STUDIES ON THE PROPERTIES OF AROMATIC PATHWAY ENZYMES OF A PSEUDOMONAD METABOLIZING TRYPTOPHAN AND 5-HYDROXYTRYPTOPHAN

by

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A THESIS
Presented to the Department of Microbiology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

June 1968

APPROVED:

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(Chairman, Graduate Council)

Acknowledgment

I would like to thank Dr. Evelyn Oginsky for her advice and help during the research program and organization of this thesis. I would also like to thank Audrey Gillespie, Joyce Schilling, and Pat Starr for invaluable help in typing the thesis. Finally, I would like to thank my thesis committee for their suggestions.

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INTRODUCTION

STATEMENT OF THE PROBLEM

A bacterium isolated by this laboratory was found capable of elaborating in much greater quantity certain degradative enzymes during growth on L-tryptophan or L-5-hydroxytryptophan than during growth on L-glutamate. Either set of degradative enzymes would convert L-tryptophan to anthranilate and L-alanine, or L-5-hydroxytryptophan, by a similar set of conversions, to 5-hydroxyanthranilate and L-alanine. The question posed by these observations is: are the enzymes of these pathways when induced by one compound identical to the set induced by the other?

This thesis attempts to answer this question, by comparative studies of the enzymes induced by either compound. The second enzyme on the pathway, formamidase, was in most cases used as a model system with studies designed to determine whether the 5-hydroxytryptophan-induced formamidase was identical to the tryptophan-induced formamidase.

Soil has been an historically important site for the isolation of organisms with particular nutritional and metabolic abilities, as it provides a large number of diverse types able to degrade a variety of organic and inorganic compounds. Isolation procedures usually utilize the Enrichment Culture technique (1), in which a sample of soil is incubated in liquid culture, under appropriate environmental conditions, with the organic compound to be degraded as sole or main carbon source. After growth has occurred, manifested by an increase in turbidity, a second and perhaps third transfer is made in the same medium to dilute out non-growing organisms. The culture is then streaked out on solid medium of the same basic composition. Individual colonies are isolated, and the isolates are tested for the ability to degrade the compound. Conditions of incubation will determine the spectrum of organisms isolated; if the medium consists of ammonium salts, phosphates, and a carbon source, and aerobic conditions are maintained, pseudomonads will predominate (2).

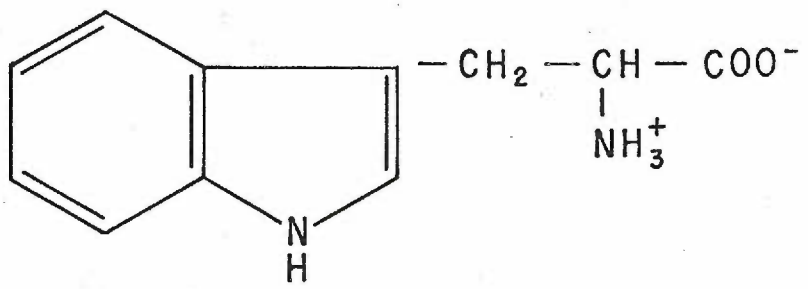
METABOLIC PATHWAYS OF L- AND D-TRYPTOPHAN DEGRADATION

A. Sequence of the L-tryptophan Pathways

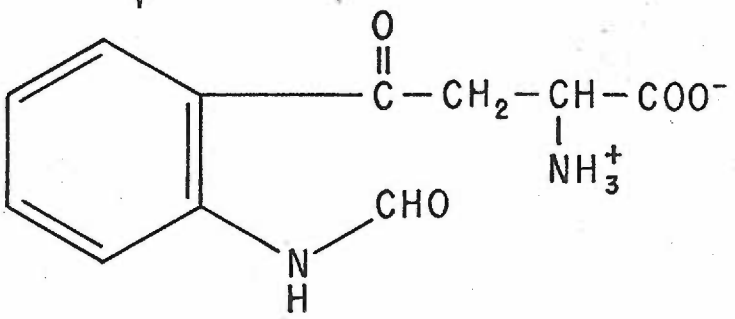
Using the enrichment culture technique, several microbiologists have isolated and studied pseudomonads capable of degrading L-tryptophan (3,4,5,6). The metabolism of L-tryptophan has been found to proceed by either of two major pathways (Figs 1, 2, and 3). The pathways (3,7) start with the same two interconversions: L-tryptophan (I) is oxidized by an enzyme known as tryptophan oxygenase to formyl-L-kynurenine (II) (8), which is, in turn, hydrolyzed by formamidase to formate and L-kynurenine (III). From this point the pathways diverge. In the

Figure 1

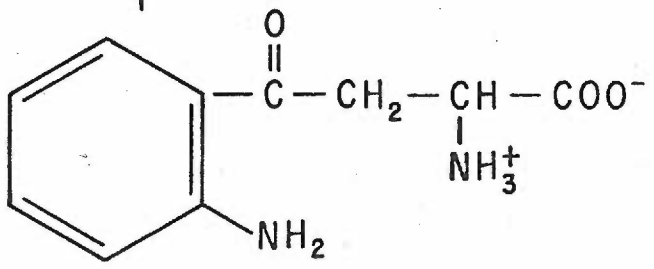
The degradation of L-tryptophan. Conversion of L-tryptophan to L-kynurenine.



I. L-TRYPTOPHAN



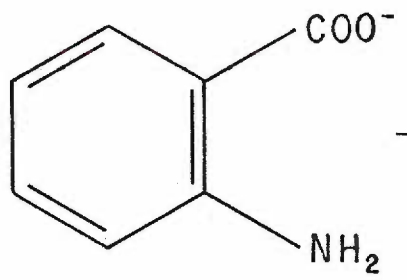
II. FORMYL-L-KYNURENINE

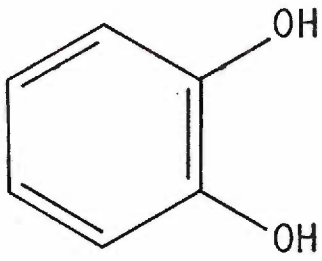


III. L-KYNURENINE

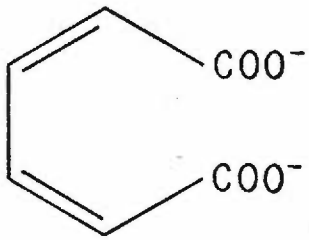
Figure 2

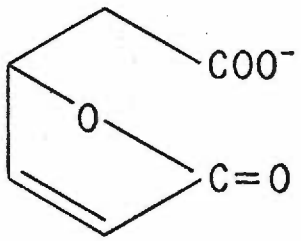
The degradation of tryptophan. Conversion of L-kynurenine to succinate and acetyl CoA by bacteria utilizing the aromatic pathway.

III → 

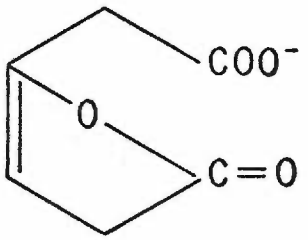
→ 

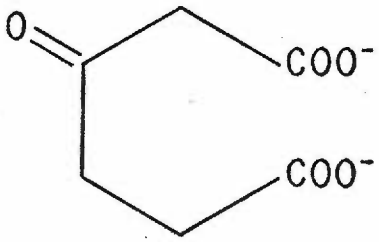
IV ANTHRANILATE V CATECHOL

V → 

→ 

VI CIS, CIS MUCONATE VII (+) MUCONOLACTONE

VII → 

→ 

VIII β-KETOADIPATE ENOL-LACTONE

IX β-KETOADIPATE

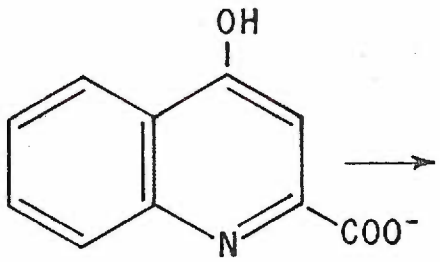
↓

SUCCINATE
ACETYL CoA

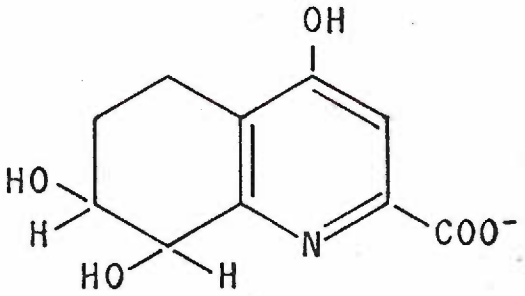
Figure 3

The degradation of L-tryptophan. The conversion of L-kynurenine to metabolic products by bacteria utilizing the quinolinic pathway.

