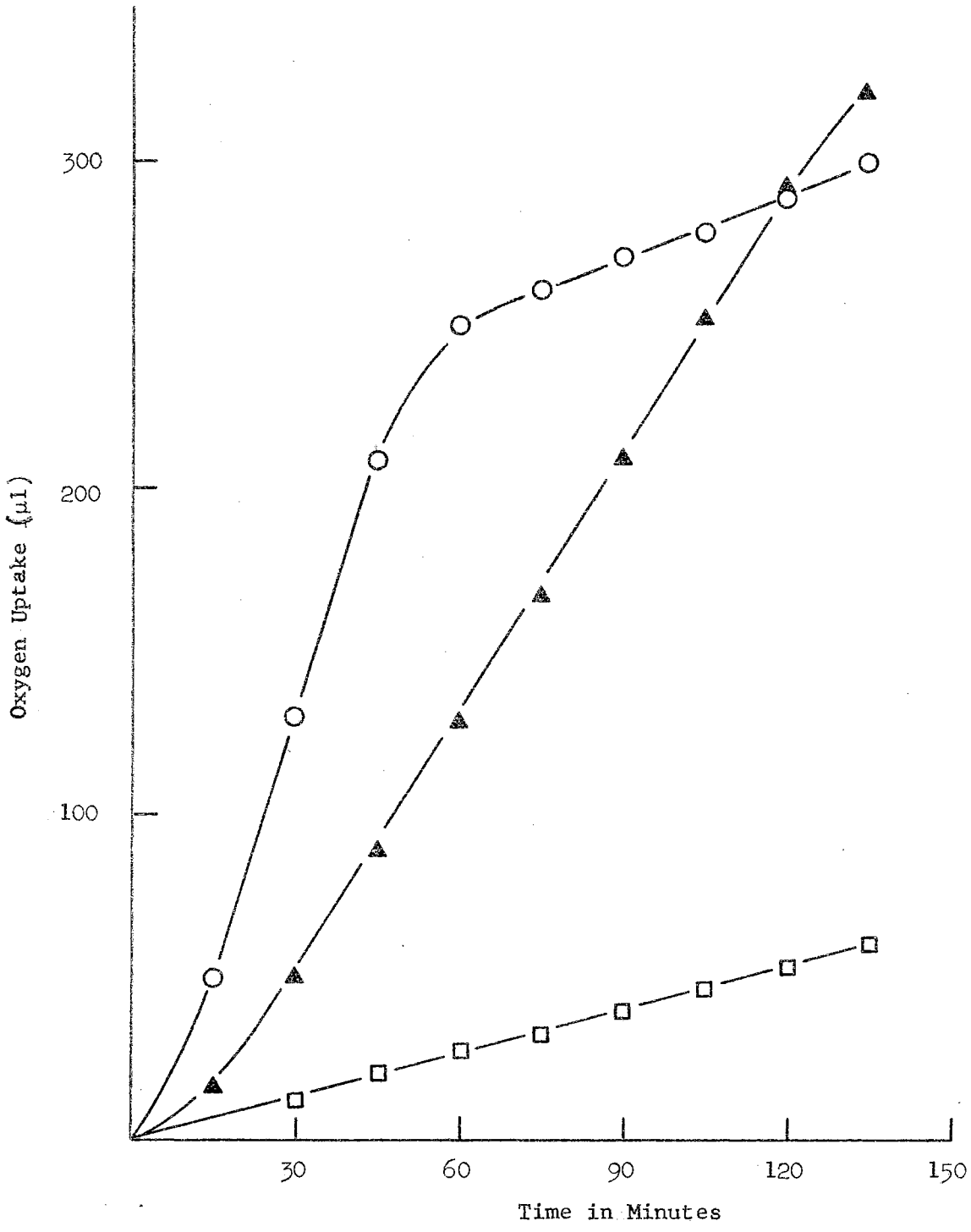

300
200
100
Oxygen Uptake (µl)
30
60
90
120
150
Time in Minutes

TABLE IV

OXIDATION OF VARIOUS AROMATIC COMPOUNDS BY A FLAVOBACTERIUM SPECIES AFTER GROWTH ON ANTHRANILIC ACID

Compound	Cells Induced(+)	Cells Adapted after a Lag Period(+)	Cells Did Not Adapt(-)*
Catechol		+**	
Protocatechuic Acid		+	
Salicylic Acid	+		
Gentisic Acid	+		
Phenol		+	
Aniline			-
Benzoic Acid		+	
Resorcinol			-
o-Nitrophenol			-
o-Nitrobenzoic Acid			-
o-Nitrobenzaldehyde			-
m-Hydroxybenzoic Acid		+	
p-Hydroxybenzoic Acid			-
2,3-Dihydroxybenzoic Acid		+	
2,4-Dihydroxybenzoic Acid			-
2,6-Dihydroxybenzoic Acid			-

* The oxidation studies were conducted for a maximum of 4 hours.

** Shows a slight but consistent increase in oxygen uptake over endogenous respiration.

endogenous respiration, but a typical oxidation-time curve was not obtained. Attempts to obtain a cell extract that would oxidize anthranilic acid from anthranilic acid-grown cell were unsuccessful.

Growth on kynurenine

Growth experiments were conducted to substantiate that this species of Flavobacterium does not utilize D-kynurenine. The total growth obtained in 1.2×10^{-3} M DL-kynurenine is approximately equal to that obtained in 6×10^{-4} M L-kynurenine (Figure 11). Similar results were obtained using 1.6×10^{-3} M DL-kynurenine and 8×10^{-4} M L-kynurenine. Measurable growth, as determined spectrophotometrically, was not obtained on a synthetic medium containing 8×10^{-4} M D-kynurenine after 72 hours of incubation at 37 C on a reciprocating shaker.

Oxygen consumption and carbon dioxide production during oxidation of metabolic intermediates

Experiments to quantitate the oxygen consumed and carbon dioxide liberated during the oxidation of D-tryptophan, L-tryptophan, L-kynurenine, DL-kynurenine, anthranilic acid, salicylic acid, gentisic acid, L-alanine, and D-alanine are summarized in Table V.

The organism requires approximately 5.8 μmoles of oxygen to oxidize 1 μmole of D-tryptophan and 5.0 μmoles oxygen to oxidize 1 μmole of L-tryptophan. Approximately 5 μmoles of carbon dioxide are liberated from each tryptophan isomer. More oxygen is consumed during oxidation of the D-isomer, but equal quantities of carbon dioxide are liberated. L-Kynurenine oxidation requires 3.7 μmoles of oxygen and 3.9 μmoles of carbon dioxide are released. The oxidation of anthranilic acid requires 3.0 μmoles of oxygen and 3.1 μmoles of carbon dioxide are liberated. Oxygen consumption on salicylic acid and gentisic acid are 2.5 and 2.1 μmoles

Figure 11

Growth of a Flavobacterium species using D-, L-, and DL-kynurenine as the carbon and energy source. ■, 1.6×10^{-3} M L-kynurenine; □, 1.6×10^{-3} M DL-kynurenine; △, 8×10^{-4} M L-kynurenine; ○, 1.2×10^{-3} M DL-kynurenine; ▽, 6×10^{-4} M L-kynurenine; ◇, 6×10^{-4} M DL-kynurenine; ●, 8×10^{-4} M D-kynurenine.

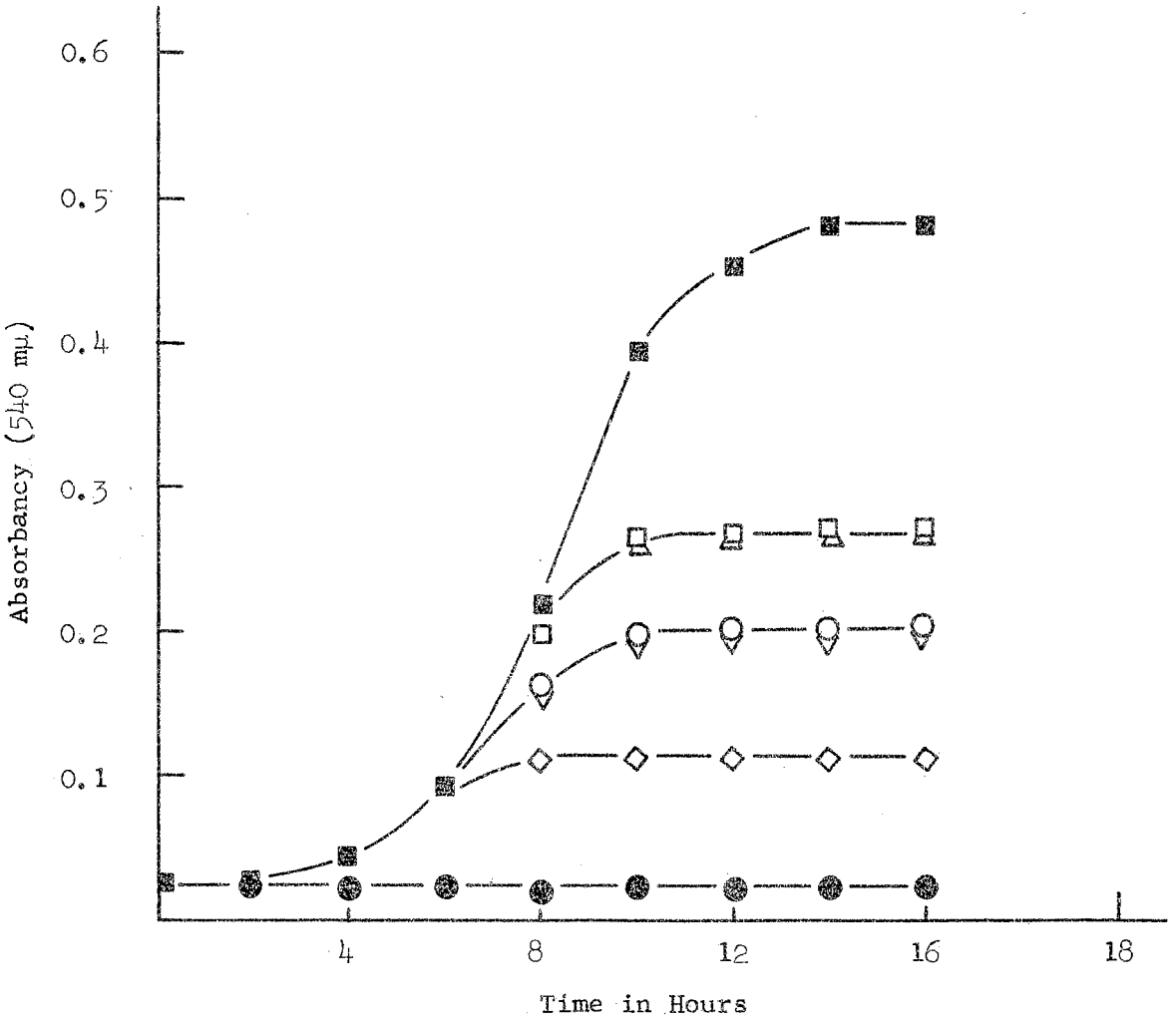

0.6
0.5
0.4
0.3
0.2
0.1
Absorbancy (540 mμ)
4
8
12
16
18
Time in Hours

TABLE V

OXIDATION OF INTERMEDIATES OF D-TRYPTOPHAN DISSIMILATION BY D-TRYPTOPHAN-GROWN CELLS OF A FLAVOBACTERIUM SPECIES

Substrate	μM Oxygen* Uptake per μM of Substrate	μM CO_2 Produced* per μM of Substrate
D-Tryptophan	5.84	5.27
L-Tryptophan	5.00	5.09
L-Kynurenine	3.75	3.92
DL-Kynurenine	2.00	1.83
Anthranilic Acid	3.03	3.12
Salicylic Acid	2.50	--
Gentisic Acid	2.10	--
L-Alanine	1.11	0.84
D-Alanine	1.16	0.98

* All values are corrected for endogenous oxygen uptake and carbon dioxide production.

of oxygen respectively. D- or L-alanine requires 1.1 μmoles of oxygen and 1 μmole of carbon dioxide is liberated.

The difference in oxygen consumption during the oxidation of D- or L-tryptophan is difficult to reconcile since the isomers are of the same oxidative level. Behrman and Stella (6) isolated several pseudomonads and assigned them metabolic pathways on the basis of sequential induction studies. When 10 isolates with the racemase-aromatic pathway were grown on L-tryptophan, they consumed an average of 7.7, 8.3 and 4.3 μmoles O_2 per μmole L-tryptophan, D-tryptophan and anthranilic acid respectively. The respective ranges were 6.2 to 8.2, 6.6 to 9.8 and 3.5 to 5.7. No explanation was offered for the difference in oxygen consumption between the two tryptophan isomers.

The differences in the total oxygen consumed during oxidation of D- and L-tryptophan could result from a variation in the extent of substrate oxidation. One explanation may be that the D-isomer is oxidized by two pathways, one of which involves the intermediary formation of the L-isomer, thus accounting for results obtained during the enzyme induction experiments, and a second pathway which involves more extensive oxygen utilization. Stanier and Hayaishi (88) found one strain that degraded L-tryptophan via a "mixed" pathway with the concomitant formation of equimolar quantities of anthranilic and kynurenic acids. However, the data presented in this study suggests that only one pathway is operative in this organism.

Identification of degradation intermediates

Chromatographic analysis of filtrates from washed cell suspensions oxidizing D- or L-tryptophan were undertaken to identify intermediates. Filtrates from D- or L-tryptophan-induced cells were analyzed during

oxidation of L-tryptophan. L-Tryptophan and anthranilic acid were detected in the filtrate. Filtrates from cells induced to D- or L-tryptophan tested during oxidation of D-tryptophan showed only one ninhydrin positive spot corresponding to tryptophan. Changing the substrate concentrations, cell turbidities, and sampling intervals did not alter the results.