III →

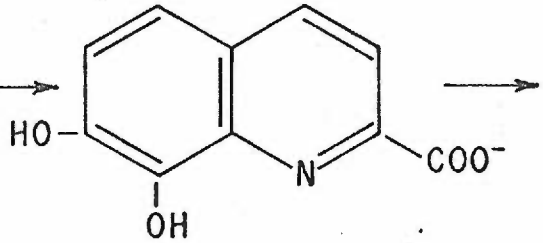


X KYNURENIC ACID

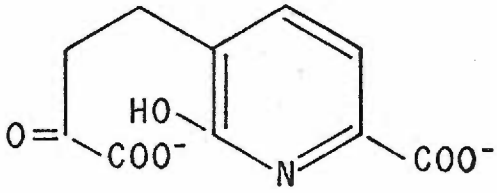


XI 7,8-DIHYDRODIOL-KYNURENIC ACID

XI →

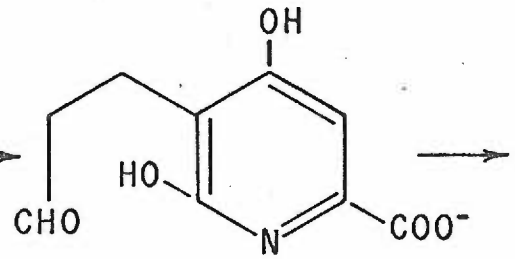


XII 7,8-DIHYDROXYKYNURENIC ACID

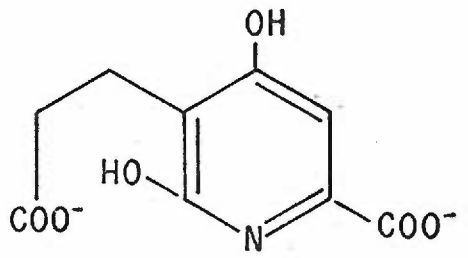


XIII 5-(γ-CARBOXY-γ-OXOPROPYL) 4,6-DIHYDROXY-PICOLINATE

XIII →

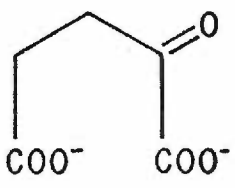


XIV 5-(β-FORMYLETHYL)-4,6-DIHYDROXYPICOLINATE

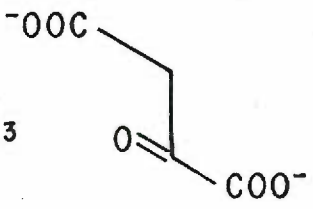


XV 5-(β-CARBOXYETHYL)-4,6-DIHDROXYPICOLINATE

XV →



NH_3



α-KETOGLUTARATE

AMMONIA

OXALACETATE

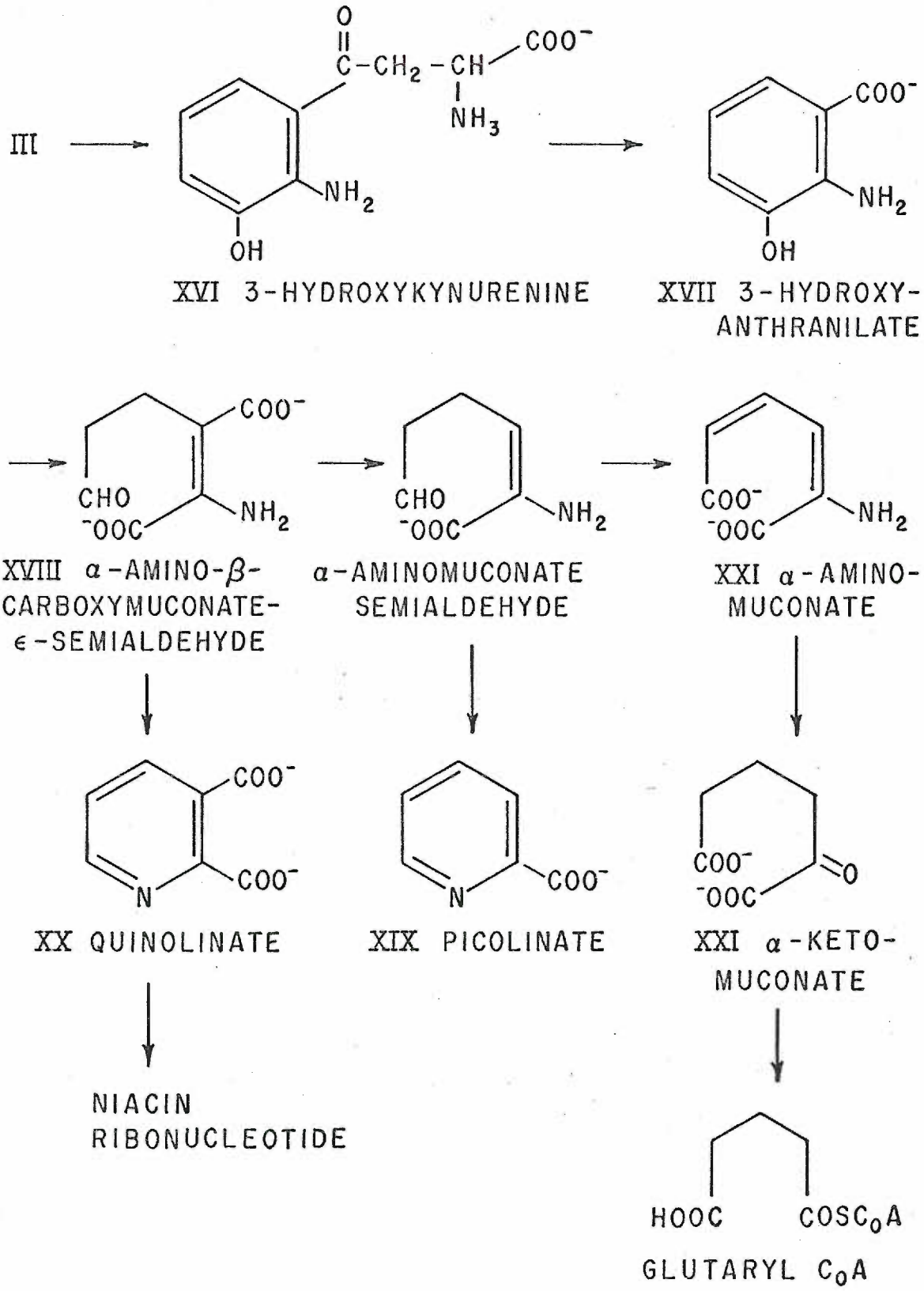
aromatic, or anthranilate, pathway (Fig. 2) III is converted by means of kynurenine to anthranilate (IV) and L-alanine. An oxidase converts IV into catechol (V). Catechol is oxidized to cis, cis muconate (VI), which is eventually converted via muconolactone (VII) and the enol lactone (VIII) to β-ketoadipate (IX). The β-ketoadipate, in the presence of coenzyme A (CoA) and ATP, yields succinate and acetylCoA (9).

In the quinolinic pathway (Fig. 3), kynurenine (III) is transaminated with α-ketoglutarate to yield, after ring closure, kynurenic acid (X) (10). Other known intermediates, in order, include 7,8- dihydroxykynurenic acid (XII) (12,13), 5-(γ-carboxy-γ-oxopropyl)-4, 6-dihydroxypicolinate (XIII) (11), 5-(β-carboxyethyl)-4, 6-dihydroxypicolinate (XV) (11), and finally α-ketoglutarate, oxalacetate and ammonia.

In higher organisms, for example, Neurospora and mammals, there is still another pathway (Fig. 4) leading from kynurenine producing on the one hand niacin, and on the other, carboxylic acids which may serve as energy sources. Thus III, in the presence of NADPH, is oxidized to 3-hydroxykynurenine (XVI) by a mixed-function oxygenase (14) found in liver (15). Next the alanyl chain is removed forming 3-hydroxyanthranilate (XVII) (16,17), by a kynureninase-like enzyme (18). The next stable compound formed is α-amino-β-carboxymuconate semialdehyde (XVIII) (19). This is converted by way of an unstable intermediate to picolinate (XIX), which is relatively inert metabolically (19) and quinolinate (XX) (20), a precursor of niacin ribonucleotide (21). Oxidation of XVIII yields α-aminomuconate (XXI) (22), which is further degraded to citric acid cycle intermediates (21). Thus α-amino-β-carboxymuconate semialdehyde is a branch point, leading to either energy-yielding pathways or vitamin biosynthetic pathways.

Figure 4

The degradation of L-tryptophan. The conversion of L-kynurenine to end-products by eukaryotic organisms.


III
XVI 3-HYDROXYKYNURENINE
XVII 3-HYDROXY-ANTHRANILATE
XVIII α-AMINO-β-CARBOXYMUCONATE-ε-SEMIALDEHYDE
α-AMINOMUCONATE SEMIALDEHYDE
XXI α-AMINO-MUCONATE
XX QUINOLINATE
XIX PICOLINATE
XXI α-KETO-MUCONATE
NIACIN RIBONUCLEOTIDE
GLUTARYL CoA

Pseudomonads do not appear to utilize tryptophan as a source of pyridine nucleotides, although there is a report (22) that the related xanthomonads do convert tryptophan to niacin. Rather, the pseudomonads use the aromatic or quinolinate pathways to derive energy and carbon sources. Most strains use only one pathway (4), while some other strains may have incomplete pathways (4) or one functioning pathway with a fraction of another (23).

There is a case of a pseudomonad, Pseudomonas savastanoi, using a different route of tryptophan metabolism, oxidizing the alanyl side chain to acetate (6) to produce indolacetic acid. Most pseudomonads use the aromatic or quinolinic pathway.

B. Sequence of D-tryptophan Pathways

Some pseudomonads oxidize D-tryptophan. Most of these organisms are found to metabolize L-tryptophan by the quinolinic pathways (5). In these organisms D-kynurenine is an intermediate in D-tryptophan metabolism; an inducible oxidase converts it to kynurenic acid (23,24). Thus in distinction to the degradation of L-kynurenine, this reaction is oxidative deamination, rather than transamination. There is only one reported strain of pseudomonas, Pseudomonas sp (ATCC, 14676), metabolizing D-tryptophan by way of the aromatic pathway. Here the D-tryptophan is first racemized to L-tryptophan (6,26), while D-kynurenine is not metabolized. A similar phenomenon is found in the related Flavobacteria, where the racemization of D-tryptophan has been shown to proceed by oxidation to indolepyruvate, followed by transamination to L-tryptophan (27). The racemization enzymes are inducible by growth on D-tryptophan (28).

A mammalian intestinal enzyme system can convert D-tryptophan to D-kynurenine (29,30). A tryptophan oxygenase acting on D-tryptophan isolated from this source (31) appears to have some activity on L-tryptophan as well. As the reaction was measured by kynurenine formation, there is either a formamidase present with equal substrate specificities, or the enzyme acting on tryptophan is not similar to tryptophan oxygenase.

C. Enzymes of the Aromatic Pathway

The enzymes of the aromatic pathway in various organisms have been studied in some detail. Certain properties, like cofactor requirements, are the same for all sources, while other properties, like response to inhibitors, differ. Tryptophan oxygenase (E.C. 1.13.1.12. L-tryptophan: oxygen oxidoreductase) often called tryptophan pyrrolase, has been studied in the most detail. It was first characterized after extraction from rat liver by Knox and Mehler (32), who termed it tryptophan peroxidase-oxidase, and thought it to be a two step reaction. The enzyme is now known to be an oxygenase, as one of the substrates is molecular oxygen (33), both atoms of which are incorporated. The product of oxidation was found to be formyl-L-kynurenine (32). This enzyme obtained from Pseudomonas fluorescens stain TR-23 has been reported (34) to be competitively inhibited by 5-hydroxytryptophan, which it will not oxidize. It is competitively inhibited by para-chloromercuribenzoate (pCMB) (35), and, like the mammalian enzyme (32), is activated by ascorbate or dithiothreitol. Purified preparations of tryptophan oxygenase from both pseudomonads (Pseudomonas strain NTCC 11, 299) and rat liver are sensitive to cyanide and show light-reversible carbon monoxide

inhibition, characteristic of iron porphyrins (36). The pseudomonad enzyme from strain NTCC 11 appears to have an essential bound copper ion, as the copper remains at a constant ratio to activity through a 70-fold purification, and the enzyme is inhibited by specific copper chelators (37). Drosophila tryptophan oxygenase is also sensitive to copper chelators (38).

The second enzyme, formamidase (E.C.3.5.1.9., aryl-formylamine amidohydrolase) often called kynurenine formamidase or formylase, has not been studied extensively. The pioneering work was again done by Knox and Mehler (39) who found that their rat liver enzyme preparation had broad substrate activities. In addition to formyl-L-kynurenine, the enzyme hydrolyzed formylanthranilate, formylorthanilate, and formyl-nitroaniline at 16%, 10% and 10% of the rate of the 'natural' substrate. The Neurospora formamidase was purified 16 fold (40), and also showed a lack of specificity, in terms of both K_m and velocity relative to that with formyl-kynurenine. Results (40) obtained were:

Compound	K_m (M)	Vrel
Formylkynurenine	1.1×10^{-4}	100
Formylanthranilate	3.3×10^{-3}	24
Formyl-o-aminoacetophenone	2.8×10^{-3}	22
Formyl-o-aminonitrobenzene	2.5×10^{-3}	13
Formyl-m-aminobenzoate	7.7×10^{-3}	4

The formamidase of Drosophila, however, not only cannot hydrolyze formylanthranilate, but its activity is inhibited by that compound (41). The K_m for formylkynurenine is of the same order as the other enzymes,

3.1×10^{-4}M (41). It is inhibited completely by 10 mM sodium sulfite, but not by sodium formate or thiourea at the same concentration (41). Rabbit liver formamidase is similar to the Neurospora and rat liver enzyme in its ability to hydrolyze formylanthranilate, which proceeds at 60% the rate of formylkynurenine (47); the rate of hydrolysis of m-formaminobenzoate was about 30% the parent compound. This rabbit liver enzyme is inhibited by pCMB and this inhibition is partially reversed by reduced glutathione or cysteine (42), suggesting sulfhydryl groups are important in enzyme activity. Inhibition by 8-hydroxyquinoline is completely reversed by Zn^{++} or Co^{++}, while ethylenediaminetetraacetate (EDTA) does not inhibit the enzyme (42). Fluoride and arsenite inhibition can be reversed by phosphate (42). Fluoride does not inhibit the Neurospora enzyme (40).

Kynureninase (E.C.3.7.1.3. L-kynurenine hydrolase), the first enzyme unique to the aromatic pathway, splits L-kynurenine to anthranilate and L-alanine (43). The P. fluorescens enzyme is almost completely inhibited by 10 mM cyanide, 1 mM semicarbazide or 2 mM hydroxylamine, and has a pH optimum of 8.5 (44). The K_m for L-kynurenine by this bacterial enzyme is 3.9×10^{-5}M. This enzyme is not completely specific: the rate of hydrolysis of 5-hydroxykynurenine is 50% that of kynurenine, and that of 3-hydroxykynurenine is about 20%. Similarly the Neurospora enzyme, purified 100 fold, will hydrolyze both 3-hydroxy- and formylkynurenine, and also has a high pH optimum of about 8 (45). Kynureninases from all sources (32,44,45) appear to require pyridoxal phosphate as coenzyme and are therefore inhibited by semicarbazide and other agents binding pyridoxal phosphate.

Anthranilate oxidase is a very labile enzyme which converts anthranilic acid into catechol. Because of the lability of the enzyme, cell-free preparations oxidizing L-tryptophan generally accumulate anthranilate (7). Recent work has demonstrated requirements for reduced pyridine nucleotides and oxygen (46,47). The enzyme activity is stimulated by the addition of reduced glutathione (46,47) and ferrous ions (47). As catechol is also an intermediate in the degradation of other aromatic compounds, like mandelate and benzoate, the conversion of anthranilate into catechol can be considered the last step in the pathway unique to tryptophan degradation.

REGULATION OF THE TRYPTOPHAN PATHWAY

A. Regulation of Tryptophan Degradation

1. Regulation of pseudomonad aromatic pathway of tryptophan degradation. Both the aromatic and quinolinic pathways in pseudomonads are inducible, or adaptive (3). Thus L-tryptophan-grown cells will quickly metabolize most intermediates on the pathway, but only slowly metabolize these compounds if the cells had been grown on a metabolite not on the pathway, such as L-asparagine. Induction by some metabolites on the pathway, like anthranilate, results in cells which rapidly oxidize anthranilate and succeeding compounds, but do not rapidly attack tryptophan or kynurenine (48). These results led to the formulation of the "successive" or "sequential" adaptation hypothesis, in which the product of one enzymatic step was thought to induce the synthesis of the next enzyme, stepwise down the pathway (48).

Recent studies by Palleroni and Stanier (49) with P. fluorescens strain TR 23 have altered this concept, as applied to the tryptophan

pathway. They demonstrated low levels of tryptophan oxygenase and formamidase in noninduced cells; kynureninase was undectectible. On induction with L-tryptophan or L-kynurenine, the first three enzymes were rapidly synthesized, while an enzyme further along in the pathway was induced only after a considerable lag. Further kinetic studies demonstrated that the first two enzymes were induced coordinately, while kynureninase was synthesized more slowly. Thus part of a pathway may be induced in a sequential manner and yet some of the enzymes may be synthesized coordinately. Sequential induction of an oxygenase, formamidase, and kynurenine transaminase may occur in another pseudomonad (53), although the evidence is not altogether convincing.

Further studies by Palleroni and Stanier (49) using short periods of induction demonstrated that the first three enzymes of the aromatic pathway were induced more rapidly by L-kynurenine than by L-tryptophan. Induction studies using mutants with complete blocks at the oxygenase or formamidase enzymes showed the remaining enzyme and kynureninase were induced by L-kynurenine, and not by L-tryptophan. Thus L-kynurenine is the true inducer for the coordinately-synthesized oxygenase and formamidase enzymes, as well as kynureminase in a strain of P. fluorescens and perhaps in all pseudomonads.

The phenomenon of induction by intermediates, rather than initial substrates, has been demonstrated in some other systems. Glycerol in Escherichia coli is phosphorylated by a kinase to α-glycerol phosphate. A dehydrogenase converts this to dihydroxyacetone phosphate, an intermediate in the Embden-Meyerhof-Parnas glycolysis pathway. A mutant, producing an enzymatically inactive kinase which could be identified

immunologically, was induced to synthesize that kinase by α-glycerol phosphate, rather than by glycerol (51). Studies with another blocked mutant eliminated the possibility that still later intermediates induced the pathway (52). Hayaishi and Lin (51) suggested that as glycerol is freely permeable through the cell membranes, it is necessary for the cell to sequester it in the form of a phosphate, which is no longer as permeable. However the K_m of induction by glycerol would have to be smaller than 10^{-6}M, the K_m for glycerolkinase, in order for induction to occur, and this synthesis of enzymes for such low concentrations of substrate would be extremely inefficient. Instead, the phosphorylated compound is the inducer. They further pointed out that this also makes the induction more stringent to analogs of glycerol which might act as inducers; not only would the analog have to react with the repressor, but also have to be phosphorylated prior to that. The same phenomenon occurs in the lac operon of E. coli. The permease and β-galactosidase, as well as transacetylase, can be induced by incubation with lactose or a variety of analogs including ONPG or TMG. The permease is assayed by studying the uptake of radioactive lactose or TMG. In strain W2244, which possesses a nonfunctional β-galactosidase, neither lactose nor ONPG was found to induce the permease to any significant extent (53). The permease is, however, induced by 6-O-β-D-galactopyranosyl-D-glucose, at amounts comparable to those usually induced by TMG. So it is not the substrate which induces, but rather some other compound, presumably a later intermediate, formed from transgalactosidation by β-galactosidase. Indeed both metabolites formed from lactose and alcohols by β-galactosidase (54) and synthetic compounds like galactosyl glycerol (55) are excellent

inducers.

Induction by an intermediate has recently been demonstrated for a second amino acid degradative pathway. Histidine is degraded by four enzymes to glutamate and formamide, the first two enzymes being histidase (E.C.4.3.1.3) and urocanase. In mutants of Aerobacter aerogenes (56) and Salmonella typhimurium (57) with nonfunctional histidases, urocanate will induce the other three enzymes, while histidine will not. However, using a similar mutant of Bacillus subtilis, Magasanik (57) showed histidine did induce the degradative enzymes.

The argument for control of amino acid degradation by intermediate compounds was proposed by Palleroni and Stanier (49), who suggested why a stable pool size of tryptophan could be maintained by regulation of tryptophan degradation by kynurenine. Because of the low pool size of tryptophan and the moderate K_m of tryptophan oxygenase, 4×10^{-4}M (34), little tryptophan would be oxidized by the basal level of tryptophan oxygenase. What tryptophan was oxidized would be converted to kynurenine by the basal level of formamidase. However, the slight basal activity of the kynureninase would cause a small pool of kynurenine, which might be partially free, as the K_m of kynureninase is low, 4×10^{-5}M. An expansion of the tryptophan pool by exogenous tryptophan would quickly result in an expanded kynurenine pool, which would induce the tryptophan degradative system. Thus,

> "The primary physiological factors which channel endogenously synthesized tryptophan into protein synthesis in P. fluorescens are therefore probably maintenance of a low pool concentration and extremely high affinity of the

specific activating enzyme." (46, p. 333)

Thus the argument is based on the differences in K_m of the activating enzymes and the oxygenase (or histidase). How valid is this assumption? The K_m for tryptophan by tryptophan oxygenase is about 4×10^{-4}M. Unfortunately the K_m of the tryptophan activating enzyme in P. fluorescens has not been determined; it is assumed to be low because of the small pool size of tryptophan. In the histidine system, the K_m of histidase in most microbes is rather large, about $3\text{-}20 \times 10^{-3}$M for B. subtilis, P.fluorescens, and A. aerogenes (56) and 1.8×10^{-3}M for P. aeruginosa (58). There are no data for the K_m values of histidine activating enzymes for these organisms. The only histidine activating enzyme whose K_m was measured was that of S. typhimurium, where a pyrophosphate exchange assay yielded a figure of 1.4×10^{-4}M (59), a factor 10-100 times smaller than that presumed for histidase. Other activating enzymes have a broad range of K_m's, even for the same activating enzyme from different strains of the same organism. Thus the K_m of phenylalanine -sRNA synthetase in one strain of E. coli (hydroxamate assay) was 4.4×10^{-4}M (60), while in two other strains it was 1×10^{-5} and 6×10^{-7}M (61), by an attachment assay. The K_m for glycyl-sRNA activating enzymes from E. coli is $1\text{-}10 \times 10^{-5}$M (62), and unpublished work (cited in 56) places the K_m of histidyl-sRNA synthetase in E. coli at 5×10^{-6}M. Because of the wide variance of the activating enzyme values, no firm conclusions can be made on the validity of the assumption that the K_m of the activating enzyme is much lower than the K_m of the initial degradative enzyme in pathways where an intermediate compound is the true inducer. These must await publication of the K_m

values.

It is not possible to determine the number of cases where the amino acid is not the true inducer of the enzymes for its degradation. We have seen that this occurs in tryptophan degradation in a pseudomonad strain (49), and histidine degradation by A. aerogenes (56), S. typhimurium (57), and perhaps P. aeruginosa (58), but in another case, histidine degradation by B. subtilis (57), the amino acid itself is the inducer. As this is a relatively new discovery, not many people as yet have reexamined the induction of degradative pathways to ascertain whether the initial compound is the inducer. Indeed, as not much attention has been paid to the control of degradative pathways, in most cases there will have to be much preliminary work before this question can be posed.

Other controls of the tryptophan degradative pathways, mediated by activity of tryptophan oxygenase, have been demonstrated in pseudomonad species. One such control is the activation of tryptophan oxygenase by tryptophan (35); such a phenomenon can be observed in vitro. Activation results in spectral changes in the enzyme, and is thought to be due to reduction of part of the molecule. The other control reported is an effect of tryptophan on the $K_m^{O_2}$ of tryptophan oxygenase in Pseudomonas acetovorans (67). As the enzyme becomes saturated with tryptophan, or if α-methyltryptophan is added, the K_m for oxygen decreases, leading to greater efficiency of binding. Tryptophan also affects the binding of carbon monoxide to the molecule (64). Thus tryptophan oxygenase is allosterically controlled (65), and this control leads to more efficient degradation of tryptophan in the presence of saturating amounts of this substrate. Histidase, in P. aeruginosa (58),

the analogous enzyme in histidine degradation, also demonstrates allosteric kinetics. However, the physiological role in that case is unknown.

2. Regulation of tryptophan degradation in other organisms. The control of tryptophan degradation in other organisms varies as the function of the pathway and complexity of production of the enzymes. In E. coli where tryptophanase degrades L-tryptophan to indole, pyruvate, and ammonia, this enzyme is induced by growth on tryptophan (66) and repressed by growth with pyruvate (67). Thus an abundance of the end product which enters energy-yielding reactions will halt synthesis of the prior degradative enzyme. In mammals and yeast where niacin is generated by one of the branches of its tryptophan degradative pathway, tryptophan oxygenase is inhibited allosterically by NADPH (68). Again there is allosteric control exerted on tryptophan oxygenase, this time to decrease production of a coenzyme already in abundance.