Failure to detect suspected intermediates in the filtrates during D-tryptophan oxidation is not surprising. Washed suspensions of DL-tryptophan-grown cells give the following oxidation rates calculated as μmoles of substrate oxidized per milligram protein per hour (all values were corrected for endogenous respiration): L-tryptophan, 1.60; D-tryptophan, 0.63; L-kynurenine, 1.69; and anthranilic acid, 1.51. Thus, the rate-limiting step in the oxidative degradation of D-tryptophan is the reaction(s) which converts D-tryptophan into the L-isomer and/or the transport of the D-isomer across the cell barrier. Since these are relatively slow processes, sufficient accumulation of intermediates to permit detection did not occur during D-tryptophan degradation. However, since the isomeric change is not necessary and/or the transport across the cell barrier is faster with L-tryptophan, the rate limiting reaction is the utilization of anthranilic acid. Thus, during rapid L-tryptophan oxidation, anthranilic acid accumulates in the filtrate.

Identification of amino substituted benzenoid intermediates in cell filtrates

Experiments were conducted to demonstrate degradation intermediates (Table VI) using a color test specific for amino substituted aromatic rings. DL-Tryptophan-grown cells were incubated with D- or L-tryptophan on a reciprocating shaker at 37 C, samples withdrawn, the organisms

TABLE VI

ACCUMULATION OF AMINO SUBSTITUTED BENZENOID COMPOUNDS IN FILTRATES OF CELLS DEGRADING D- OR L-TRYPTOPHAN*

Sample time in Minutes	Filtrates from Cells Degrading L-Tryptophan (Absorbancy)	µg of Amino Substituted Compound Accumulated**	Filtrates from Cells Degrading D-Tryptophan (Absorbancy)	µg of Amino Substituted Compound Accumulated**
0	0.02	0.48	0.01	0.24
15	0.17	14.40	0.01	0.24
30	0.31	105.60	0.02	0.48
45	0.50	172.80	0.02	0.48
60	0.62	213.60	0.02	0.48
75	0.60	206.40	0.02	0.48
90	0.60	206.40	0.02	0.48
105	0.27	93.60	0.02	0.48
120	0.03	12.00	0.02	0.48
135	0.02	0.48	0.02	0.48

* 0.0268 gm substrate

** Calculated using anthranilic acid as a standard.

immediately removed by filtration through a Millipore filter (type HA), and the filtrate assayed for amino substituted aromatic compounds.

Filtrates from cell suspensions degrading L-tryptophan showed the presence of an amino substituted aromatic ring. The color intensity increased to the maximum level after 60 minutes, then rapidly decreased until no color was evident after 135 minutes. A color reaction indicating a diazo coupling with the amino substituted ring was not evident in cells degrading D-tryptophan, indicating that the entrance of D-tryptophan into the cell and/or conversion to the L-isomer is the rate limiting reaction and anthranilic acid does not accumulate in the filtrate.

Oxidation of D-tryptophan by cell-free extracts

Extracts, obtained by X-press disintegration of DL-tryptophan-grown cells, oxidized D-tryptophan to indolepyruvic acid in the presence of certain electron acceptors. The reaction could be followed manometrically using phenazine methosulfate as the electron acceptor (Figure 12). The addition of 2,6-dichlorophenolindophenol enabled the reaction to be measured spectrophotometrically as the decrease in extinction at 600 mμ consequent upon reduction of the dye (Figure 13). D-Tryptophan was readily oxidized, but L-tryptophan would not serve as a substrate for the oxidase (Figure 12, 13). Extracts from L-tryptophan-grown cells were devoid of any oxidase activity. Diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin mononucleotide, flavin adenine dinucleotide, or methylene blue would not function as electron acceptors in the oxidase system.

The cell extract from DL-tryptophan-induced cells oxidizes several D-amino acids but has no detectable activity toward L-amino acids. The D-isomers of tyrosine, histidine, phenylalanine and methionine, as well

Figure 12

Oxidation of D-tryptophan by extracts of DL-tryptophan-grown cells of a Flavobacterium species. The main compartment of the Warburg flasks contained 100 μmoles of Tris buffer (pH 7.4) and 1.0 ml of extract (containing 15 mg of protein) in a total volume of 2.0 ml. The center well contained 0.2 ml of 20% KOH (w/v). After thermal equilibration at 37 C for 10 minutes, 10 μmoles of D-tryptophan and 0.2 ml of 1% phenazine methosulfate (w/v) were added from the side arms. The oxygen uptake (μliters) was recorded. ● , complete system; ■ , phenazine methosulfate omitted; ▲ , D-tryptophan omitted; ○ , phenazine methosulfate and D-tryptophan omitted; ◨ , L-tryptophan replaced D-tryptophan; △ , indolepyruvic acid replaced D-tryptophan.

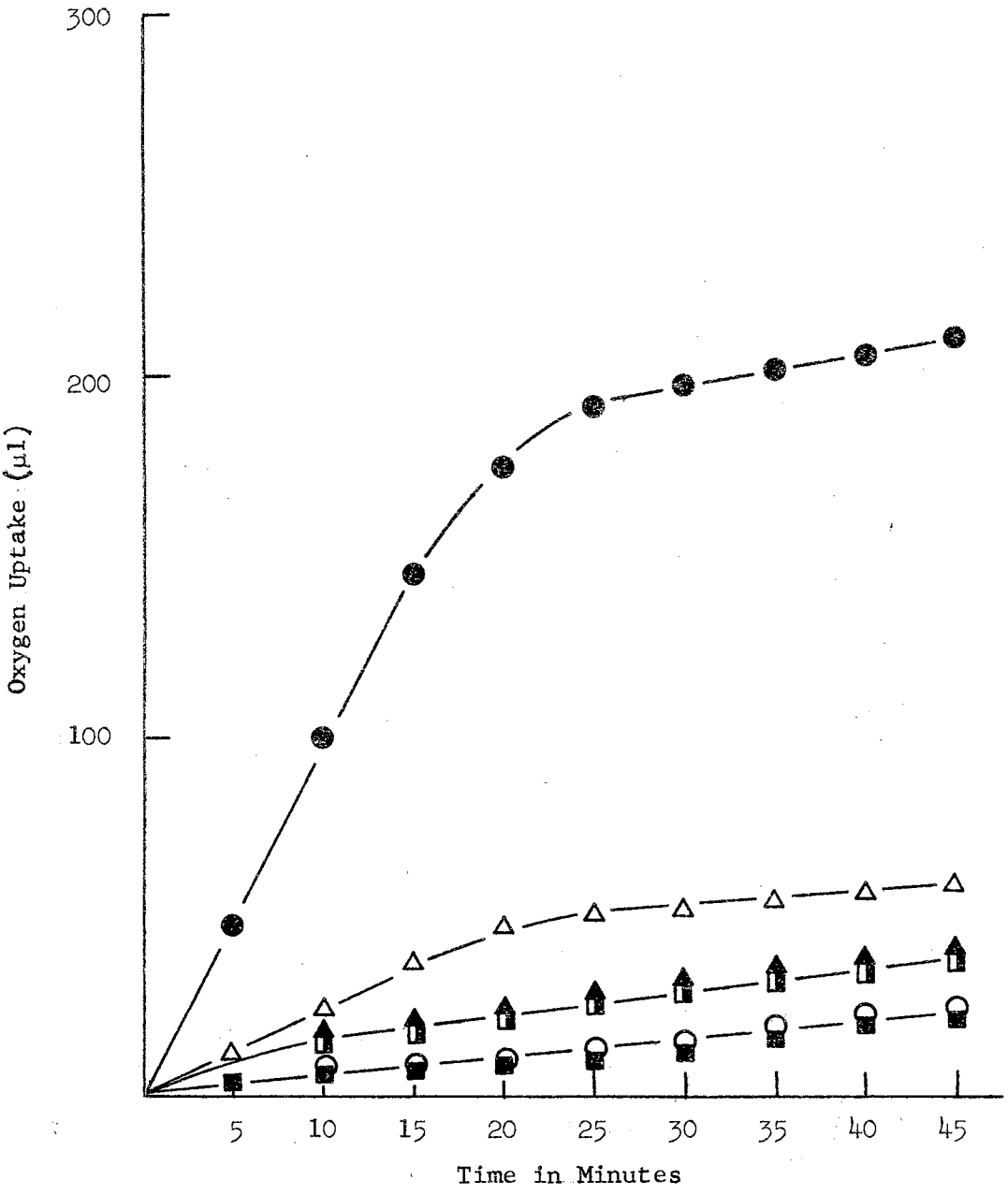
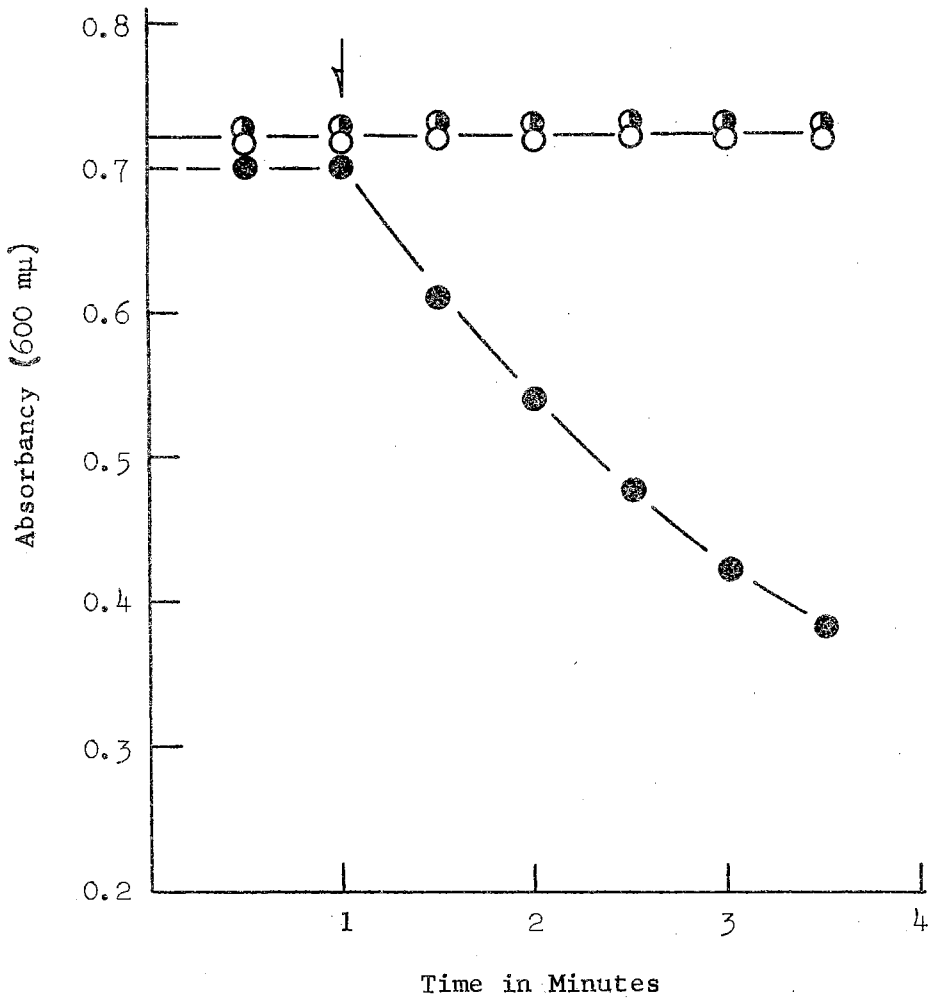

300
200
100
Oxygen Uptake (µl)
5
10
15
20
25
30
35
40
45
Time in Minutes

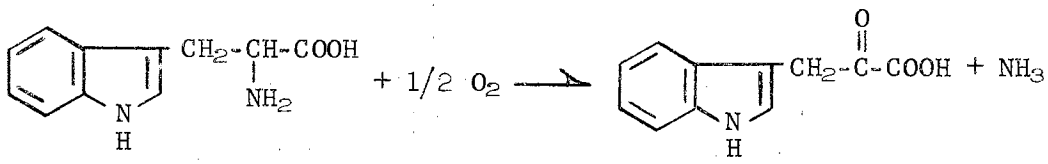
Figure 13

Reduction of 2,6-dichlorophenolindophenol catalyzed by extracts of DL-tryptophan-grown cells. The complete system (●) contained: 200 μmoles of Tris buffer (pH 7.4), 5 μmoles of sodium cyanide, 0.12 μmoles of 2,6-dichlorophenolindophenol, and 0.1 ml of cell extract (containing 1.64 mg of protein) in a total volume of 3.0 ml. D-Tryptophan (10 μmoles) was added at the point indicated by the arrow. The reduction of 2,6-dichlorophenolindophenol was measured as the decrease in extinction at 600 mμ with time. Control cuvettes contained the above system but omitted cell extract (○) or D-tryptophan (◑).


0.8
0.7
0.6
0.5
0.4
0.3
0.2
Absorbancy (600 mμ)
1
2
3
4
Time in Minutes

as tryptophan, are attacked at various rates while the D-isomer of serine, alanine, glutamic acid, aspartic acid and arginine are oxidized slowly or not at all (Table VII).

The stoichiometry of the oxidation varied slightly depending on the amino acid substrate, but this can be reconciled since the oxidase(s) is a component of a crude extract. D-Tryptophan oxidation consumed 0.70 to 0.75 mole O_2 per mole of substrate. The theoretical uptake for oxidative deamination is 0.5 mole O_2 per mole of substrate. The first product of the oxidase-D-tryptophan reaction is indolepyruvic acid, and the oxygen uptake in the indolepyruvic acid control indicated some auto-oxidation of indolepyruvic acid (Figure 12), thus accounting for the slightly higher value. The lability of indolepyruvic acid in solution has been studied by Kaper and Veldstra (47) and Wildman, Ferri, and Bonner (99). These authors concluded that indolepyruvic acid undergoes spontaneous decomposition in solution. Results from several experiments indicated that when the oxygen uptake of the indolepyruvic acid control was substracted from the uptake of the D-tryptophan system (Figure 12), the difference varied from 0.55 to 0.70 mole O_2 per mole substrate. Thus, the stoichiometry of the oxidation of D-tryptophan by the oxidase was established to be:



D-Tryptophan Indolepyruvic Acid

Oxygen uptake in the presence of added phenazine methosulfate and the yellow color of the extract suggest that the oxidase is a flavoprotein. However, the addition of known flavin compounds did not show significant

TABLE VII

SPECIFICITY AND ACTIVITY OF CRUDE D-AMINO ACID OXIDASE PREPARATIONS FROM DL-TRYPTOPHAN-GROWN CELLS OF THE FLAVOBACTERIUM SPECIES

D-Amino Acid	Activity*
Alanine	0
Arginine	0
Aspartic Acid	0
Glutamic Acid	0
Histidine	112
Methionine	117
Phenylalanine	132
Serine	0
Tryptophan	100
Tyrosine	130

* Activity on D-tryptophan arbitrarily assigned a value of 100; other values are relative to this rate.

oxygen uptake. An active catalase, determined by the addition of dilute H_2O_2 to the extract, is present in the crude preparations negating the nonenzymatic decarboxylation of the α-keto acid formed by the oxidase.

The enzymatic formation of indolepyruvic acid from D-tryptophan was detected by paper chromatography. The keto acid is unstable under the conditions of chromatography, so precautions were taken to insure that the spots on the chromatograms represent products of enzymatic reactions and not artifacts.

An ascending chromatogram of synthetic indolepyruvic acid using Solvent System A as the developing solvent is schematically represented in Figure 14. Development in Solvent System B produced one spot with considerable trailing. Thus, indolepyruvic acid is relatively stable in acid solution but extremely labile in alkaline solutions as previously noted by Bentley et al. (7) and Kaper and Veldstra (47).

No attempt was made to identify the decomposition products of indolepyruvic acid, but all of the spots were developed by Salkowski or Ehrlich reagents and probably contain the indole nucleus. Kaper and Veldstra (47) observed that spots B, C, and F corresponded in color and approximate position to indoleglycolic acid, indoleacetic acid and indolealdehyde respectively. The remaining spots have not been identified.

Chromatograms prepared from the deproteinized flask contents of four experimental systems are summarized in Figure 15. Spots G and H result from phenazine methosulfate -- apparently G corresponds to the reduced form and H to the oxidized form. Spot H quenches ultraviolet light and is a visible light yellow turning slightly darker after spraying with Ehrlich reagent. Spot G is a visible bright organge-red which turns maroon on aging.

Figure 14

Standard chromatogram of indolepyruvic acid and phenazine methosulfate (Solvent: isopropanol:ammonium hydroxide(sp. gr. 0.896):water(10:1:1 v/v). I, Phenazine methosulfate, spots located without developing reagent. II, indolepyruvic acid, spots located with use of Ehrlich reagent; colors indicated are those observed within 30 minutes after application of Ehrlich reagent.

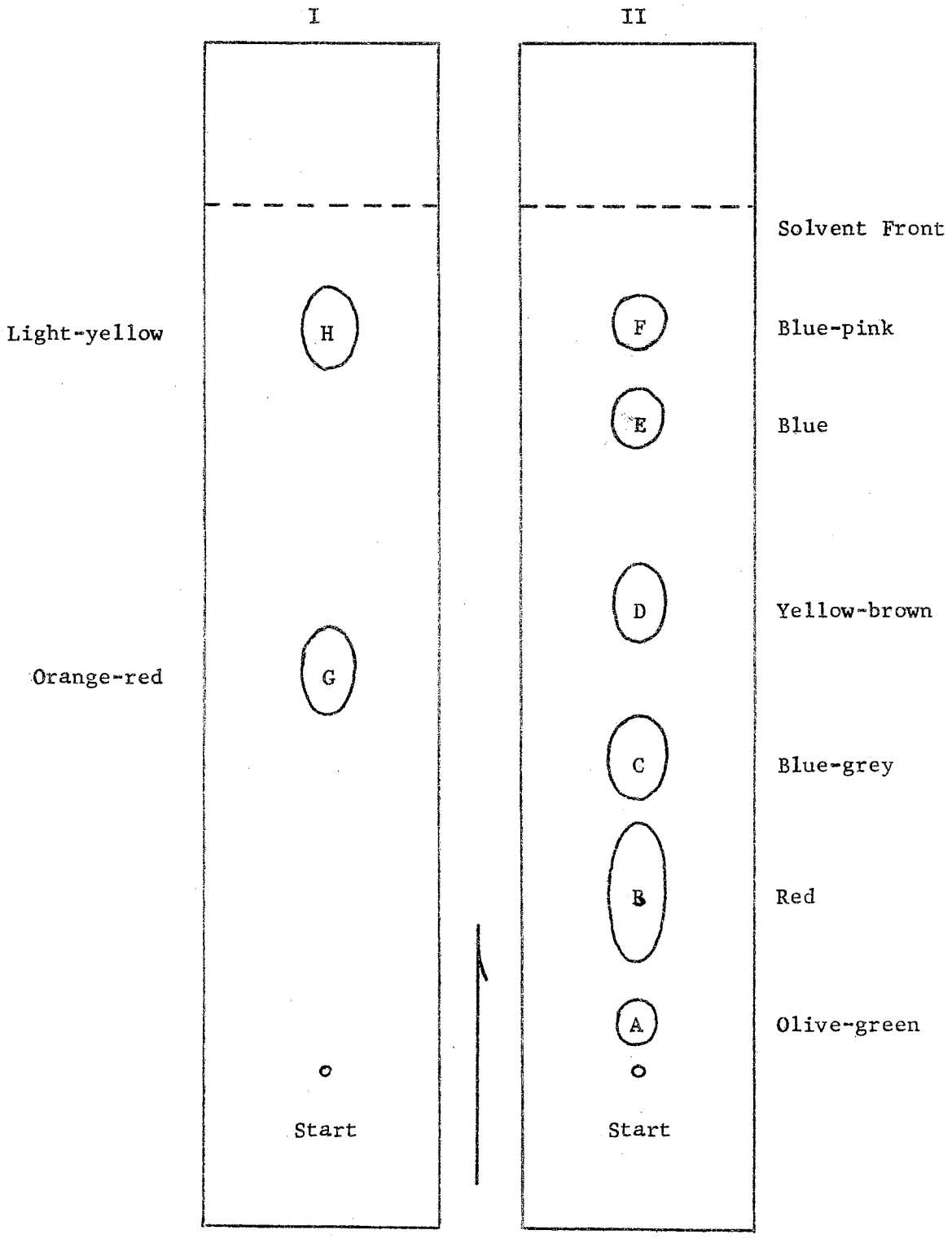
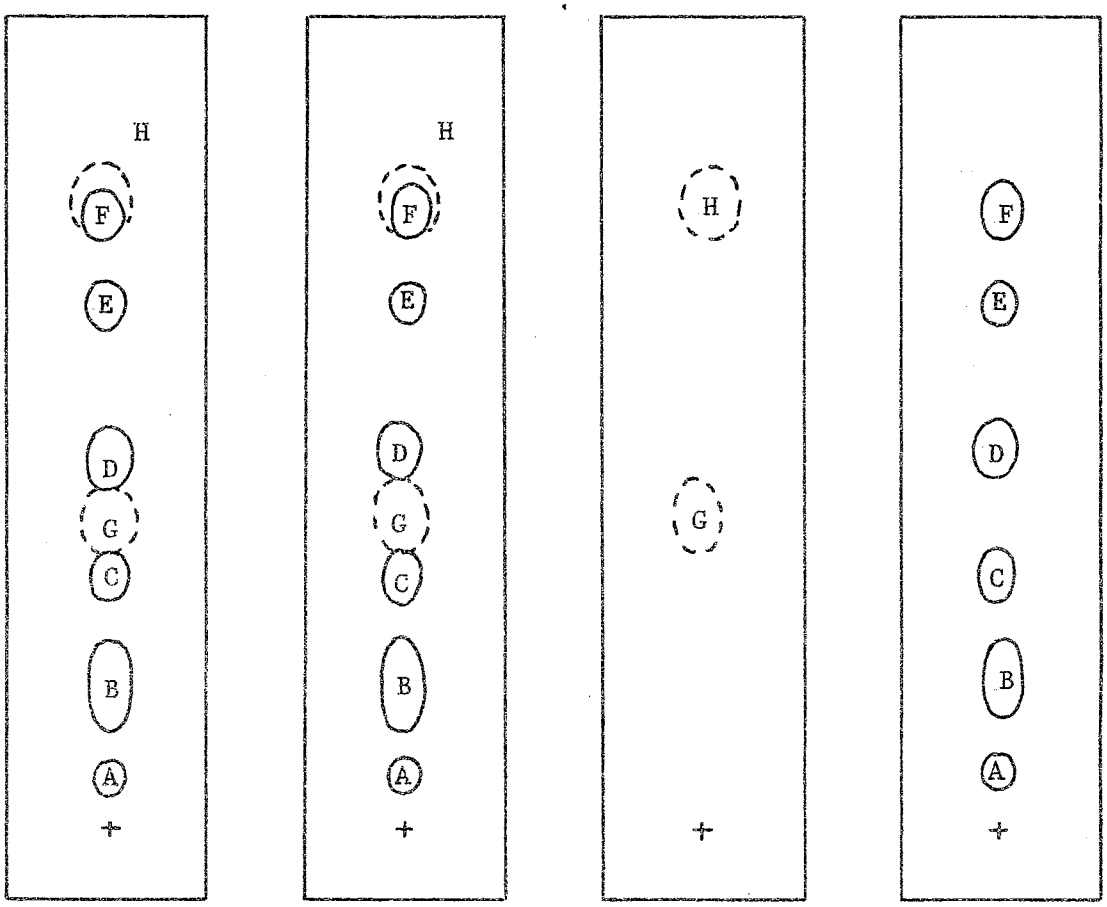

I
II
Solvent Front
Light-yellow
H
F
Blue-pink
E
Blue
D
Yellow-brown
Orange-red
G
C
Blue-grey
B
Red
A
Olive-green
Start
Start

Figure 15

Chromatograms obtained from deproteinized flask contents of four systems containing D-amino acid oxidase from DL-tryptophan-grown cells. System I contained enzyme, Tris buffer, NaCN, phenazine methosulfate and D-tryptophan (Table VII). System II contained the components of System I with D-tryptophan replaced by indolepyruvic acid. System III contained the components of System I with D-tryptophan omitted. System IV contained the components of System II with phenazine methosulfate omitted. The mixtures were incubated at 37 C and the reaction stopped by heating in a boiling water bath. The deactivated protein was removed by centrifugation and the deproteinized flask contents chromatographed using Solvent System A.



System I System II System III System IV

Spots A, B, C, D, E and F are characteristic of indolepyruvic acid in an alkaline solution. A comparison of System I containing cell extract, NaCN, phenazine methosulfate and D-tryptophan with the synthetic indolepyruvic acid and phenazine methosulfate controls (Figure 14) established that the complete pattern of spots, characteristic of indolepyruvic acid, is obtained. This indicates that indolepyruvic acid was produced from D-tryptophan (System I). System II, identical to System I with the exception that D-tryptophan was replaced by indolepyruvic acid, gave the same characteristic pattern of spots augmenting the conclusion that indolepyruvic acid is formed from D-tryptophan in System I.

The fact that two compounds possess the same R_f value is not sufficient proof that they are identical, but the occurrence of a pattern of spots, identical in R_f values and color reactions with that of authentic indolepyruvic acid, can be considered proof of the presence of the compound.

Conversion of indolepyruvic acid into L-tryptophan

Aida et al. (1) used dried cells of several bacterial species to demonstrate that indolepyruvic acid was readily converted to L-tryptophan via a transamination reaction. L-Glutamic and L-aspartic acids were the most efficient amino donors, but several L-amino acids could serve as a source of the amino group.

Cell extracts of DL-tryptophan-induced Flavobacterium catalyzed the conversion of indolepyruvic acid to L-tryptophan. Experiments established that L-glutamic acid, L-glutamine or L-aspartic acid, but not L-asparagine, would function as amino donors. Glutamic acid was more efficient than aspartic acid (Table IX). L-Tryptophan production, via the transamination reaction, was compared in a number of different systems (Table VIII).

TABLE VIII

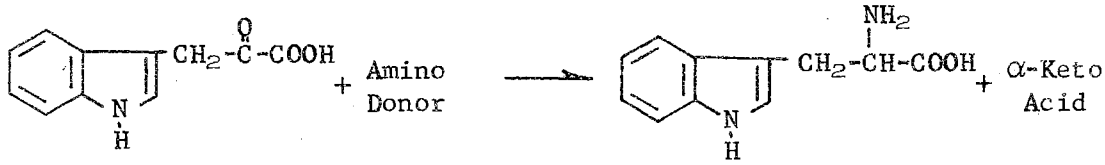
FORMATION OF L-TRYPTOPHAN BY EXTRACTS OF DL-TRYPTOPHAN-GROWN CELLS OF A FLAVOBACTERIUM SPECIES

System No.	Reaction Mixture	O_2 Uptake/ Substrate (μmoles)	Growth and Relative Location on Chromatogram
1	Complete system (D-tryptophan)	0.64	+++(Tryptophan)
2	D-Tryptophan omitted	0.00	-
3	Phenazine methosulfate omitted	0.00	-
4	L-Glutamic acid omitted	0.64	+ (Tryptophan)
5	Complete system but cell extract boiled before incubation	0.00	-
6	Complete system (indolepyruvic acid)	0.02	+++(Tryptophan)
7	Indolepyruvic acid omitted	0.00	-
8	Phenazine methosulfate and sodium cyanide omitted	0.78	- (Anthranilic)
9	L-Glutamic acid omitted	0.01	+ (Tryptophan)
10	Phenazine methosulfate, sodium cyanide and L-glutamic acid omitted	0.12	- (Anthranilic)
11	Complete system but cell extract boiled before incubation	0.03	-
12	Complete system (L-tryptophan)	0.00	+++(Tryptophan)
13	Phenazine methosulfate and sodium cyanide omitted	0.98	- (Anthranilic)
14	Complete system but cell extract boiled before incubation	0.00	+++(Tryptophan)

The main compartment of the Warburg flasks contained: 100 μmoles of Tris buffer (pH 7.4), 20 μmoles of NaCN, 20 μmoles of L-glutamic acid, and 1.5 ml of extract of DL-tryptophan-grown cells (containing 20 mg of protein) in a total volume of 2.4 ml. The center well contained 0.2 ml of 20% KOH (w/v). After thermal equilibration at 37 C, 10 μmoles of either D-tryptophan, L-tryptophan, or indolepyruvic acid and 0.2 ml of 1% phenazine methosulfate (w/v) were added from the side arms. Oxygen uptake was measured, the flask contents heated in a boiling water bath for 5 minutes and centrifuged. The supernatant solution was chromatographed employing the ascending technique with Solvent System A. The presence of L-tryptophan was demonstrated by the bioautographic technique. Growth was evaluated after 24 hours incubation. R_f values were: tryptophan, 0.16; anthranilic acid, 0.26. Oxygen uptake values were corrected for endogenous.