Further control of tryptophan degradation is demonstrated by mammalian systems and mediated by several different mechanisms. 1) There is an unknown control mechanism exerted during and after embryogenesis, reflected in the order of appearance of the enzyme activities. Formamidase is present before birth, and the activity increases about two weeks after birth (69). 2) Tryptophan oxygenase is a highly labile enzyme. In the presence of tryptophan, inactivation of the enzyme, in vitro, is less rapid (70). The turnover in vivo in the presence of tryptophan is also less rapid. 3) The enzyme may be 'induced' by corticosteroids (71). That is, after administration of

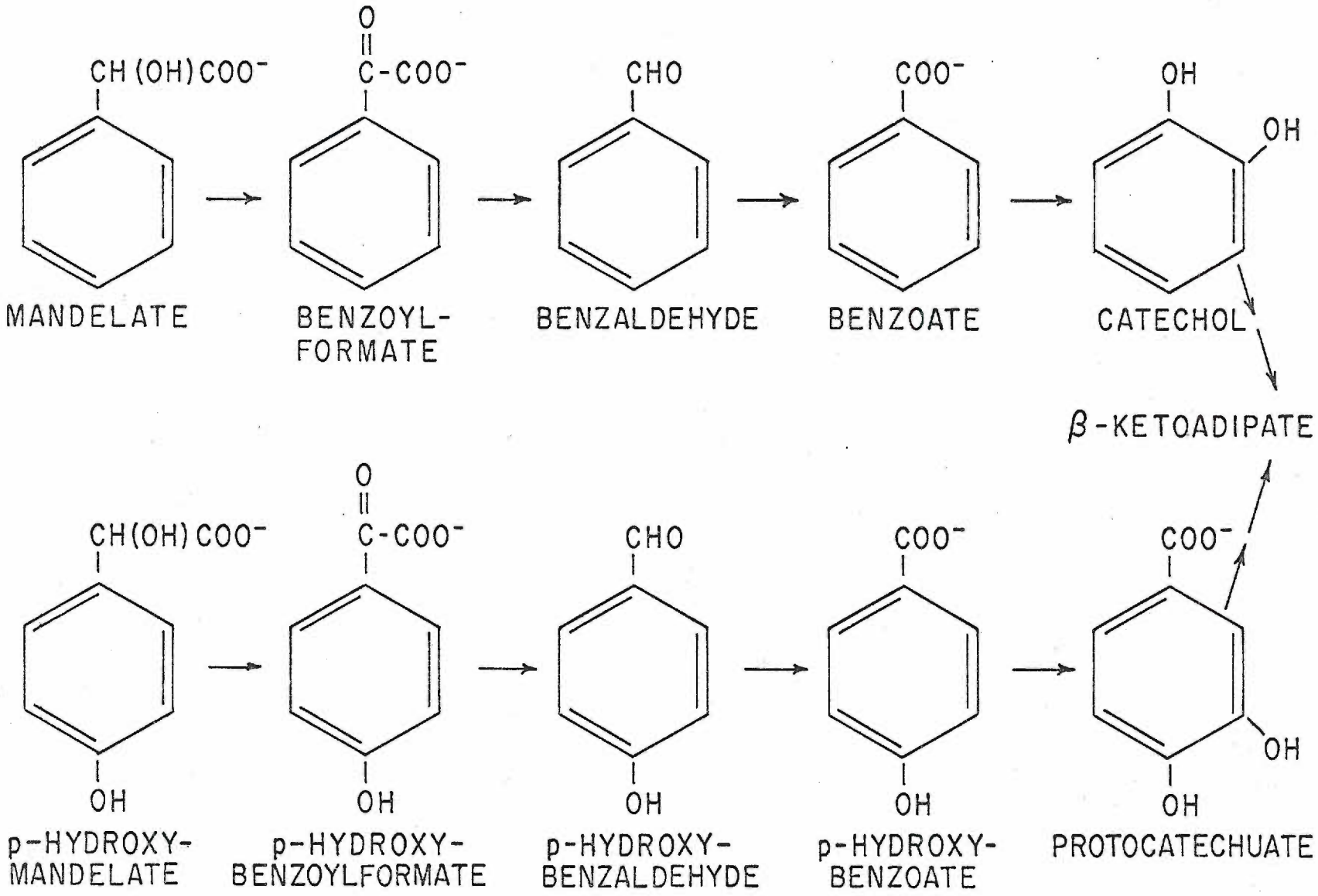
corticosteroids like cortisone, tryptophan oxygenase activity increases several fold. As cortisone does not protect the enzyme against heat inactivation (72), it is thought to produce an increase in enzyme synthesis. Treatment with tryptophan gives an apparent induction, which may merely reflect stabilization of the enzyme. 4) There is also control of tryptophan oxygenase activity exerted by a cofactor, heme. If hematin is added to a cell-free enzyme preparation, the activity of tryptophan oxygenase increases (73). The synthesis of heme may be linked to that of the enzyme (74), as the concentration of heme appears to increase on induction of tryptophan oxygenase. Thus in these control mechanisms, with the exception of cortisone induction, all control is exerted on the activity of tryptophan oxygenase. The levels of the other enzymes on the degradative pathway were not studied except indirectly (the oxygenase is assayed by kynurenine production, presupposing formamidase is always present in excess).

B. Regulation of Other Degradative Pathways

Before a discussion of the metabolism of the tryptophan analog, 5-hydroxytryptophan, this thesis will discuss briefly the regulation of degradation of two other aromatic compounds, mandelate and p-hydroxymandelate (Fig. 5). As mentioned earlier, the degradation of mandelate in Pseudomonas putida proceeds through catechol. Three enzymatic steps convert L-mandelate to benzoate, and three steps convert p-hydroxymandelate to p-hydroxybenzoate. One set of enzymes metabolizes both mandelate and p-hydroxymandelate intermediates; this is induced by growth on any of the following compounds: mandelate, p-hydroxymandelate,

Figure 5

The pseudomonad degradation pathway of mandelate and p-hydroxymandelate.


CH(OH)COO⁻
O
C-COO⁻
CHO
COO⁻
OH
OH
MANDELATE
BENZOYL-FORMATE
BENZALDEHYDE
BENZOATE
CATECHOL
β-KETOADIPATE
CH(OH)COO⁻
O
C-COO⁻
CHO
COO⁻
COO⁻
OH
OH
OH
OH
OH
p-HYDROXY-MANDELATE
p-HYDROXY-BENZOYLFORMATE
p-HYDROXY-BENZALDEHYDE
p-HYDROXY-BENZOATE
PROTOCATECHUATE

benzoylformate (75), p-chloro-, p-bromo-, or m-hydroxymandelate (76). Blocked mutants demonstrated that both L-mandelate and benzoylformate are inducers (77). The set of enzymes is repressed by short exposure to some end products, including succinate and acetate (75).

The next step of the pathway is a single step converting either benzoate to catechol, or p-hydroxybenzoate to protocatechuate. This step is mediated by an enzyme specific to each pathway (78). The conversion of catechol or protocatechuate to β-ketoadipate enol-lactone and β-ketoadipate (79) is mediated by distinct enzymatic pathways. In the mandelate pathway catechol induces catechol oxidase, and cis, cis-muconate induces the next two enzymes; the last three enzymes in both pathways are back-induced by β-ketoadipate (79).

The control of this same last sequence is different in Moraxella calcoacetica, where the pathways of mandelate and p-hydroxymandelate are identical. The two pathways converge at β-ketoadipate enol-lactone, but β-ketoadipate is not the inducer. Rather, the pathways are forward-induced by cis, cis-muconate and protocatechuate (80). This forward induction results in overlapping enzymes. If the enol-lactone hydrolase and transferase enzymes from benzoate or p-hydroxybenzoate grown cells are examined, they are found to have different heat stabilities (80), suggesting there is one set of enzymes converting β-ketoadipate enol-lactone to β-ketoadipate for benzoate grown cells, and another for p-hydroxybenzoate grown cells. The protocatechuate pathway is also used for shikimate degradation (81). Thus there is a multiplicity of enzymes in a degradative pathway. This point will be examined again in a later section of the Introduction.

METABOLISM OF 5-HYDROXYTRYPTOPHAN AND DERIVATIVES

5-hydroxytryptophan is widely distributed in nature, occurring in such diverse places as amphibian skin, venoms (82), and fungi (83). Thus one might expect to find organisms metabolizing this compound. Bacterial metabolism of 5-hydroxytryptophan intermediates will be discussed and then mammalian metabolism of hydroxylated indoles briefly reviewed. Interactions between hydroxylated intermediates and enzymes induced by tryptophan will also be considered.

A. Metabolism of Hydroxylated Indole Derivatives in Bacteria

Chromobacter violaceum is a bacterium somewhat similar in morphology to the pseudomonads. Under appropriate growth conditions a purple pigment termed violocein is produced. The pigment consists of three components: a 5-hydroxyindolyl group, α-pyrrolidine and an oxindole (84). The suggestion was made that tryptophan, which stimulates production of the pigment, is hydroxylated to 5-hydroxytryptophan, which is further oxidized and then incorporated into the pigment (85). Supporting this view was the demonstration of hydroxylating enzymes in C. violaceum which would convert L-tryptophan into 5- and 6-hydroxy indole derivatives (86). However, 5-hydroxytryptophan is not incorporated into violacein (87), nor are tryptamine, 5-hydroxytryptamine, nor 5-hydroxyindole (84). Sebek and Jagger (84) feel the hydroxylation of tryptophan to be a side reaction, and that tryptophan is degraded to indole, which is then metabolized a further step or two and then hydroxylation of the pigment or pigment precursor occurs. The 5-hydroxytryptophan does not appear to be used by the organisms; tryptophan is not used as a carbon source by the organism.

Further utilization of hydroxylated intermediates is shown by a species of Achromobacter. While the organism will oxidize anthranilate, growth on anthranilate induced degradative enzymes not for oxidation of catechol, but rather for oxidation of 5-hydroxyanthranilate and 2,5-dihydroxybenzoic acid (88). Apparently in this organism there is an additional hydroxylation, before the anthranilate is utilized. There is a strain of E. coli which might also perform this step, as its growth is stimulated by 5-hydroxyanthranilate (89). However, growth is not stimulated by anthranilate, and these effects are manifested in cells which had not been adapted to the compounds in question. It would be of interest to know whether 5-hydroxykynurenine stimulated growth, and whether anthranilate entered the cells as readily as 5-hydroxyanthranilate.

B. Metabolism of 5-hydroxytryptophan by Mammalian Enzymes

Mammalian enzymes will hydroxylate anthranilate to 5-hydroxyanthranilate (90,91). The enzymes are particle bound and require NADPH. Another fragment of metabolism of hydroxylated metabolites is found in mouse liver, where enzymes convert 5-hydroxykynurenine to 6-hydroxykynurenic acid by cyclization and decarboxylation (92), to 4,6-dihydroxyquinoline by cyclization and hydroxylation (92), or to 5-hydroxykynuramine (94). The pathway is thought (94) to arise from tryptophan and kynurenine, by hydroxylation of the latter compound.

The most investigated pathway of hydroxylated indoles in mammals is the physiologically important one leading to 5-hydroxytryptamine (serotonin). L-tryptophan is hydroxylated (95) to 5-hydroxytryptophan by an enzyme whose activity is difficult to detect. The 5-hydroxytryptophan

is then decarboxylated by enzymes found in the kidney (98); like most decarboxylases, the enzyme requires pyridoxal phosphate (97), and is inhibited by agents which inactivate this cofactor (98). The product of decarboxylation is serotonin. The decarboxylated product of tryptophan, tryptamine, has been eliminated as a precursor of serotonin by labeling studies (99). Serotonin is further metabolized by monamine oxidase (100) to 5-hydroxyindolacetaldehyde and 5-hydroxyindolacetate (101), which is excreted. The pathway is suspected to play an important role in physiological processes, but the nature of the role is obscure.

In most cases tryptophan metabolites, and those from hydroxylated compounds, are metabolized by separate enzymes. In fact, one usually inhibits the other. The pseudomonad tryptophan oxygenase inhibited by 5-hydroxytryptophan has been mentioned (34). Mammalian tryptophan oxygenase is also inhibited by tryptophan analogs. Inhibition is noncompetitive with 5-hydroxytryptophan (K_i 0.067 mM) (102). However, 5-hydroxytryptophan either induces or, more likely, stabilizes the mammalian liver enzyme (103). The kynureninase enzymes are more permissive in all cases, mammalian and bacterial enzymes hydrolyzing 5-hydroxykynurenine (34) and Neurospora hydrolyzing 3-hydroxykynurenine (45). The degradation of tryptophan by whole cells of E. coli is not inhibited by 5-hydroxytryptophan. The decomposition of indole, through anthranilate, by an unidentified strain of bacteria is similarly unaffected (104).