L-Tryptophan was produced when the extract was incubated with D-tryptophan, phenazine methosulfate and L-glutamic acid (System No. 1) or with indolepyruvic acid and an amino donor (System No. 6). When L-glutamic acid or other amino donors were omitted from these systems, a very small quantity of L-tryptophan was formed. This was probably due to transamination which occurred due to the presence of minute quantities of an amino donor in the cell extract.

L-Tryptophan was not produced in the D-tryptophan containing systems unless an electron acceptor such as phenazine methosulfate was added. Thus, the direct racemization of D-tryptophan does not occur. The oxidation of D-tryptophan results in the formation of indolepyruvic acid and the conversion of indolepyruvic acid to L-tryptophan can be represented by:



Indolepyruvic Acid L-Tryptophan

The rate of the overall conversion process can be quantitated by measuring oxygen uptake or the production of anthranilic acid. The crude extract contained the necessary enzymes to convert L-tryptophan to anthranilic acid and alanine (in quantitative yields). Alanine was identified by paper chromatography. The rate of conversion of L-tryptophan to anthranilic acid and alanine was much faster than the conversion of indolepyruvic acid to L-tryptophan (Figure 16).

L-Glutamic and L-aspartic acids can serve as the amino donor in the indolepyruvic acid-L-amino acid transaminase system (Table IX). The

Figure 16

The formation and oxidation of L-tryptophan by cell extracts of DL-tryptophan-grown Flavobacterium species. ●, 12 μmoles L-tryptophan; ▽, 15 μmoles indolepyruvic acid and 30 μmoles L-glutamic acid; ○, 15 μmoles indolepyruvic acid; ◑, 15 μmoles indolepyruvic acid and 30 μmoles L-alanine; □, 30 μmoles L-glutamic acid; ▲, endogenous.

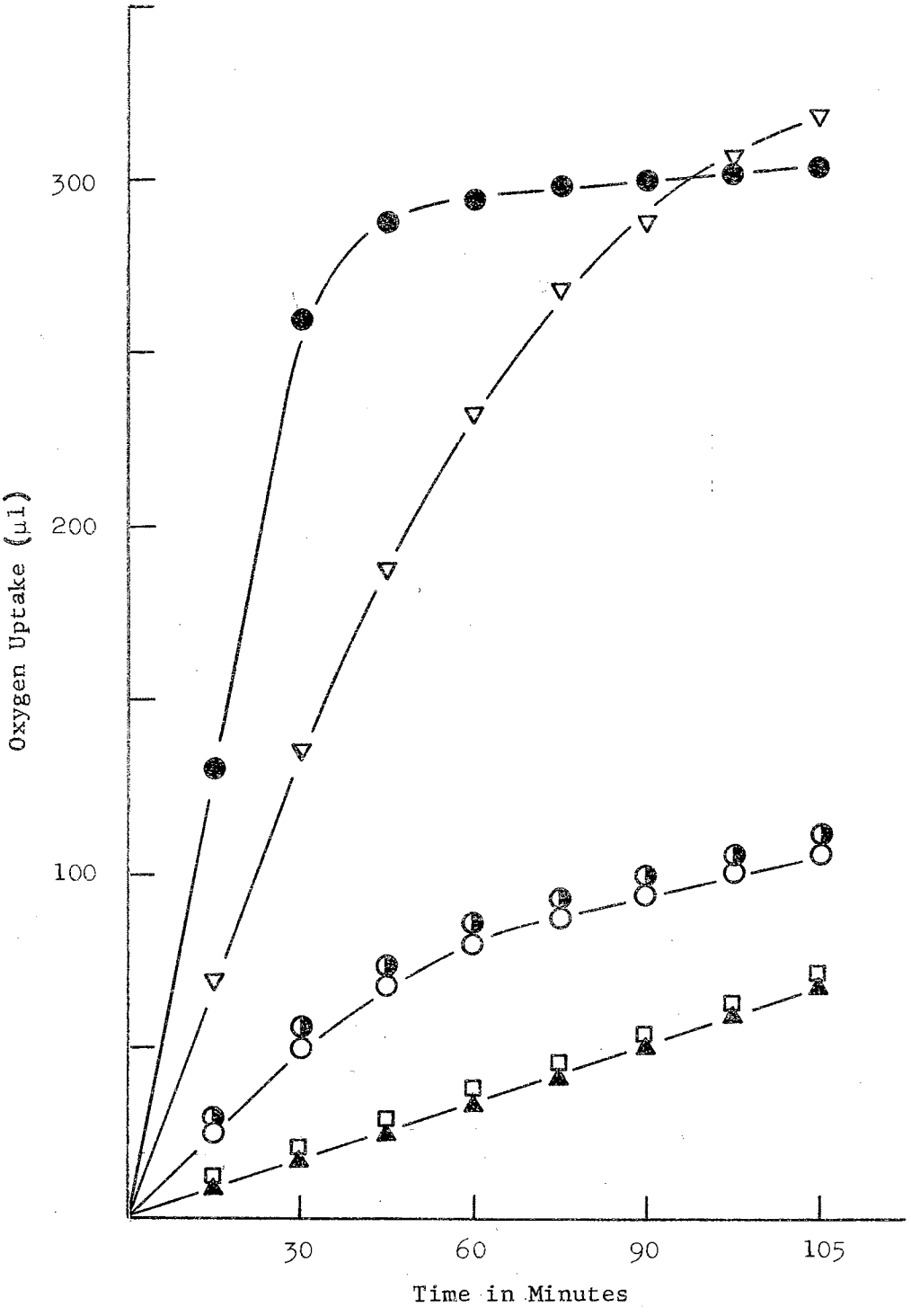

300
200
100
Oxygen Uptake (µl)
30
60
90
105
Time in Minutes

TABLE IX

INDOLEPYRUVIC ACID-L-AMINO ACID TRANSAMINASE ACTIVITY
FROM FLAVOBACTERIUM CELL EXTRACTS

Indolepyruvic Acid μMoles	Amino Donor	O_2 Consumed μMoles	Anthranilic Acid Formed μMoles	Percent Yield
2.5	L-Glutamic	2.59	2.40	96
5.0	L-Glutamic	5.00	4.38	87
7.5	L-Glutamic	7.47	6.84	91
10.0	L-Glutamic	9.98	8.52	85
2.5	L-Aspartic	1.11	0.80	32
5.0	L-Aspartic	1.95	1.20	24
7.5	L-Aspartic	2.50	2.16	28
10.0	L-Aspartic	3.34	2.42	24
5.0	L-Alanine	Trace	Trace	0
5.0	D-Glutamic	Trace	Trace	0
5.0	D-Alanine	Trace	Trace	0

The formation of L-tryptophan was determined by quantitating the anthranilic acid produced and by calculating the oxygen consumed during oxidation of L-tryptophan to L-kynurenine. The rate of formation of anthranilic acid using L-glutamic acid as the amino donor is plotted in Figure 17. The activity of the transaminase system varied depending upon the particular enzyme preparation, and stringent efforts to obtain a reproducible system were unsuccessful.

Growth on indolepyruvic acid

Growth studies were conducted using indolepyruvic acid as the carbon and energy source, and limited growth was obtained (Figure 18). The growth on indolepyruvic acid could be due to utilization of indolepyruvic acid, but since auto-oxidation of the acid is rapid, it is soon destroyed and growth ceases. Also, growth could be due to the limited use of a "break-down" product of the acid. Supplementation of the indolepyruvic acid medium with succinic acid did not enhance the ability of the organism to use indolepyruvic acid for growth. Addition of other carbon and energy sources, such as L-glutamic acid, pyruvic acid and L-alanine, gave similar results. Growth in the indolepyruvic acid and succinic acid system lagged behind the succinic acid control indicating that this concentration of indolepyruvic acid was slightly toxic to Flavobacterium species. Kaper and Veldstra (47) noted that incubating indolepyruvic acid with either a growing culture or a washed cell suspension of Agrobacterium tumefaciens produced erratic growth results due to the instability of the acid and stated that low concentrations must be used since a concentration higher than 0.002 percent was toxic.

Oxidation of L-tryptophan by cell extracts

Cell extracts of DL- or L-tryptophan-grown cells oxidized L-trypto-

Figure 17

The formation of anthranilic acid from indolepyruvic acid and L-glutamic acid using cell extracts of DL-tryptophan-grown cells. Six ml of extract (containing 73.8 mg protein), 180 μmoles of Tris buffer (pH 7.4), 30 μmoles of indolepyruvic acid, 60 μmoles L-glutamic acid and water to a total volume of 9 ml were incubated with shaking at 37 C. At indicated time intervals, samples were withdrawn, deproteinized and analyzed for anthranilic acid. ○, complete system; ●, L-glutamic acid omitted.

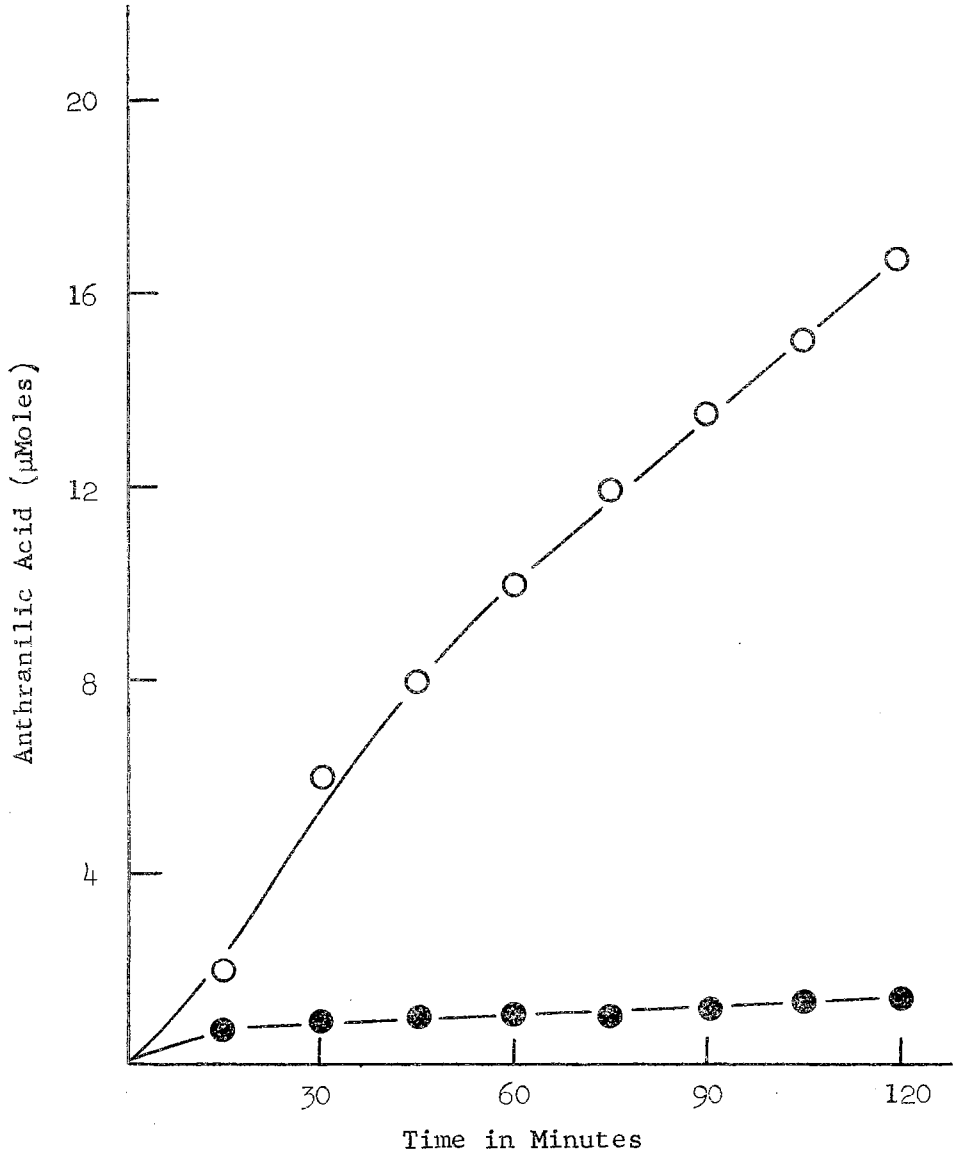
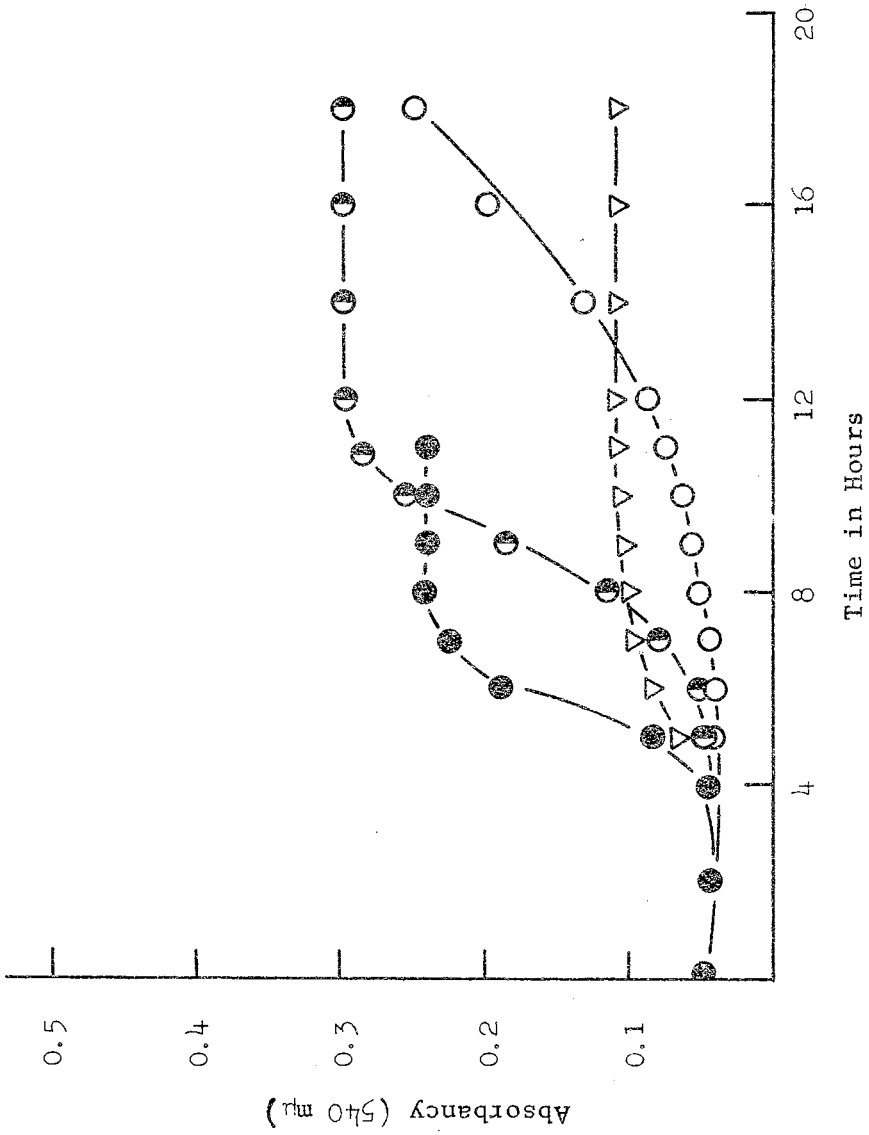

20
16
12
8
4
Anthranilic Acid (μMoles)
30
60
90
120
Time in Minutes

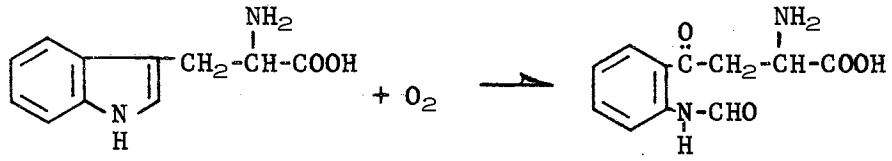
Figure 18

Growth of a Flavobacterium species on indolepyruvic acid in the presence and absence of succinic acid. ○, 1.2 x 10^{-3} M D-tryptophan; ▽, 6 x 10^{-2} M indolepyruvic acid; ●, 2 x 10^{-3} M succinic acid; ◑, 6 x 10^{-2} M indolepyruvic acid plus 2 x 10^{-3} M succinic acid.

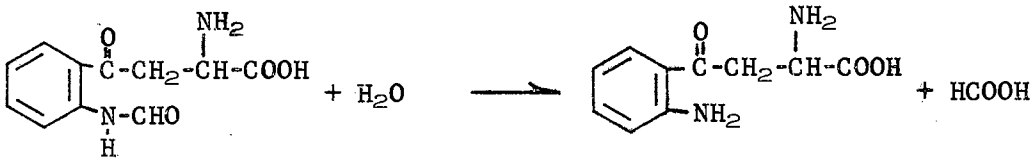

Absorbancy (540 mμ)
0.5
0.4
0.3
0.2
0.1
4
8
12
16
20
Time in Hours

phan to L-kynurenine with the utilization of 1 mole O_2 per mole of L-tryptophan. Occasionally kynurenine could be demonstrated by paper chromatography (Solvent System B, R_f 0.45), but normally kynurenine was rapidly hydrolyzed to anthranilic acid and alanine. No oxygen was utilized during this hydrolysis.

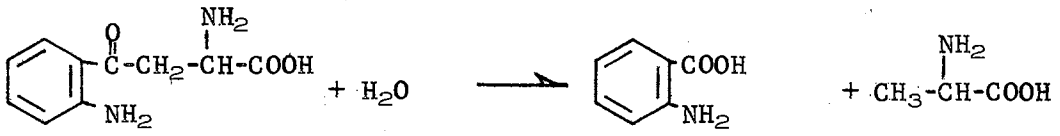
The conversion of L-tryptophan to N-formyl-L-kynurenine proceeds by cleavage of the indole nucleus between carbons number 2 and 3 and requires 1 mole of molecular oxygen. N-Formyl-L-kynurenine is then hydrolyzed with water to form L-kynurenine and formic acid. Formic acid may be degraded to carbon dioxide and water. L-Kynurenine is hydrolyzed to L-alanine and anthranilic acid. The overall sequence can be represented by:



L-Tryptophan N-Formyl-L-kynurenine



N-Formyl-L-kynurenine L-Kynurenine Formic Acid



L-Kynurenine Anthranilic L-Alanine

Proposed scheme of D-tryptophan degradation

The schematic illustration of D-tryptophan degradation by the Flavobacterium species is illustrated in Figure 19. The oxidative deamination of D-tryptophan to indolepyruvic acid requires one-half mole of O_2 per mole substrate, and the corresponding imino compound is an intermediate. Transamination of the α-keto acid with an amino donor forms L-tryptophan. L-Tryptophan is oxidized to anthranilic acid and alanine as previously described. Anthranilic acid is oxidized, with the uptake of one-half mole O_2, to salicylic acid, which is oxidized, with the uptake of one gram atom of oxygen, to gentisic acid. The oxidation of gentisic acid requires 2 moles O_2 per mole of substrate. L-Alanine is degraded with the uptake of 1 mole O_2 and release of 1 mole carbon dioxide per mole of alanine.

Influence of tryptophan analogs on the oxidation of L- and D-tryptophan

The influence of tryptophan analogs on the oxidation of D- or L-tryptophan by a washed suspension of DL-tryptophan-grown cells was studied. DL-Tryptazan, DL-oxindolealanine, DL-indolelactic acid, indoleacetic acid, indolepropionic acid, tryptamine, tryptophol, DL-5-hydroxytryptophan, and D- and L-5-hydroxytryptophan were utilized in these experiments.

DL-Tryptazan inhibits oxidation of both D- and L-tryptophan by the washed cell suspension (Figure 20). The degree of inhibition is a function of the inhibitor/substrate ratio suggesting that tryptazan is a competitive inhibitor. An inhibitor/substrate ratio of 1:1 inhibited the oxidation of L-tryptophan 24 percent while the rate of D-tryptophan oxidation was reduced 54 percent. Higher inhibitor/substrate ratios showed a more pronounced inhibition with both substrates. The cells were

Figure 19

Proposed scheme of D-tryptophan degradation by a species of Flavobacterium. X, an unknown electron acceptor which can be replaced by phenazine methosulfate or 2,6-dichlorophenolindophenol; amino donor, L-glutamic acid, L-aspartic acid or L-glutamine.

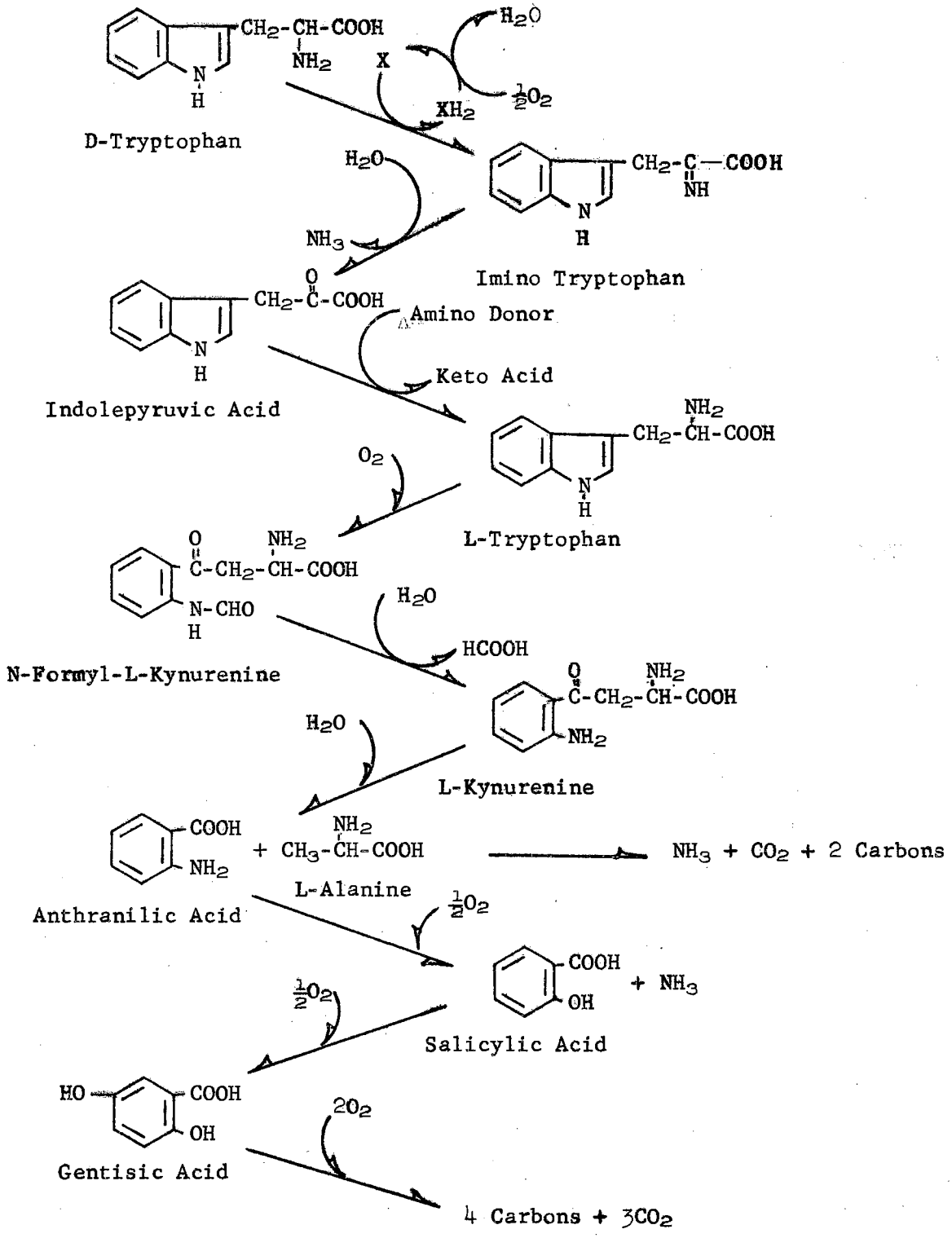
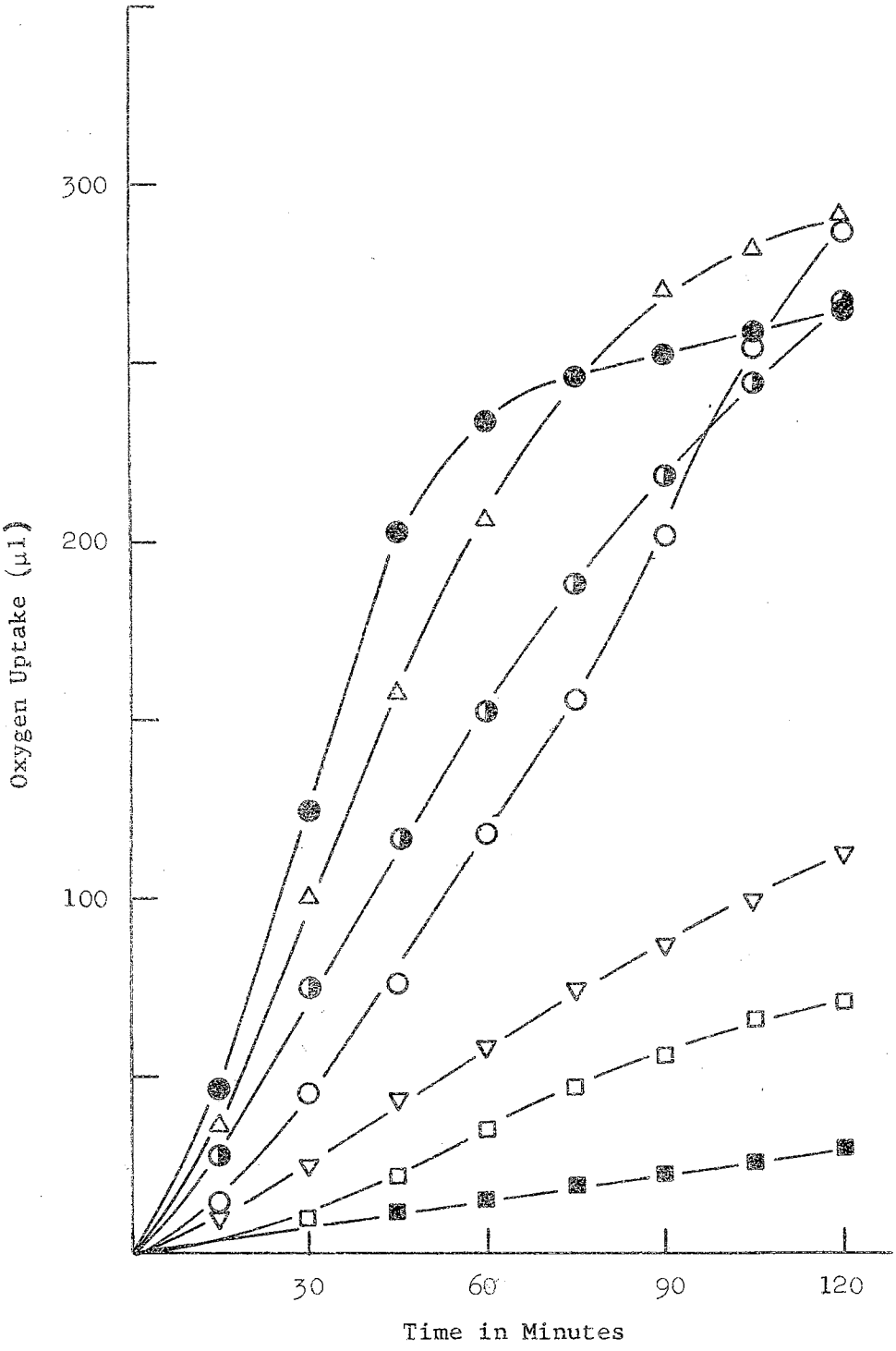

D-Tryptophan
Imino Tryptophan
Indolepyruvic Acid
Amino Donor
Keto Acid
L-Tryptophan
N-Formyl-L-Kynurenine
L-Kynurenine
L-Alanine
Anthranilic Acid
NH3 + CO2 + 2 Carbons
Salicylic Acid
Gentisic Acid
4 Carbons + 3CO2

Figure 20

The oxidation of D- and L-tryptophan in the presence of DL-tryptazan by a washed cell suspension of a Flavobacterium species. ●, 2 μmoles L-tryptophan; △, 2 μmoles L-tryptophan and 2 μmoles DL-tryptazan; ◑, 2 μmoles L-tryptophan and 6 μmoles DL-tryptazan; ○, 2 μmoles D-tryptophan; ▽, 2 μmoles D-tryptophan and 2 μmoles DL-tryptazan; □, 2 μmoles D-tryptophan and 6 μmoles DL-tryptazan; ▩, endogenous.