METABOLISM OF SOIL ISOLATE 3311 ON TRYPTOPHAN AND 5-HYDROXYTRYPTOPHAN

A. Metabolic Properties of the Organism

In this laboratory a gram negative bacterium, designated isolate 3311, was isolated by soil enrichment with 5-hydroxytryptophan as sole

carbon source (105). This pseudomonad could also grow on simple media with L-tryptophan as carbon source. Intact cells harvested from growth with either substance as carbon source oxidized tryptophan rapidly and extensively, in manometric studies; cell-free extracts metabolized tryptophan only as far as anthranilate. On the other hand, 5-hydroxytryptophan was metabolized rapidly only by 5-hydroxytryptophan-grown organisms; as above, degradation was extensive when whole cells were employed, but proceeded only as far as 5-hydroxyanthranilate with cell-free extracts. Tryptophan-grown cells oxidized 5-hydroxytryptophan only after several hours' incubation. A long lag also occurred when extracts of such cells were incubated with 5-hydroxytryptophan. The lag period, which was shown not to involve protein synthesis, was then followed by a period of increasing rate of metabolism of 5-hydroxytryptophan; the product of metabolism was found to be 5-hydroxyanthranilate. One possible explanation for the lag was the acute inhibition by 5-hydroxytryptophan of the formamidase activity of tryptophan-grown cells; the formamidase activity of 5-hydroxytryptophan-grown cells was much less sensitive to such inhibition (105).

B. Possible Multiplicity of Enzymes in Isolate 3311

As the pathways are so similar, the question of whether there was a separate set of enzymes for the 5-hydroxytryptophan system was posed. Either the set of enzymes induced by tryptophan has enough freedom to metabolize and be induced by 5-hydroxytryptophan, or there is a separate set of enzymes induced by growth on 5-hydroxytryptophan, also capable of metabolizing the unhydroxylated set of intermediates. The best approach to this problem seemed to be to concentrate studies on one of

the enzymatic steps. Formamidase was chosen because a) its measured activity was many times higher than that of the other two enzymes, under all conditions, b) preliminary studies described above suggested differences in properties, and c) the assay is extremely simple; no cofactors are required.

1. Cases where one enzyme catalyzes reactions with both a substrate and a substrate analog. There is a variety of evidence with other systems that hydroxylated or the similar fluoridated compounds are metabolized by the parent enzyme system. For example, this seems to be the case for 4-fluorophenylalanine and phenylalanine hydroxylase (106), a conversion of 2-fluorobenzoate to 3-fluorocatechol and 2-fluoromuconate (107), 4-fluoroproline and proline hydroxylase (108), γ-hydroxyglutamate and decarboxylation (109), amination (110), and transamination (111). Furthermore, as cited above, mandelate and p-hydroxymandelate are metabolized three steps by the same set of inducible enzymes (75).

Why then examine the possibility of two distinct formamidases? First, the type of inhibition of the formamidases by 5-hydroxytryptophan was different and secondly, there was preliminary evidence from heat stability studies that there were at least two distinct formamidase sites, if not enzymes. So preliminary evidence favored two enzymatic sites: were they carried on one protein or two?

2. Cases where two enzymes catalyze a common step. In biosynthetic pathways where there is a common metabolic step or steps leading to the synthesis of two or more essential compounds, the

regulation of the common step(s), through regulation of synthesis of the enzymes, is usually controlled by repression (112). In some cases two end products must be present for repression of the enzyme, so called concerted repression (113). Where there is a multiplicity of end products and no concerted repression, there is usually a multiplicity of enzymes, each repressed by one of the end products. This latter seems to be a common control mechanism in the Enterobacteriaceae. In addition, the enzymes are usually inhibited by end products, affording a quicker control of the system. Thus in the biosynthetic pathway for aromatic compounds in E. coli, responsible for the production of tryptophan, phenylalanine, tyrosine and catalytic amounts of other aromatic compounds, there are three different enzymes for the first step unique to the pathway. Each enzyme is repressed, and two are inhibited, by a different amino acid end product (114,115). This same enzyme, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase, in most pseudomonads is inhibited only by tryosine (116,117) which suggests a single enzyme. The mechanism of control is unknown; however, this inhibition by tyrosine cannot be the only controlling factor or the cells would not grow as they do, on media supplemented with tyrosine and lacking the other amino acids. It is possible, although unlikely, that the other amino acids are synthesized by different pathways (117). Alternately, the other enzymes might be repressed under the growth conditions (117), as there is generally a "major" enzyme in multiple catalyzed steps with the others present in smaller amounts. Or, it might be that phenylalanine and tryptophan control the enzymes responsible for tyrosine formation, and thus control the common sequence under most growth conditions. In

fact in Pseudomonas strain ATCC 11299a phenylalanine synthesis is controlled by both phenylalanine and tyrosine; phenylalanine inhibits prephenate dehydratase while tyrosine stimulates it (118).

In a later common sequence in the same pathway, chorismate mutase catalyzes a step leading to the synthesis of both tyrosine and phenylalanine. In this case in both E. coli and A. aerogenes (119) there are two enzymes, each controlled by repression and inhibition by one amino acid. Two distinct loci in B. subtilis are responsible for production of three chorismate mutases (120). The enzyme occurs in an aggregate with other tryptophan biosynthetic enzymes (121). Other similar examples of multiply-catalyzed steps include the aspartokinases of E. coli (122), leading to the synthesis of four amino acids, and the glutamine-dependent carbamyl phosphate synthetase of Saccharomyces cerevisiae (123), leading to the synthesis of arginine and pyrimidines.

All the above examples of multiple enzymes catalyzing on enzymatic step are in biosynthetic pathways. The sole example encountered in a literature search for multiplicities of degradative enzymes has been discussed earlier, the benzoate and p-hydroxybenzoate degradative pathways in Moraxella (80). The paucity of reported cases of such multiple degradative enzymes is due in part to a general lack of concern for the control mechanisms in degradative enzymes. There is an awakening of interest, reflected in several recent papers of pseudomonad degradative pathways (58,63,76,77,78,79). The mechanisms of control of these pathways reported are still at a very general level, without, in most cases, the careful analysis of specific examples which has characterized the studies in biosynthetic systems. The sole control mechanisms reported

are induction and either end product (e.g. 75) or catabolite (124) repression, the phenomena being distinct (125). Catabolite repression involves repression of degradative pathways by energy-yielding compounds not necessarily convertible to end products of the pathway. End-product repression might be a little more selective in which pathways are shut off, perhaps being the 'fine adjustment' while catabolite repression is the 'coarse adjustment.' For example, pyruvate, an end product, represses tryptophanase and D-serine deaminase (67), while succinate and acetate repress the early steps in catechol degradation (75).

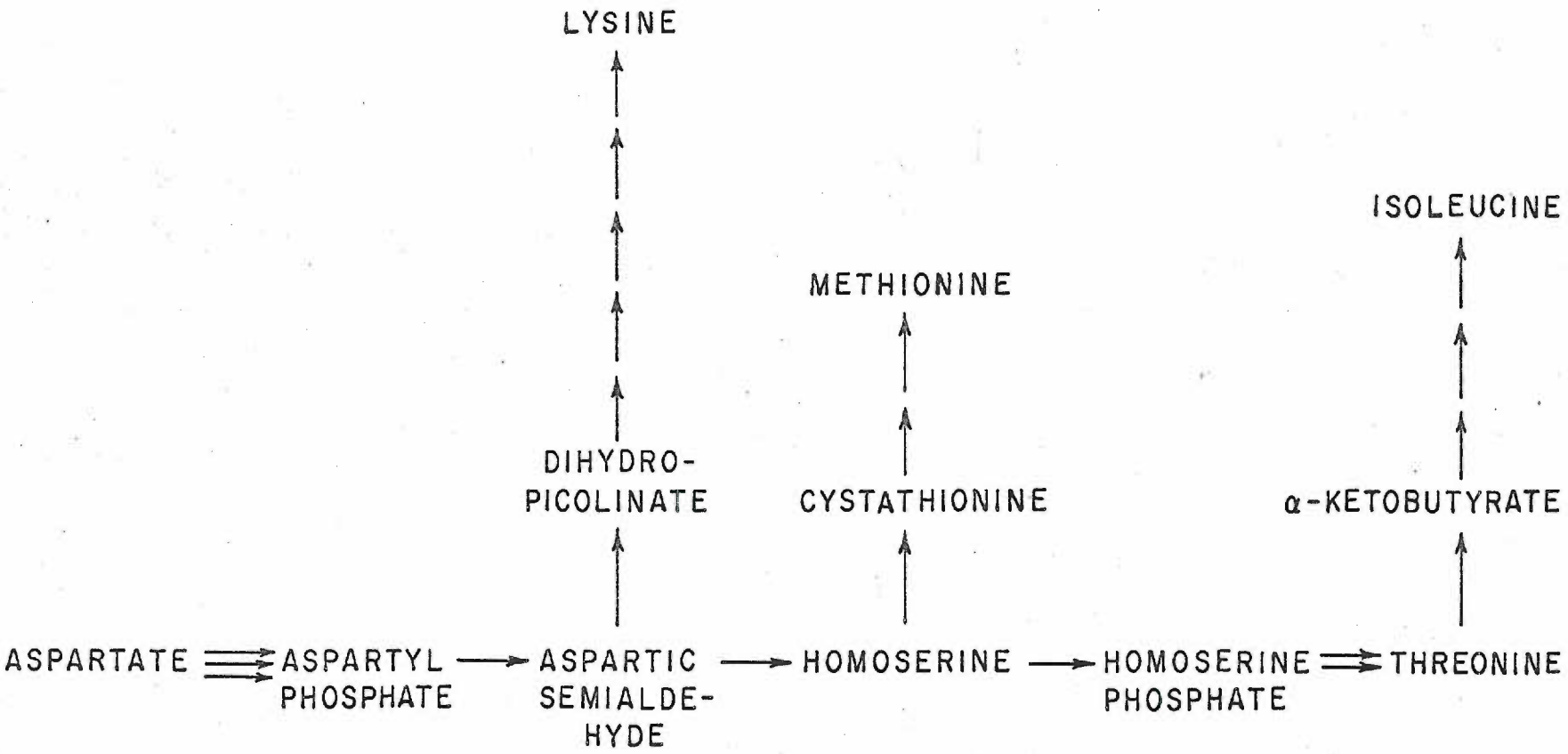
The problem in degradative systems differs from that of biosynthetic systems: for biosynthetic pathways the organism must insure that each important product be synthesized, if not supplied by the environment, while for degradative pathways, the pathway basically provides an efficient source of energy and intermediates for biosynthesis. There would seem to be no need to maintain synthesis of the degradative enzymes if the substrate were not present. However, the condition of an intermediate compound inducing the pathway alters the situation somewhat. Now there must be enough enzyme activity at all times to metabolize the initial substrate as far as the inducing intermediate. This phenomenon has been demonstrated by Palleroni and Stanier who found in their Pseudomonas strain a low basal level of tryptophan oxygenase and formamidase in the absence of exogenous tryptophan or kynurenine. Furthermore, even when a metabolic block existed between tryptophan and kynurenine, to eliminate endogenously-produced kynurenine, some activity of the remaining enzyme was demonstrable. This activity might be the result of either incomplete repression of a single enzyme system metabolizing

tryptophan to kynurenine, or the presence of a second enzyme system. In this particular system there is the possibility that some remnants of a quinolinic system remain. This might include the oxygenase and formamidase, but certainly would not include a kynureninase, which is demonstrable under basal level conditions. There are no reports of studies in which organisms with both pathways have been examined for multiple oxygenases or formamidases.