300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

unable to oxidize tryptazan.

DL-5-Hydroxytryptophan in an inhibitor/substrate ratio of 1:1 and 3:1 did not influence the microbial oxidation of L-tryptophan but drastically curtailed oxidation of D-tryptophan (Figure 21). A 1:1 inhibitor/substrate ratio reduced the rate of D-tryptophan oxidation by 60 percent. Increasing the ratio to 4:1 and 7:1 showed some inhibition of L-tryptophan oxidation. The inhibition by DL-5-hydroxytryptophan was dependent on the inhibitor/substrate ratio. Similar results were obtained using DL-oxindolealanine as the antimetabolite (Figure 22). Neither DL-5-hydroxytryptophan nor DL-oxindolealanine was oxidized by the washed cell suspensions.

When D-5-hydroxytryptophan was added to L-tryptophan oxidizing cells, an inhibitor/substrate ratio of 1:1 and 4:1 did not influence the rate of oxidation, but a high inhibitor concentration (8:1) showed a 90 percent inhibition (Figure 23). L-5-Hydroxytryptophan drastically curtailed the oxidation of L-tryptophan in a 4:1 ratio (70 percent), and increasing the inhibitor/substrate ratio to 8:1 showed a more pronounced inhibition.

L-5-Hydroxytryptophan significantly inhibited the oxidation of D-tryptophan (Figure 24). An inhibitor/substrate ratio of 1:1 reduced the rate of oxidation by 54 percent. Higher ratios caused greater rate reduction. An inhibitor/substrate ratio of 1:1 using D-5-hydroxytryptophan as the inhibitor did not influence the oxidation of D-tryptophan. Ratios of 4:1 and 8:1 inhibited the rate by 15 and 39 percent respectively.

Tryptamine, indoleacetic acid, indolepropionic acid and tryptophol were selected as inhibitors lacking an asymmetric carbon on the side chain. Tryptamine in a maximum inhibitor/substrate ratio of 6:1 did not influence the oxidation of either D- or L-tryptophan.

Figure 21

The oxidation of D- and L-tryptophan in the presence of DL-5-hydroxytryptophan by a washed cell suspension of a Flavobacterium species. ●, 2 μmoles L-tryptophan; △, 2 μmoles L-tryptophan and 2 μmoles DL-5-hydroxytryptophan; ◑, 2 μmoles L-tryptophan and 6 μmoles DL-5-hydroxytryptophan; ○, 2 μmoles D-tryptophan; ▽, 2 μmoles D-tryptophan and 2 μmoles DL-5-hydroxytryptophan; □, 2 μmoles D-tryptophan and 6 μmoles DL-5-hydroxytryptophan; ▩, endogenous.

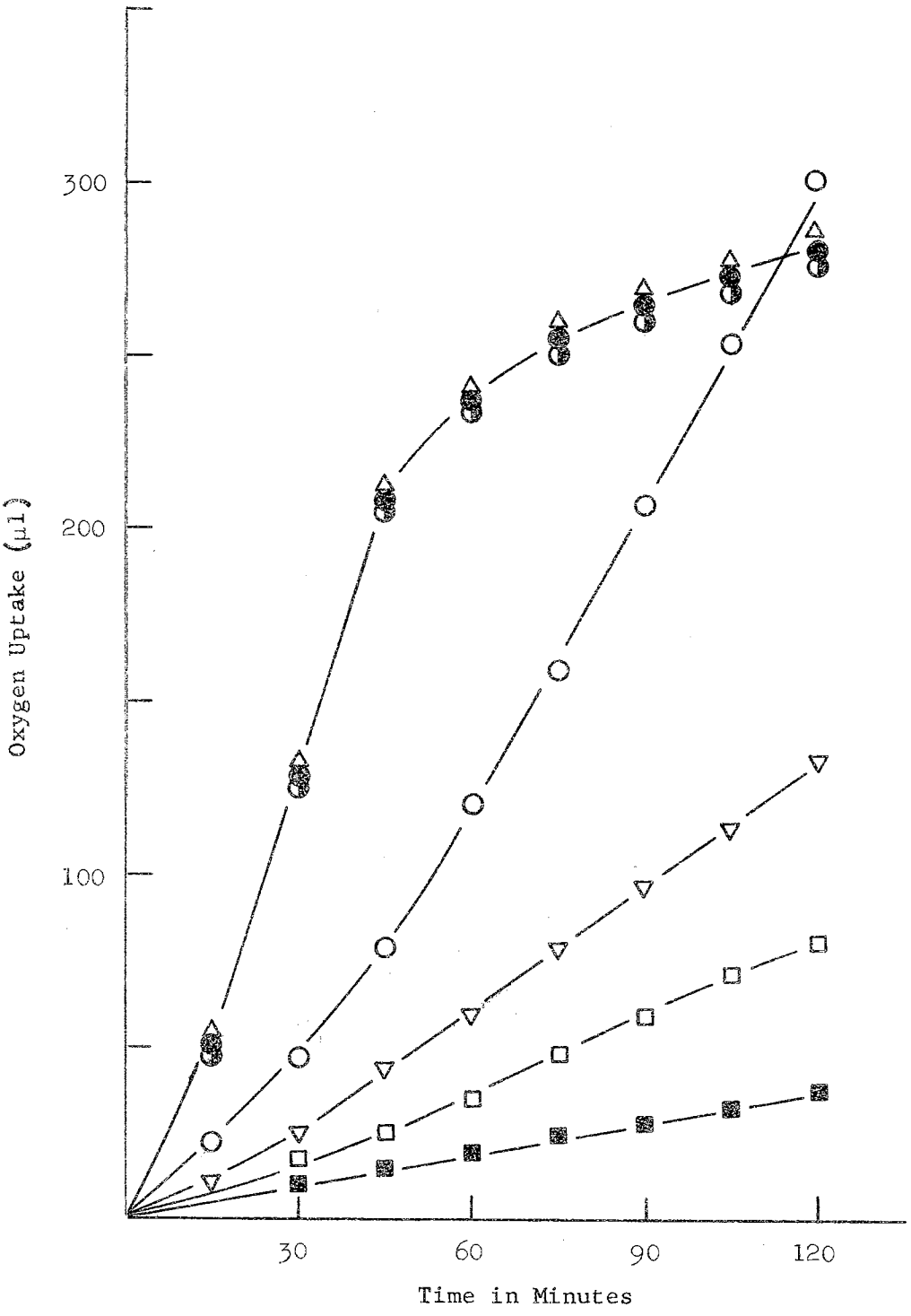

300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

Figure 22

The oxidation of D- and L-tryptophan in the presence of DL-oxindolealanine by a washed cell suspension of a Flavobacterium species. ●, 2 μmoles L-tryptophan; △, 2 μmoles L-tryptophan and 2 μmoles DL-oxindolealanine; ◑, 2 μmoles L-tryptophan and 6 μmoles DL-oxindolealanine; ○, 2 μmoles D-tryptophan; ▽, 2 μmoles D-tryptophan and 2 μmoles DL-oxindolealanine; □, 2 μmoles D-tryptophan and 6 μmoles DL-oxindolealanine; ■, endogenous.

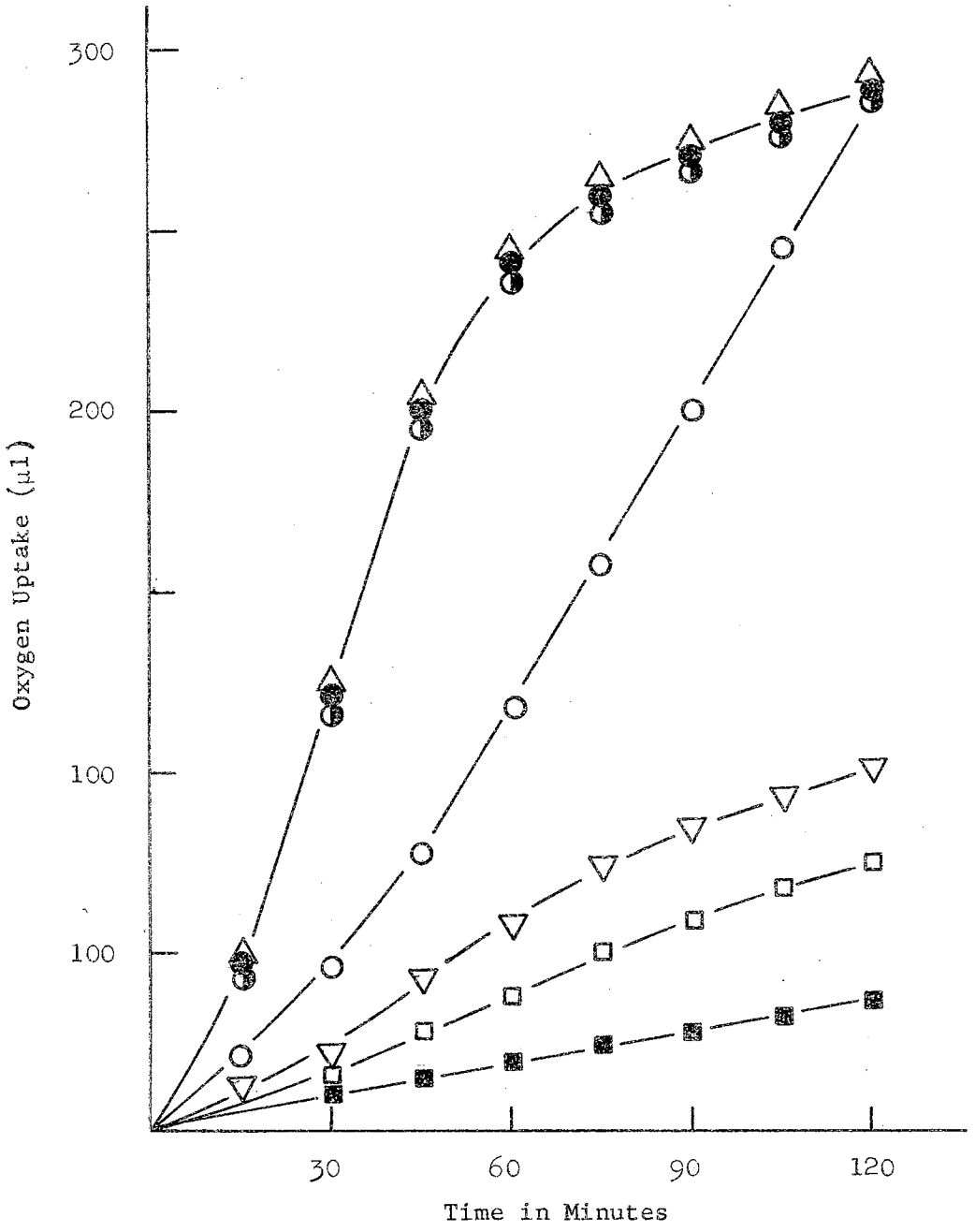

300
200
100
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

Figure 23

The oxidation of L-tryptophan in the presence of D- and L-5-hydroxytryptophan by a washed cell suspension of a Flavobacterium species. ●, 2 μmoles L-tryptophan; △, 2 μmoles L-tryptophan and 8 μmoles L-5-hydroxytryptophan; ○, 2 μmoles L-tryptophan and 16 μmoles L-5-hydroxytryptophan; ▩, 2 μmoles L-tryptophan and 2 μmoles D-5-hydroxytryptophan; □, 2 μmoles L-tryptophan and 8 μmoles D-5-hydroxytryptophan; ▲, 2 μmoles L-tryptophan and 16 μmoles D-5-hydroxytryptophan; ◑, endogenous.