3. Cases of one enzyme with more than one active site. Another possibility which might occur in the 5-hydroxytryptophan-tryptophan utilization scheme is one enzyme with two sites, each with its own K_m, V_m and substrate specificity, due either to the conformation or amino acid composition at that site. Thus the heat lability of the 5-hydroxytryptophan-induced formamidase might be due to a heat labile site, yet the proteins be the same. The most investigated systems of proteins with two sites, "dicephalic" proteins (126), are on biosynthetic pathways which have been discussed before. In these cases two or more enzymatic activities, both on the same pathway but not necessarily catalyzing adjacent reactions, remain together through extensive purification. Genetic and physiological studies reveal that the two enzyme activities are controlled together; they are repressed, and sometimes but not always, inhibited together. Both can be lost as the result of a single mutation. The most thoroughly studied examples of this type occur in the pathway of E. coli leading from aspartate to lysine, threonine, isoleucine, and methionine (Fig. 6). Aspartokinase converts aspartate to aspartyl phosphate, a precursor for all the amino acid end products. Homoserine dehydrogenase converts homoserine, which would

Figure 6

The pathway from aspartate to lysine, methionine, threonine and isoleucine in E. coli K12, illustrating the two steps where multiple enzymes have been characterized.


LYSINE
ISOLEUCINE
METHIONINE
DIHYDRO-
PICOLINATE
CYSTATHIONINE
α-KETOBUTYRATE
ASPARTATE
ASPARTYL
PHOSPHATE
ASPARTIC
SEMIALDE-
HYDE
HOMOSERINE
HOMOSERINE
PHOSPHATE
THREONINE

otherwise be transformed to methionine, to homoserine phosphate, a precursor of threonine and isoleucine.

Further analysis revealed that when aspartokinase was repressed by lysine, the remaining aspartokinase activity was not inhibited by lysine; similarly when either aspartokinase or homoserine dehydrogenase was repressed by threonine plus isoleucine, the remaining activity was not inhibited by threonine. There appeared to be multiple enzymes under separate control (112). The purified threonine-controlled aspartokinase (aspartokinase I) was found to have homoserine dehydrogenase activity; both activities were inhibited by threonine (127). Mutations induced simultaneous loss or acquisition of both activities. Later work on this threonine-controlled system showed (128) the specific activity of both to be constant over a 600-fold purification. One of the substrates of the dehydrogenase both inhibited the aspartokinase and protected it against heat inactivation. Thus, the two enzymatic sites interact.

Recently a second aspartokinase was found in E. coli K12 to have homoserine dehydrogenase activity (129). Both activities (aspartokinase II and homoserine dehydrogenase II) were repressed by methionine. Both multiple proteins, aggregates I and II, are large with molecular weights of > 300,000 and about 160,000 respectively. This molecular weight may decrease on mutation. There are two classes of mutants of aggregate I which have aspartokinase activity and lack dehydrogenase activity (126). Mutants of the first class have the same molecular weight but have lost the interaction between sites. Mutants of the second have a molecular weight of 40,000. These have lost the

sensitivity to threonine, suggesting this is a property of regulatory chains.

A similar regulatory system is found in the chorismate mutase step of the synthesis of phenylalanine and tyrosine in A. aerogenes and E. coli (119). There are two chorismate mutases, one repressed slightly by phenylalanine (chorismate mutase P), and one strongly repressed by tyrosine (chorismate mutase T). The chorismate mutases are separable by DEAE chromatography. Chorismate mutase T was eluted with prephenate dehydrogenase, the first enzyme leading specifically to tyrosine synthesis; the two enzymes were affected simultaneously by mutation. Similarly chorismate mutase P traveled with prephenate dehydratase, the first enzyme leading specifically to phenylalanine synthesis, and the two were also affected together by mutation. A similar linkage of chorismate mutase P and prephenate dehydratase has been proposed for pseudomonad enzymes (130). Fungi also appear to have such complexes of enzymes, since some of the enzymes on the tryptophan synthetic pathway are found together on sucrose gradients (131). The presence of this phenomenon spread through so many different organisms implies that the control exerted thereby is efficient. Other examples of this type of control include indolglycerol phosphate synthetase and N-5-phosphoribosyl-anthranilate synthetase (132,133,134) in E. coli and A. aerogenes where two or more separate structural genes code for the enzymes; yeast aspartotranscarbamylase and carbamylphosphatase (135,136), cystathione synthetase and L-serine and L-threonine dehydratases (137), and the phosphatase and dehydrase coded by the B gene of histidine synthesis in S. typhimurium (138).

The dangers in concluding that two enzyme activities are on the same protein are demonstrated by Munkres (139,140) who concluded on the basis of 1) simultaneous change of both activities by single mutations, 2) inability to separate the activities by column fractionation, 3) immunological properties and 4) amino acid compositions, that Neurospora malate dehydrogenase and aspartate aminotransferase activities were on the same protein. Work published (4) one year later revealed separation of the activities by column fractionation procedures, as well as by ammonium sulfate fractionation (141).

There are other reports of single proteins having different activities, but these activities are not as physiologically related. In other cases the separate activities demonstrated are very similar, and could well be catalyzed by the same enzymatic site. Perhaps the most important set of enzymes which catalyze two reactions are those where one activity is increased at the expense of the other. The first example of this is the mammalian enzyme glutamic dehydrogenase. This molecule is in the state of equilibrium with molecules with fewer subunits. Reagents which favor disaggregation, like NADH, increase alanine dehydrogenase activity, while those favoring aggregation, like NAD or ATP, increase the glutamic dehydrogenase activity (142). Mercury salts appear to produce disaggregation (143) in these enzymes. Mammalian glycerate-2,3-diphosphatase and phosphoglycerate mutase (144,145) are similar in that mercuric chloride will increase the specific activity of purified phosphatase at the expense of the mutases while glutathione will reverse the effect. The phenomenon does not occur with pCMB treatment, nor with other heavy metal ions like Cd^{++} or Pb^{++} (145).

The last example of this is also a mammalian enzyme, the intestinal tryptophan oxygenase which can apparently oxidize D- or L-tryptophan. Evidence favoring more than one site participation is afforded by different pH activity curves with D- or L-tryptophan (31). Conditions favoring L-tryptophan oxidation lowered D-tryptophan oxidation, and vice versa.

Other examples of single proteins with perhaps two activities include: mammalian fructose 1,6-diphosphatase/sedoheptulose-1,7-diphosphatase (146,147), pseudomonad glutaminase/asparaginase (148), and mammalian pyrimidine deoxyribosyl transferase/deoxythymidine phosphorylase (149). So there is evidence that a protein may have two enzymatic sites, although the distinction between one protein with two sites and two aggregated proteins has not yet been made in many cases.

APPROACH TO THE PROBLEM

A. Purification of the Formamidase Enzymes

The ideal method for determining whether the formamidases induced by growth on tryptophan or 5-hydroxytryptophan are the same protein would be to purify each formamidase and compare the pure proteins with respect to physical and chemical properties: molecular weight, number of subunits, turnover number, immunological individuality and cross reactivity, and amino acid content and perhaps sequence of each subunit. Unfortunately the 5-hydroxytryptophan-induced activity had been found to be extremely heat labile, rendering exploration into purification procedures very difficult (105). As described later in this thesis, attempts were made to purify the tryptophan-induced formamidase, in order to obtain a pure preparation for biochemical and immunological studies. Note that the

exact inducer of the system is not known for certain, as there are no blocked mutants between tryptophan or kynurenine. For convenience, tryptophan and its hydroxylated derivative are used as the inducers, although the true inducer may be metabolically derived from these compounds. It was hoped that establishment of a procedure for the purification of the tryptophan-induced formamidase, resulting in at least markedly increased specific activity, if not unequivocal purity, would permit comparative studies with the 5-hydroxytryptophan-induced enzyme. For example, the behavior of 5-hydroxytryptophan-induced formamidase in column fractionation procedures known to effect purification of the tryptophan-induced formamidase should provide some information about similarities or differences of the enzymes. However, as described in a later section of the thesis, the tryptophan-induced formamidase was not purified to an extent satisfactory for delicate physical-chemical techniques. Therefore, other approaches became necessary.

B. Genetic Studies

The question of one set of enzymes or two is amenable to genetic studies. This has been used successfully in the mandalate/p-hydroxymandelate system where a mutant was obtained that would grow neither on mandelate nor on p-hydroxymandelate (75), strongly suggesting that the same set of enzymes acted on both substrates. Similarly in the study of aspartate transcarbamylase and homoserine dehydrogenase (127) and other dicephalic proteins, both enzymatic activities were affected by a single mutation, indicating one protein with two enzymatic activities was involved. Thus a study was initiated with isolate 3311 to obtain a mutant which could not use tryptophan as sole carbon source, in order to

test it for growth on 5-hydroxytryptophan. The results obtained with various mutagenic agents in combination with several screening methods are reported in this thesis. Mutations which were isolated appeared to revert easily and the testing procedure was both subjective and not very reproducible.

At the same time another genetic study was in progress to isolate bacteriophage capable of lysogenizing 3311, in order to transduce the genes responsible for tryptophan and 5-hydroxytryptophan degradation. To accomplish that aim, a number of soil pseudomonads were isolated and tested for phage production on many possible indicator strains, including 3311. Similarly, phage production by 3311 was tested using all the other isolates as indicator strains. These phage then were to be used in transduction tests between 3311 and either mutants of 3311 defective in tryptophan and 5-hydroxytryptophan degradation or other indicator strains unable to degrade either compound. Transductants for tryptophan-metabolizing genes were to be tested for the ability to metabolize 5-hydroxytryptophan. If one set of genes were responsible for a single set of enzymes induced by either tryptophan or 5-hydroxytryptophan, then those transductants able to metabolize tryptophan would always be able to metabolize 5-hydroxytryptophan. If the genes are different, producing different enzymes, the two metabolic activities might separate on transduction to some extent. However, as reported in a later section of this thesis, the bacteria isolated did not carry phage active on 3311, nor vice versa. A study of some metabolic properties of 3311 and the other pseudomonads suggested they were not closely related taxonomically, and were therefore unlikely to interact genetically.

C. Induction Studies

A study of the physiological control of the enzyme synthesis induced by tryptophan and 5-hydroxytryptophan was done to determine whether these processes differed for the two inducers. This was accomplished by a study of the kinetics of induction, the specific activities of maximally induced enzymes, and an examination of two metabolites which might be expected to repress the enzyme synthesis. This procedure has been demonstrated to have use in the mandelate and p-hydroxymandelate pathways in another pseudomonad: since the levels of enzymes were about the same after induction by either compound and were repressed about the same extent by the same compounds, and also affected simultaneously by a presumed single mutation, the enzymes were considered to be the same (75).

Induction studies were done by varying the amount of inducer as well as the period of time used for induction, in order to determine 1) whether the induction period was the same for the tryptophan- and 5-hydroxytryptophan-induced enzymes, 2) whether the enzymes attained the same specific activities on maximal induction, and 3) whether the order of induction of the enzymes was parallel for the two systems. If there were gross differences in the induction pattern, particularly with respect to point 3), it would be likely that entirely different enzymes were induced. A second possibility would be that 5-hydroxytryptophan had to undergo many enzymatic changes before it could induce all the enzymes of the early pathway.

In addition to the induction experiments, key intermediates in the early series of steps, namely succinate and alanine, were examined for ability to repress one or both formamidases, as had been the case in

the mandelate pathway (75). The results of these studies are reported in a later section of this thesis.