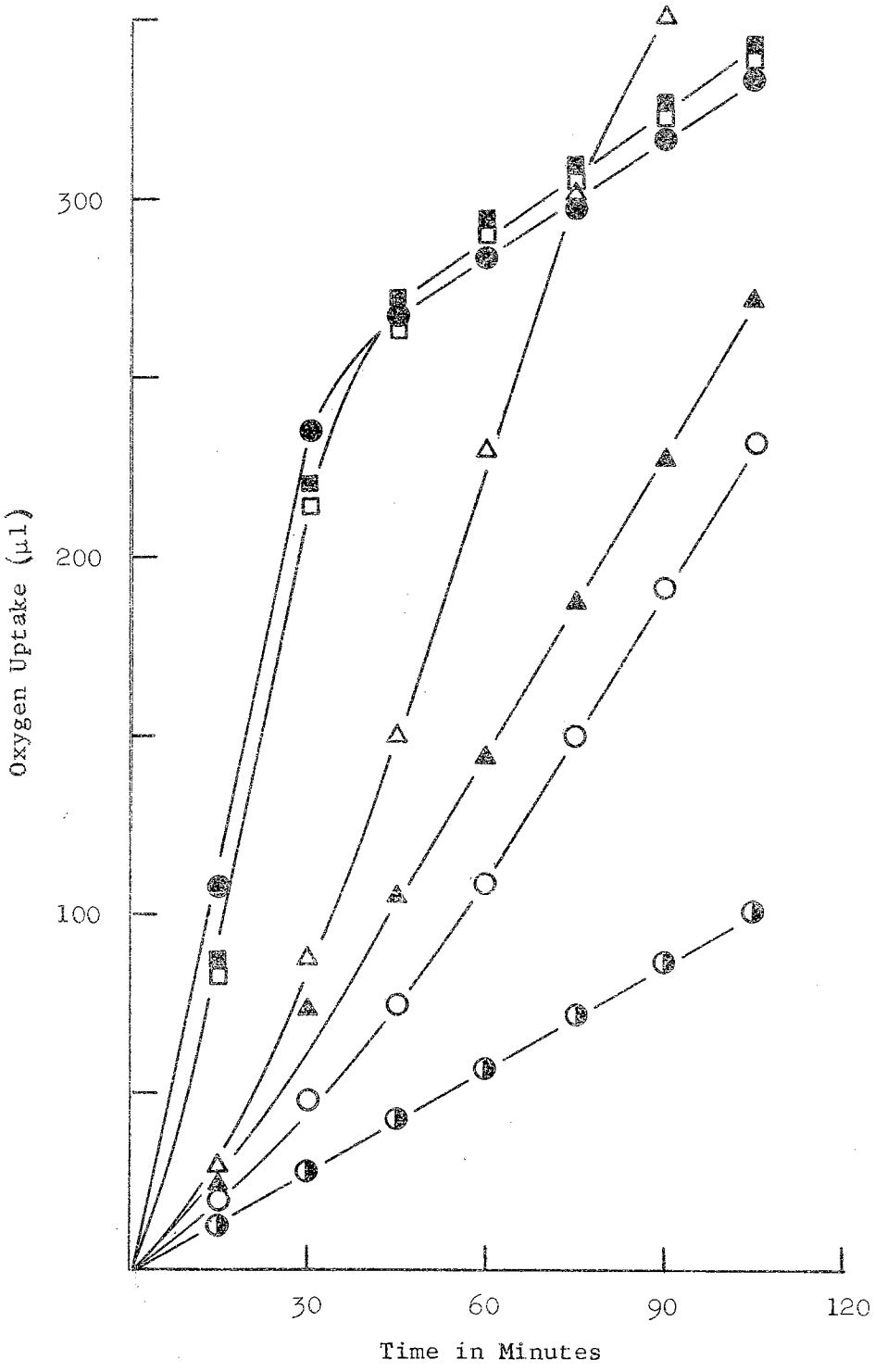
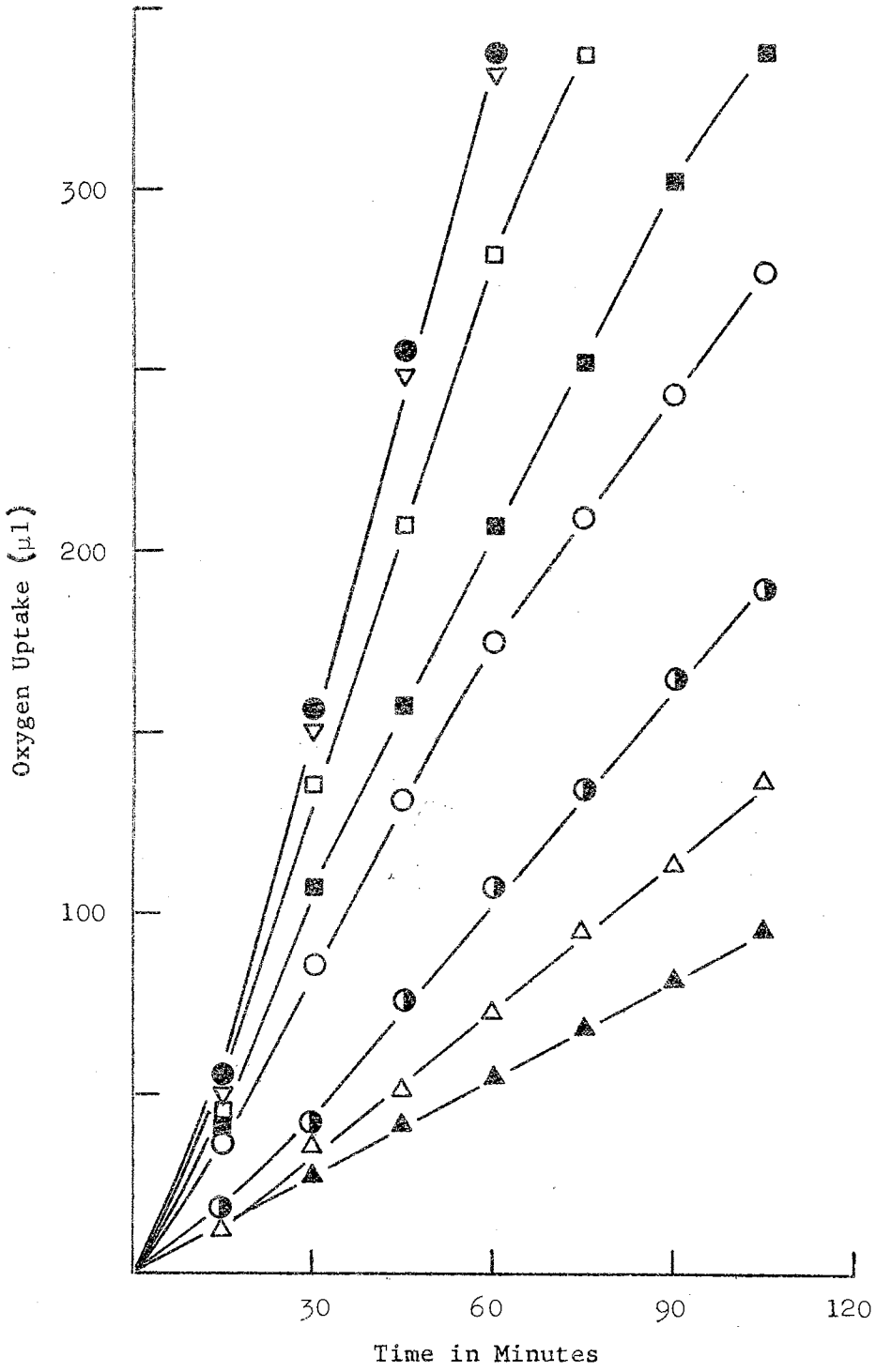

300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

Figure 24

The oxidation of D-tryptophan in the presence of D- and L-5-hydroxytryptophan by washed cells of a species of Flavobacterium. ●, 2 μmoles D-tryptophan; ○, 2 μmoles D-tryptophan and 2 μmoles L-5-hydroxytryptophan; ◐, 2 μmoles D-tryptophan and 8 μmoles L-5-hydroxytryptophan; △, 2 μmoles D-tryptophan and 16 μmoles L-5-hydroxytryptophan; ▽, 2 μmoles D-tryptophan and 2 μmoles D-5-hydroxytryptophan; □, 2 μmoles D-tryptophan and 8 μmoles D-5-hydroxytryptophan; ▩, 2 μmoles D-tryptophan and 16 μmoles D-5-hydroxytryptophan; ▲, endogenous.


300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

Tryptophol in low inhibitor/substrate ratios (1:1, 4:1) did not inhibit D-tryptophan oxidation. A 1:1 ratio did not inhibit L-tryptophan oxidation but a 4:1 inhibitor/substrate ratio showed a slight inhibition (30 percent) of L-tryptophan oxidation (Figure 25). Higher ratios (6:1, 8:1) inhibited oxidation of both D- and L-tryptophan.

A 4:1 ratio of indolepropionic acid/substrate inhibited the oxidation of L-tryptophan by 43 percent and D-tryptophan by 63 percent.

The effect of indolepropionic acid, indoleacetic acid, tryptophol, tryptamine and L-5-hydroxytryptophan on D-tryptophan oxidation by extracts of DL-tryptophan-grown cells was studied.

Tryptophol in an inhibitor/substrate ratio of 3:1 showed a 35 percent inhibition (Figure 26A). When the ratio is 8:1, inhibition is nearly complete. Tryptophol in low inhibitor/substrate ratios did not inhibit oxidation of D-tryptophan by washed cell suspensions (Figure 25), but did inhibit oxidation by the extracts. These findings suggest that tryptophol is not readily transported across the cell osmotic barrier since the primary site of tryptophol inhibition appears to be intracellular. Tryptamine in an inhibitor/substrate ratio of 3:1 inhibited the oxidation of D-tryptophan in cell extracts by 42 percent (Figure 26B). A similar inhibitor/substrate ratio did not inhibit the oxidation of D-tryptophan by whole cells suggesting that tryptamine also is not readily taken up by the cell.

The inhibition of D-tryptophan oxidation in cell extracts by L-5-hydroxytryptophan present in an 8:1 ratio was only 40 percent (Figure 26C). A L-5-hydroxytryptophan/D-tryptophan ratio of 1:1 gave a 58 percent inhibition in whole cells (Figure 24) indicating that the primary site of L-5-hydroxytryptophan action was the inhibition of uptake of D-tryptophan

Figure 25

The oxidation of D- and L-tryptophan in the presence of tryptophol by a washed resting cell suspension of a Flavobacterium species. ● , 2 μmoles L-tryptophan; △ , 2 μmoles L-tryptophan and 2 μmoles tryptophol; ◐ , 2 μmoles L-tryptophan and 8 μmoles tryptophol; ○ , 2 μmoles D-tryptophan; ▽ , 2 μmoles D-tryptophan and 2 μmoles tryptophol; □ , 2 μmoles D-tryptophan and 8 μmoles tryptophol; ▩ , endogenous.

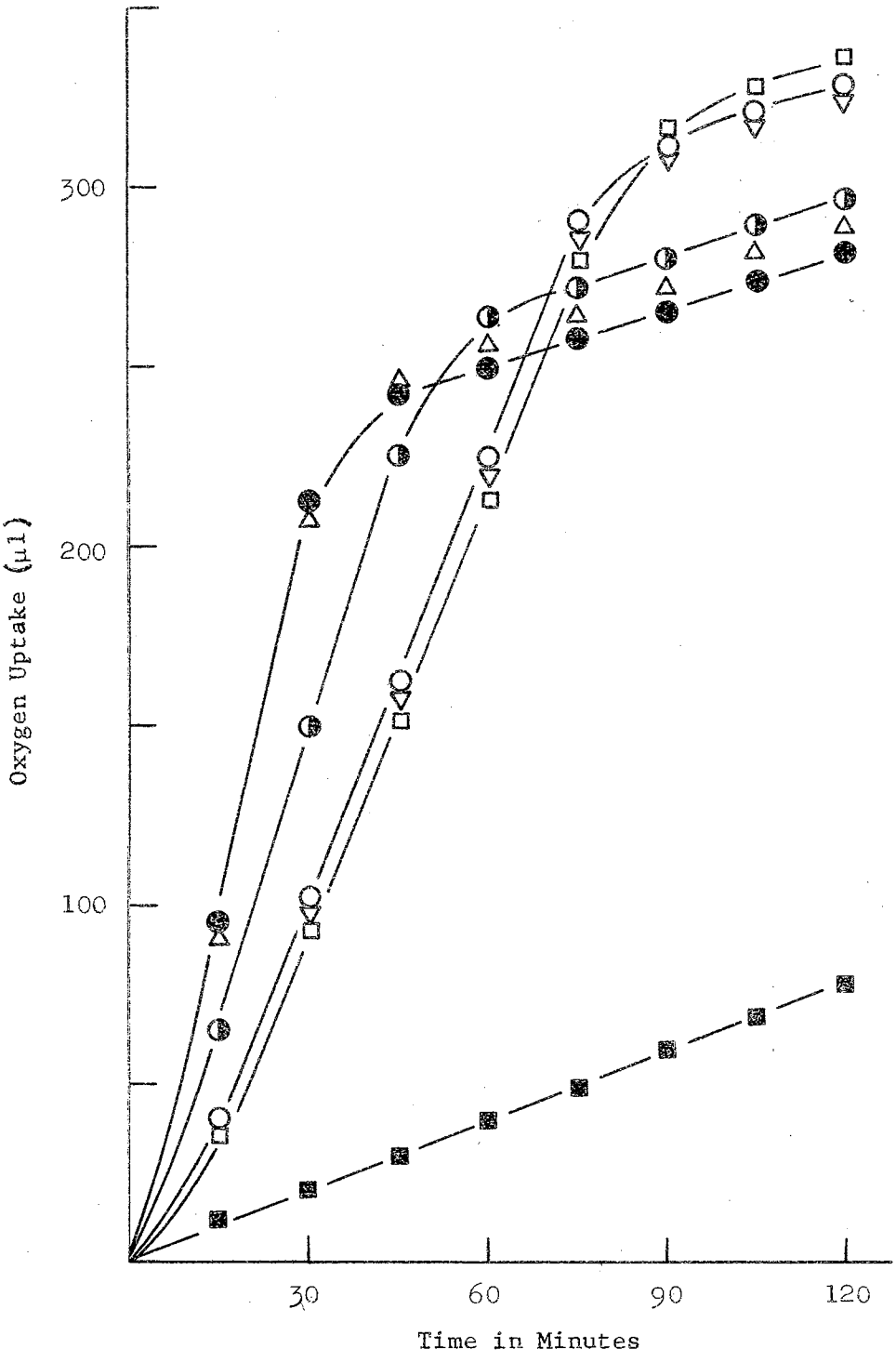
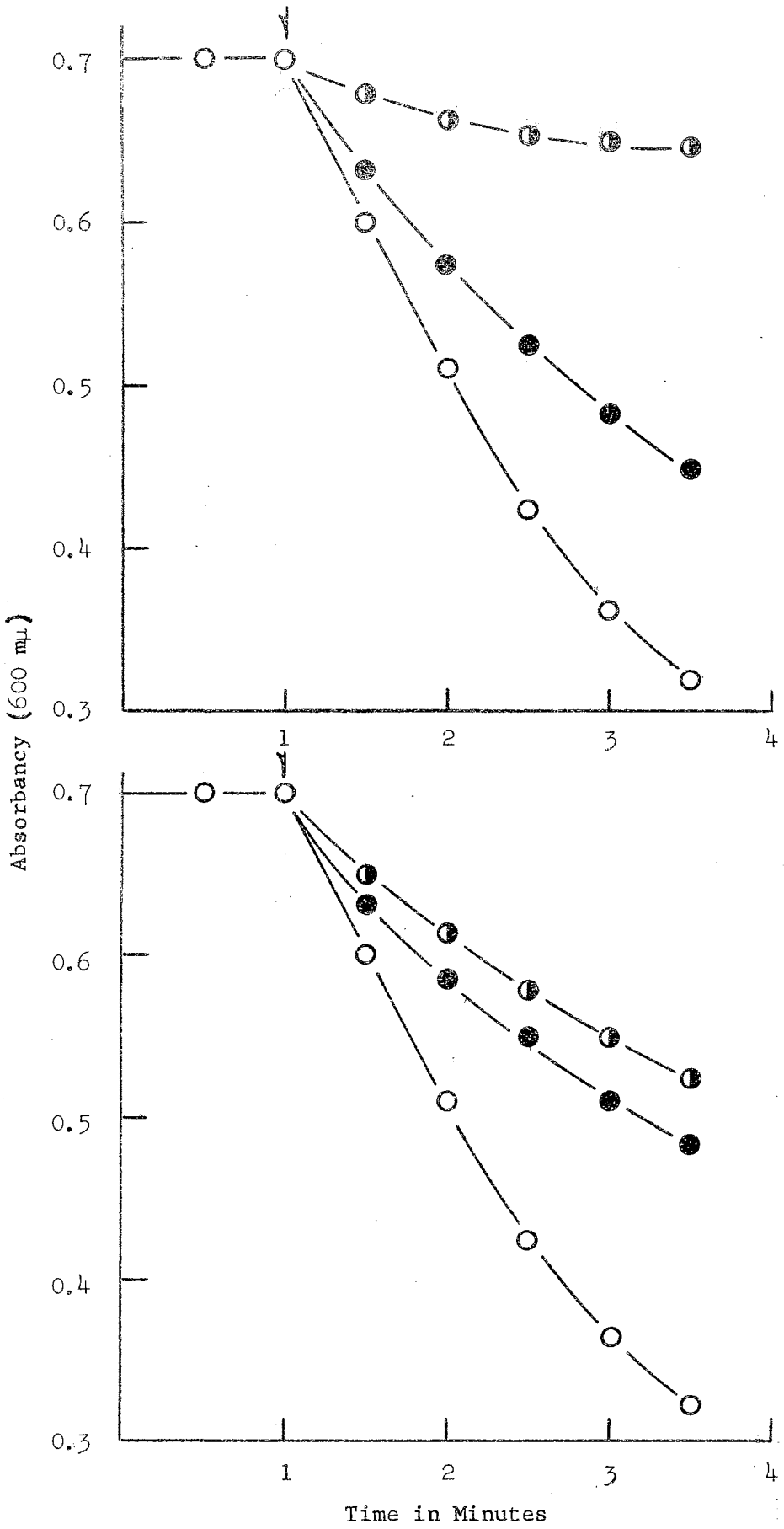
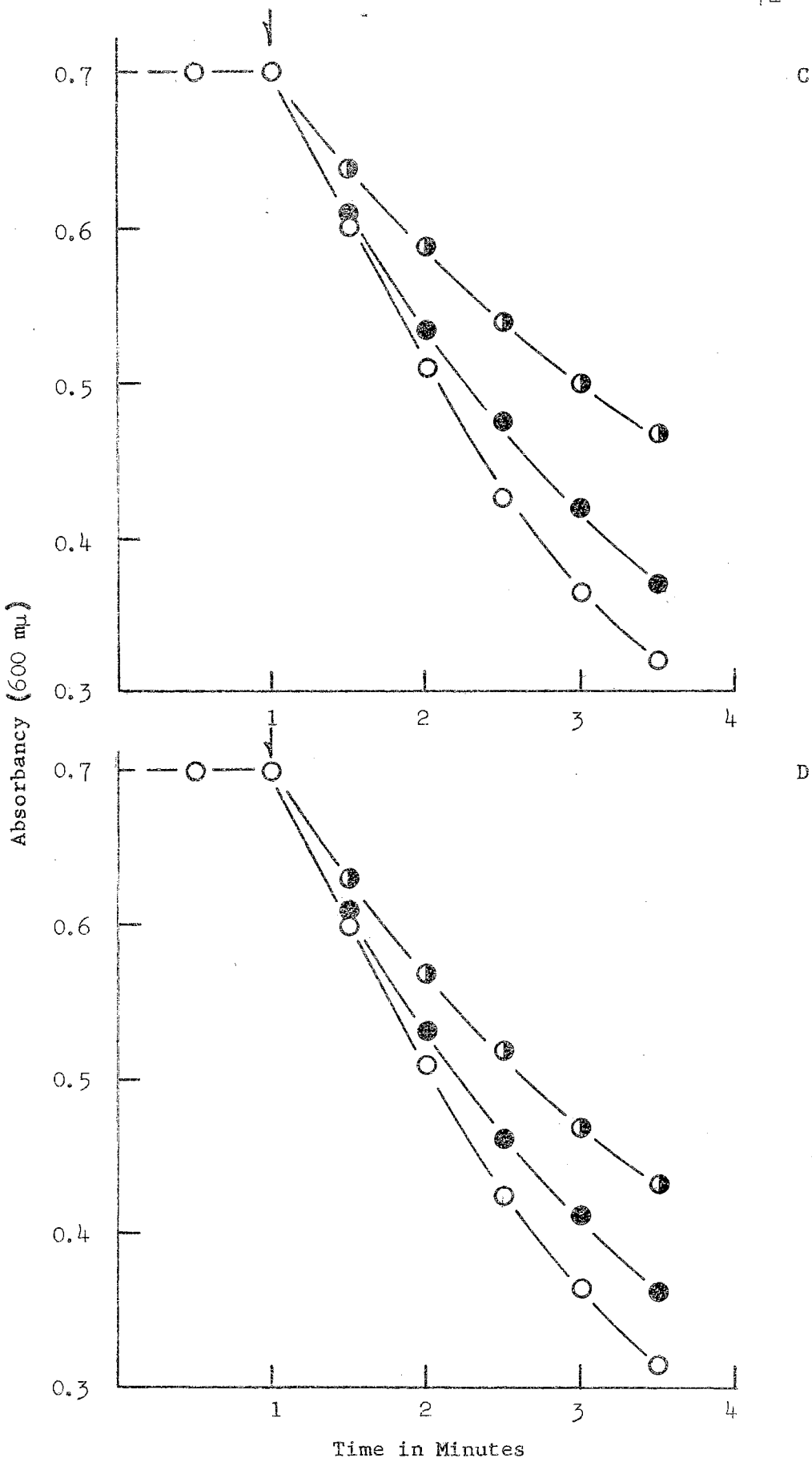

300
200
100
Oxygen Uptake (µl)
30
60
90
120
Time in Minutes

Figure 26

Oxidation of D-tryptophan in the presence of inhibitors by extracts of DL-tryptophan-grown cells. The complete system (○) contained 200 μmoles Tris buffer (pH 7.4), 0.12 μmoles of 2,6-dichlorophenolindolphenol and 0.1 ml of cell extract (containing 1.76 mg of protein) in a total volume of 3.0 ml. D-Tryptophan (10 μmoles) was added at the point indicated by the arrow. The reduction of 2,6-dichlorophenolindophenol was measured as the decrease in extinction at 600 mμ with time. Control cuvettes (not shown) were prepared. Dye reduction was not evident in any of the inhibitor controls. ● , contained a 3:1 inhibitor/substrate ratio; ◑ , contained an 8:1 inhibitor/substrate ratio; A, tryptophol inhibitor; B, tryptamine inhibitor; C, L-5-hydroxytryptophan inhibitor; D, indolepropionic acid inhibitor.


A
B
0.7
0.6
0.5
0.4
0.3
1
2
3
4
Absorbancy (600 mμ)
Time in Minutes


C
D
0.7
0.6
0.5
0.4
0.3
Absorbancy (600 mμ)
1
2
3
4
Time in Minutes

by the cells.

Addition of indolepropionic acid during D-tryptophan oxidation by cell extracts in an inhibitor/substrate ratio of 3:1 and 8:1 gave 11 and 29 percent inhibition respectively (Figure 26D). A 4:1 ratio inhibited D-tryptophan oxidation by 63 percent in the washed cell suspension suggesting this analog also inhibits the uptake of D-tryptophan.

Under the conditions of these experiments, tryptophan analogs may (a) inhibit the microbial oxidation of both D- and L-tryptophan, (b) inhibit oxidation of D-tryptophan but not the L-isomer, or (c) exert no influence on the oxidation of either tryptophan isomer. The results show that the primary site of action may be associated with the uptake system or with the internal enzymatic oxidation mechanism depending on the analog.

CHAPTER V

SUMMARY AND CONCLUSIONS

The utilization of D-tryptophan as the sole carbon, nitrogen and energy source by a species of Flavobacterium has been investigated. Growth experiments indicate the organism utilizes L-tryptophan more readily than the D-isomer and total growth is greater per unit of L-tryptophan than with D-tryptophan. The difference in growth yield apparently is dependent on the efficiency with which the two compounds are used since both tryptophan isomers are at the same oxidation level.

D-Tryptophan utilization can be "sparked" by addition of an easily assimilated substrate such as L-tryptophan, succinic acid, pyruvic acid or D-alanine. This substrate supplies a readily available carbon and energy source which facilitates the utilization of D-tryptophan.

Differences in the permeability of cells or intracellular structures to L- and D-tryptophan could result in a difference in energy expenditure. The transport of D-tryptophan across cell membranes could require the expenditure of more energy than required for L-tryptophan; thus, less energy would be available for other metabolic functions and a lower cell yield obtained when the organism is grown on D-tryptophan.

The oxidation of D-tryptophan by washed cells proceeds approximately 50 percent as rapidly as oxidation of the L-isomer. The dissimilation of D-tryptophan requires one gram atom of oxygen per molecule more than is consumed during oxidation of the L-isomer. Similar quantities of carbon

dioxide are evolved during oxidation of D- or L-tryptophan.

D-Tryptophan catabolism proceeds via L-tryptophan, L-kynurenine and anthranilic acid. Anthranilic acid is then oxidized to salicylic acid and gentisic acid. Many compounds containing the indole or quinoline nucleous were eliminated as intermediates.

Extracts of DL-tryptophan-grown cells oxidize D-tryptophan if phenazine methosulfate or 2,6-dichlorophenolindophenol is added as an electron acceptor, and one gram atom of oxygen is consummed per mole of D-tryptophan. Flavinadenine nucleotide, flavin mononucleotide, triphosphopyridine nucleotide and diphosphopyridine nucleotide will not function as electron acceptors in the experimental system. The extract will also oxidize several other D-amino acids. Extracts of L-tryptophan-induced cells are devoid of D-amino acid oxidase activity.

The product of oxidation of D-tryptophan by cell extracts is indolepyruvic acid. Oxidative deamination of D-tryptophan, not linked with the pyridine nucleotides, would result in liberation of the energy of deamination in the form of heat (17). Therefore, the formation of ammonia per se by oxidative deamination would lead to no net gain of useful energy to the cell since there may be no coupling mechanisms available for synthesis of adenosine triphosphate at this step.

The catabolism of D-tryptophan by Flavobacterium requires an amino donor. Cell extracts catalyze transamination of indolepyruvic acid to L-tryptophan using L-glutamic acid, L-aspartic acid or L-glutamine as the amino donor. Synthesis of the essential donor would require energy and result in correspondingly lower growth yields on D-tryptophan as compared to the L-isomer.

D-Tryptophan utilization begins with a dehydrogenation reaction.

The resulting compound, imino tryptophan, is spontaneously hydrolyzed non-enzymatically to indolepyruvic acid, thus destroying the asymmetric center of the tryptophan molecule. Indolepyruvic acid is transaminated to L-tryptophan which is oxidized with one mole of molecular oxygen to N-formyl-L-kynurenine. The kynurenine derivitive is hydrolyzed by water to formic acid and kynurenine. The formic acid is dismutated to carbon dioxide and water, while kynurenine is hydrolyzed to anthranilic acid and L-alanine. Anthranilic acid is oxidized to salicylic acid and then to gentisic acid. Gentisic acid is further degraded with the uptake of 2 moles O_2 per mole of acid and three moles of carbon dioxide are evolved.

Data from whole cells and cell extracts show that tryptophan analogs may inhibit the oxidation of both D- and L-tryptophan and that the site of action of each analog varies.

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VITA

Jerry Roy Martin

Candidate for the degree of

Doctor of Philosophy

Thesis: METABOLISM OF D-TRYPTOPHAN BY A FLAVOBACTERIUM SPECIES

Major Field: Microbiology

Biographical:

Personal Data: Born at Abilene, Kansas, February 27, 1933, the son of Roy Arnold and Mary Tullis Martin; married to Jill Elizabeth Rebman on August 20, 1961.

Education: Graduated from Salina High School, Salina, Kansas, in 1951; received the Bachelor of Science degree, with a major in fishery biology, from Utah State University, Logan Utah, in 1955; completed requirements for the Doctor of Philosophy degree in August, 1964.

Professional Experience: Served as a research assistant at Oklahoma State University from 1958 to 1964 in the Department of Microbiology.

Professional and Honorary Societies: Phi Sigma, Sigma Xi, Phi Lambda Upsilon, Phi Kappa Phi and Missouri Valley Branch of the American Society for Microbiology.