D. Kinetic Studies

An analysis of the kinetics of each formamidase in the presence of inhibiting agents was carried out. The ability of anthranilate or alanine to protect against several of these agents was also examined. The possibilities to be distinguished by these studies include 1) one enzyme with a single catalytic site induced by either tryptophan or 5-hydroxytryptophan. The site would have slightly different properties depending on the inducer, to agree with previous kinetic data. This could happen in either of two ways - by an event occurring in the presence of the inducer at protein synthesis such as incorporation of 5-hydroxytryptophan into the protein or incomplete chain synthesis, or by alteration of a formed enzyme, as in the case of glutamine synthetase (150). Another possibility is that of 2) one enzyme with two catalytic sites having different properties due to differences in configuration or amino acid sequence. Each site would catalyze the hydrolysis of formyl-L-kynurenine and 5-hydroxyformyl-L-kynurenine, with only one of the two sites expressed, the site determined by the presence of the inducer during synthesis. Earlier in the Introduction, examples of proteins with two activities, where one activity is increased only at the expense of the other, are given. The solution might be 3) two different proteins each induced by only one of the inducers, but with similar activities on formyl-L-kynurenine and 5-hydroxyformyl-L-kynurenine.

A study of the K_m and V_m of the formamidases, coupled with studies of inhibition and on heat inactivation, can rule out some of these

possibilities, depending on the data. If the K_m and V_m values were the same, and the response to all inhibitors were similar, an argument for a single enzyme with a single site could be made. This argument has been proposed, for example, for the hydroxylation of phenylalanine by an enzyme thought to be tyrosine hydroxylase (151). In this case the K_i for phenylalanine as inhibitor of tyrosine hydroxylation was the same as its K_m for its own hydroxylation; the same condition held for tyrosine as inhibitor and substrate. Furthermore, the hydroxylation reaction with either substrate was inhibited equally by two different inhibitors, and, finally, purification procedures maintained a constant ratio of activity with the two substrates. A similar case is demonstrated for two inducible dehydrogenases in a pseudomonad, where "growth on D-glucarate induces a dehydrogenase which in all kinetic and physical respects examined appears identical to that induced by hydroxyproline" (152), the properties being mobility in sucrose gradient, pH optima, K_m's for two substrates and an analog, relative rates with substrate analogs, and heat inactivation studies.

The heat lability of the 5-hydroxytryptophan-induced formamidase, relative to that induced by tryptophan, makes the possibility of one protein with a single site unlikely. Only by formulating an hypothesis, untestable at this time, namely that the presence of 5-hydroxytryptophan in addition to inducing the same protein, alters the tertiary or quaternary structure of the molecule, giving it altered properties, could this alternate be saved. The new hypothesis, however, makes no definite predictions about the properties of the molecule, for example it may or may not be the same immunologically, depending on whether antigenic

sites get exposed or buried, and it may or may not act the same way with inhibitors. It is very difficult to test this hypothesis at any level of purity of the enzymes, for it would have to be shown that the amino acid sequence is exactly the same for both formamidases. However, the hypothesis that the primary structure is altered by the inclusion of 5-hydroxytryptophan could be tested by studying the uptake of labeled 5-hydroxytryptophan into proteins.

The second alternative concerns one protein with two enzymatic sites. Tests with inhibitors which bind to the active site would give either the same or different sensitivities, depending on how similar the sites were. However, inhibitors not binding to the active site but non-competitive inhibitors, might always inactivate either formamidase to the same extent. More rigorous criteria for one protein with two sites include movement of the two activities together on ion exchange columns (119) or sucrose gradients (131), a constant ratio of activities through extensive purification (128, 132, 137, 144, 147, 148), and simultaneous inactivation by mutation (127, 133, 136). Any one of these criteria is not sufficient proof. The constant ratio of activities will exclude some dicephalic proteins whose sites are not equally stable, and it is possible for a similar protein to be purified with any enzyme. Also inadequate is the simultaneous loss by mutation, as the mutation may be polar, or be in an operator region, and in both cases affect several genes, or it may affect a regulatory chain common to two or more distinct proteins. Even the demonstration of two activities on one purified protein is not a sure criterion, as there is no unequivocal criterion for protein purity.

In the last case, where two distinct protein molecules are proposed, one would expect to find differences in response to noncompetitive inhibitors, in contrast to the last case. Thus the problem is resolved, at this level of protein heterogeneity, to an examination of inhibitors thought to act noncompetitively. This approach was taken by Mathews (153) who wished to determine whether the dihydrofolate reductase produced by E. coli infected with phage was the one the uninfected organism produced i.e., did the phage carry genetic information coding for its own enzyme. He examined the enzymes, in crude preparations, produced by the bacterium infected with several different strains of phage, as well as preparations derived from the uninfected bacterium. On the basis of pH optima, pyrimidine specificity, aminopterin and trimethoprim sensitivity, urea sensitivity, and heat inactivation of the various enzyme preparations, he was able to conclude with a high degree of certainty that the enzymes were different.

The results of the studies with inhibitors are presented in a later section of this thesis.

MATERIALS AND METHODS

GENERAL GROWTH PROPERTIES AND TECHNIQUES FOR ENZYME ASSAYS

A. Basal Medium and Stock Culture

Isolate 3311 was maintained in stock culture on slants of basal medium (Table I), containing as carbon source 0.01% L-tryptophan, 0.1% L-glutamate, or 0.08% DL-5-hydroxytryptophan, and solidified with 1.5% Difco Bacto-Agar. The slants were kept under refrigeration and the organisms transferred at approximately two-month intervals. The basal medium was supplemented by the addition of 10 mg yeast extract (Difco, Detroit, Mich.) per 100 ml medium for some experiments. This increased the yield of cells without affecting the specific activity of the enzyme significantly.

The carbon source was dissolved in distilled water. The solution was adjusted to pH 7, brought to 1/5 final volume and sterilized. Kynurenine, 5-hydroxytryptophan or anthranilate were sterilized by Millipore filtration (0.45 μ pore size); tryptophan and glutamate solutions were autoclaved as above. The concentrations of carbon sources used in the media were 0.1 - 0.2% sodium L-glutamate (pfs, Sigma Chemical Co., St. Louis, Mo.), 0.1% L-tryptophan or 0.2% DL-tryptophan (Nutritional Biochemicals Corp., Cleveland, Ohio), and 0.08% DL-5-hydroxytryptophan (Nutritional Biochemicals Corp.).

B. Growth of the Organism

1. Growth of the organism in basal medium. An experiment was done to determine the relative growth rate of isolate 3311 on tryptophan, 5-hydroxytryptophan, or glutamate as carbon source in the unsupplemented

TABLE 1

Pseudomonas Basal Medium (105)

Constituent	g/100 ml Distilled water
K_2HPO_4	0.15
KH_2PO_4	0.05
$MgSO_4 \cdot 7H_2O$	0.041
$(NH_4)_2SO_4$	0.05

Reagent grade salts are dissolved in 4/5 final volume and autoclaved at 121 C for 10 min.

basal medium. Consequently, 0.1 ml of an overnight culture grown on basal-0.1% L-glutamate medium was added to each of three 500-ml flasks containing 250 ml each of basal with 5 x 10^{-3} M L-tryptophan, DL-5-hydroxytryptophan, or L-glutamate. The flasks were shaken on a Gyratory shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 30 C, and 10 ml samples were withdrawn every half hour for a determination of the optical density (turbidity) at 520 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer.

2. Growth of the inoculum. All incubations unless otherwise noted were performed at 30 C either in an incubator (for plates) or shaker. For all enzymatic studies described in this thesis, the inoculum used was obtained from stationary growth cultures on basal-glutamate medium. Erlenmeyer flasks containing 10 - 20 ml of basal medium with 0.2% L-glutamate were inoculated with a culture from a stock slant, and were shaken for 20 - 24 hr. The viable count was 2 - 3 x 10^9 cells/ml. If the organisms were to be induced on solid medium, 0.3 - 0.4 ml of the culture were spread on each plate and the plates incubated until harvest time. If the organisms were to be induced in liquid culture (200 - 1500 ml per flask), 2 - 10 ml of the inoculum were pipetted into each flask.

In studies with mutagenic agents, 17-hr overnight cultures grown as above were subjected to the mutagenesis treatment. Generally one 125 ml flask containing 10 ml of basal-0.2% glutamate medium was used per mutagen. The 17-hr culture was centrifuged at 3,000 g for 15 min, and resuspended in 10 ml of sterile saline. The viable count of the saline suspension was usually 0.8-2 x 10^9 cells/ml.

3. Induction of the cells. For the series of experiments where the inducer concentration or time of induction was varied, the basal medium was solidified with 0.85% Oxoid ionagar #2 (Colab, Chicago Heights, Ill.) and supplemented with 0.1% L-glutamate. The purity of this agar often allows more reproducible specific activities of degradative enzymes (154).

For the rest of the induction experiments, Difco Bacto-Agar, 1.5% was used to solidify the medium. Thirteen plates were poured from 200 ml medium.

In both cases the plates were allowed to harden at room temperature several hours before inoculation. After inoculation, the plates were incubated for 16.5 hr, unless otherwise noted. Cells were harvested by suspension of the surface growth into a total volume of 40 - 50 ml of sterile saline per 200 ml of growth medium. This suspension was centrifuged at 4,000 g for 15 min. The cells were resuspended in 30 ml of sterile saline per 13 plates and centrifuged again. They were finally taken up in 5 - 10 ml of 0.01 M potassium phosphate buffer, pH 7.0.

Cells grown in flasks were harvested after 16 - 20 hr growth. There were about 3 - 4 x 10^9 cells/ml after growth. The cells were centrifuged at 3,000 g for 30 min, suspended in 30 - 120 ml sterile saline, recentrifuged at 4,000 g for 15 min, and suspended in 10 - 20 ml buffer. If more than 2 liters of culture were to be harvested, the cells were spun down in a Sorvall SS-4 centrifuge with a Szent-Gyorgyi & Blum continuous flow system at 14,500 g.

C. Sonication

The cells cooled to 4 C in an ice bath were sonicated with the

water-cooled standard probe of a Bronwill-Blackstone ultrasonic apparatus (Rochester, New York) at a dial setting of 60. All subsequent steps with the enzyme preparations were performed using either an ice bath or refrigeration. Sonication was performed for 5 - 20 min, depending on the cell density and volume of the solution. For the cells harvested from 200 ml of media and suspended in 5 ml of buffer, sonication was carried out for 5 min. For the large amount of cells harvested from 6 - 9 liters of medium, sonication was carried out for 15 - 20 min.

The sonicate was spun at 2 C for 10 min at 20,000 g. This procedure, centrifugation at 2 C for 10 min at 20,000 g, was standard for every subsequent centrifugation with the cell-free preparation. The supernatant solution, i.e. the cell-free or crude preparation, was then treated with protamine sulfate, as described below. For assays in induction studies, the crude preparation was used for enzymatic assays.

D. Protamine Sulfate Treatment

In order to precipitate nucleic acids, a solution of protamine sulfate (Krishell Laboratories, Portland, Oreg.) adjusted to pH 7.0 with 1 M KOH, was added to the cell-free preparation. In general, 0.1 volume of 1% protamine sulfate was added to the extract harvested from 200 ml medium; this ratio was adjusted depending on the density of bacteria to as much as 0.2 volume of a 3% solution. The protamine solution was added dropwise while the cell-free suspension was slowly mixed by a magnetic mixer. The protamine-treated enzyme preparation was then allowed to remain undisturbed for 10 min. The preparation was centrifuged and the precipitate, containing primarily protamine-precipitated nucleic acids and some co-precipitated proteins, was discarded. The

supernatant solution was used for the studies with inhibitors. However, the studies on enzyme purification were performed on material which had been subjected to a second identical protamine fractionation, followed by ammonium sulfate fractionation.

E. Ammonium Sulfate Fractionation

In order to effect partial purification of the tryptophan-induced formamidase enzyme, the preparation was subjected to two or three fractionation steps with ammonium sulfate and the material, which precipitated at 35%, 50 or 55%, and 70% saturation, analyzed. The ammonium sulfate, either as solid crystals or as a cold, saturated solution, was slowly added to the protamine supernate with gentle stirring. This ammonium sulfate-treated solution was stirred for another 10 min, and then allowed to remain an additional 20 min without stirring. The suspension was centrifuged, the supernatant fluid was drawn off for further treatment, and the precipate was suspended in 2 - 3 ml of 0.1 M potassium phosphate buffer at pH 7.0. The supernatant fluid could then be treated with the next higher concentration of ammonium sulfate. The supernatant solution from 70% saturated ammonium sulfate was discarded. The resuspended ammonium sulfate fractions, and in particular that fraction insoluble in 50 or 55% saturated ammonium sulfate, constitute the "purified fractions" used in further purification procedures.

F. Protein Assays

Protein concentrations were determined colorimetrically with the Reiner and Cheung modification (155) of the Folin-Ciocalteau procedure, using a Bausch & Lomb Spectronic 20 spectrophotometer and crystallized

bovine serum albumin (A grade, Calbiochem, Los Angeles, Calif.) as protein standard. The standard curve at 620 mμ obtained with bovine serum albumin was linear from 25 to 150 - 200 μg protein. The standard was run with every determination.

G. Enzyme Assays

All assays were followed spectrophotometrically at 360 mμ using a Beckman DU Spectrophotometer with 1 cm silica cuvettes. The substrates and semicarbazide were adjusted to pH 7 with a KOH if necessary. Formyl-L-kynurenine solutions and the enzyme preparations were held in ice baths. Other reactants were at room temperature. There was a 5 min preincubation of the enzyme with semicarbazide in the buffer-water mixture in two of the assays. The reaction was initiated by adding the substrate and starting the timer (Time-It, calibrated at 0.01 min, Precision Scientific Co., Chicago, Ill.). The solution was mixed with a gentle stream of bubbles from an air line. In most studies with inhibitors, it was not necessary to mix the components together; however, this was done with the concentrated urea solutions.

All assays are based on the appearance or disappearance of kynurenine. Reaction rates are reported as the optical density difference x 10^3 at 360 mμ per min. The activities are expressed in millimicromoles kynurenine formed or degraded per min, by dividing the rate by 1.467 (based on a molar extinction coefficient of 4.4×10^3 calculated from material presented in (32)). Both rates and activities are expressed as positive numbers. Specific activity is defined as the activity per milligram protein in the reaction mixture.

1. Formamidase. The enzyme extract, 0.15 - 1.5 ml, was pre-incubated 5 min at room temperature with phosphate buffer, water and semicarbazide (Blue Label, Calbiochem), before the addition of 0.3 ml of formyl-L-kynurenine (B grade, Calbiochem) to initiate the reaction. The semicarbazide was added to inhibit kynureninase activity. The blank cuvette contained all components except the enzyme. If the blank did not contain formylkynurenine, the initial optical density of the reaction cuvette would be high, due to absorption by formylkynurenine and the slight amount (less than 1%) of kynurenine present. The reaction would be somewhat more difficult to follow.

Standard Enzyme Assay

	In 3 ml
K phosphate buffer, pH 7.0	16.7 mM
Semicarbazide HCl	10 mM
Formyl-L-kynurenine	0.5 mM
Enzyme extract	0.15 - 1.5 ml
Distilled water	to volume

The experiments to determine the K_m of the enzyme were performed with a series of substrate concentrations.

Optical density readings were taken about 30 sec after the addition of substrate and read at about 12 sec intervals for 1.5 - 2 min. The rates therefore represent initial rates; they were linear in the region examined, and proportional to enzyme concentration.

2. Kynureninase. Kynurenine was assayed spectrophotometrically with L-kynurenine sulfate (A grade, Calbiochem) as substrate and

with omission of the semicarbazide treatment and preincubation time. The decrease in optical density was followed with readings taken every 20 sec or so for 1 - 2 min; the decrease was linear in that interval. The blank cuvette contained only buffer and water in order to more efficiently utilize the enzyme preparation.

Standard Enzyme Assay

	In 3 ml
K phosphate buffer, pH 7.0	16.7 mM
L-kynurenine sulfate	5.0×10^{-2} mM
Enzyme extract	0.5 - 2.0 ml
Distilled water	to volume

The rather low substrate concentration was chosen in order to keep the initial optical density about 0.300; low rates would be difficult to assay with higher optical densities. However, the enzyme was not fully saturated with substrate; doubling the substrate concentration produced an increase in rate of 17%. Added pyridoxal phosphate was not required; the enzyme activity was not increased by the addition of 10 µg of pyridoxal phosphate (phosphoric acid ester, Nutritional Biochemicals Corp.).

3. Tryptophan oxygenase. After preincubation of the enzyme preparation for 5 min at room temperature with semicarbazide, buffer and water, L-tryptophan (Nutritional Biochemicals Corp.) was added to initiate the reaction. Optical density readings were continued from about 30 sec after substrate addition over 2 - 7 min. The high activity of formamidase in these preparations made this assay practical. Whenever

there was any doubt whether formamidase was in excess, 0.1 ml of a guinea pig liver preparation, purified by the method of Knox & Mehler (32), was added to the reaction. The preparation, which was kept frozen until use, had high formamidase activity, and lacked the more labile tryptophan oxygenase.

Standard Enzyme Assay

	In 3 ml
K phosphate buffer, pH 7.0	16.7 mM
Semicarbazide HCl	10 mM
L-tryptophan	1.0 mM
Enzyme extract	0.3 - 1.5 ml
Distilled water	to volume

EXPERIMENTS ON PURIFICATION OF THE TRYPTOPHAN-INDUCED FORMAMIDASE

A. Paper Electrophoresis of the Crude Material

Paper electrophoresis was used to determine the isoelectric point of the tryptophan-induced formamidase as an aid in choosing appropriate fractionation conditions. The sample, 10 µl of guinea pig preparation or 20 µl of bacterial preparation, was applied to Whatman 3MM strips, 1 x 13 in, and electrophoresed in the cold room for 3 - 17 hr, using a 6 ma current and voltage of approximately 260 v (Buchler Instrument Co., Fort Lee, N. J.). Buffers used included 0.075 M Tris (2-amino-2-hydroxymethyl) -1,3-propanediol HCl (Trizma base, Sigma Chemical Co., St. Louis, Mo.), 0.0083 M phosphate, and 0.05 M acetate. The buffers were prepared by the method of Gomori (156), diluted, and the pH examined and adjusted with one of the stock solutions if necessary. After electrophoresis the strips were air dried and then lightly sprayed with 5mM

formyl-L-kynurenine solution. The strips were again allowed to dry at room temperature, and the papers were examined under ultraviolet light (Mineralight model R-51, Ultraviolet Products, Inc., San Gabriel, Calif.). The bright blue fluorescence of kynurenine identified the location of the enzyme. Protein localization was done using Beckman protein dye B-1 and fixative B-2.

B. Column Chromatography

Columns prepared by Mr. Gunther Weiss, glassblower, of the University of Oregon Medical School, had the following dimensions: 0.9 x 20 cm, 1.9 x 20 cm, and 1.9 x 40 cm, and were equipped with a standard female taper. A 3-foot extension tube with a male standard taper was added to the top of the column when the bed was to be poured. The bottom of most columns was fitted with a teflon plug to support the bed, and a ball joint which permitted the column to be attached to Beckman connectors, allowing insertion of 1/8 in polyethylene tubing. This tubing carried the eluents from the column to the fraction collector (Gilson G. M. E. Fractionator model VL, Middleton, Wisc.). The columns were designated to reduce mixing as much as possible. Another set of columns, used for preliminary experiments only, did allow for considerably more mixing. All but the most exploratory operations in column chromatography were carried out in a 4 C cold room, unless otherwise specified.

Columns were packed in the usual manner. The column was first partially filled with buffer, a thin layer of spun glass gently placed on the bottom of the column, and a half-inch layer of 3 mm glass beads carefully layered over the spun glass. After the extension tube was

fitted into place, buffer was added to within several inches of the top of the tube, and allowed to slowly pass through the column. The resin or supporting medium was then added at the top of the tube in the form of a homogenous slurry in buffer. This slurry was allowed to slowly settle through the length of the extension tube and column, while more was added as needed at the top. All of the Sephadex or kieselguhr had to be added in a thick slurry in a single step; both settled so rapidly as to significantly decrease the rate of flow, so that more of the slurry could not be added without leading to density differences. After the supporting medium had settled, the extension tube was removed. The column bed was washed overnight with buffer to allow it to settle and the flow rate to stabilize. The next day the flow rate was calculated. If it was not constant, the column was washed further.

The sample was then applied to the top of the column, and the elution procedure started. Fractions were generally collected at timed intervals, 15 to 45 min/tube, although some early experiments were done using volume collections. A Beckman Accu-Flo pump was used to obtain constant flow rates with DEAE cellulose or kieselguhr. Flow rates were within the range of 4 - 20 ml per hr, depending on the type of supporting medium and column size.

1. DEAE cellulose chromatography. Diethylaminoethyl (DEAE) cellulose (Selectacel type 40, Schleicher & Schuell, Keene, N. H.) was prepared by batchwise washing a sample of DEAE cellulose in several volumes of 1 M K_2HPO_4 pH 9, until the effluent was colorless. The resin was then filtered on a Buchner funnel fitted with Whatman #5 filter paper. Fines were removed in the wash treatment and also during

the following two steps. The resin was then washed extensively on the filter with distilled water, and with several volumes of 0.003 M potassium phosphate buffer pH 7.0. The resin was brought into suspension in the buffer and adjusted to pH 7.0 with HCl. This was the stock suspension of DEAE cellulose and stored in the cold room as such. When the DEAE cellulose was to be used in a different buffer system, the amount of resin to be used was extensively washed with the new buffer until the pH of the effluent was that of the buffer. Further equilibration of the resin with the buffer occurred when the poured column was washed with the buffer.

A set of preliminary experiments were done with DEAE cellulose in phosphate buffer, using batchwise increases of KCl to elute the enzyme. The enzymes could be recovered from the resin in such a manner. The same lot of resin was used in all experiments.

Elution of the columns poured with DEAE cellulose was done, in all major experiments, with a linear gradient of NaCl in 0.01 M Tris-HCl, at pH 8.0 produced with a mixing chamber consisting of two equal cylinders (100 or 500 ml) attached in sequence through a stopcock (157). For the gradient, equal volumes of buffer containing NaCl were added to each cylinder. The near cylinder contained the most dilute NaCl solution and was the mixing chamber. It was clamped over a magnetic mixer and was stirred vigorously with a stirring bar. The far cylinder contained the solution at the highest salt concentration of the gradient. The elution procedure was as follows: The enzyme was added to the top of the bed of the column, and then the column was washed, usually with 100 - 200 ml of buffer containing the salt concentration at the low end

of the gradient, to wash off contaminating proteins. The gradient was started by adjusting the levels of buffer in the cylinders to the desired height and by opening the stopcock between the cylinders, allowing the buffer to be pumped through the column. Approximately 200 - 250 ml of buffer were used in the gradient. The column was finally washed with the high salt concentration buffer for a total of 100 - 150 ml. Detailed descriptions of the individual experiments are contained in the Results section.

2. Sephadex chromatography. Suspensions of Sephadex G-75, G-100, and G-200 beads (Pharmacia, Uppsala, Sweden) were prepared by adding 20 g of each to 500 - 600 ml of 0.01 M Tris-HCl pH 7.2, and stirring for several days. Columns were poured by the one-step method, described above. Tris buffer (0.01 M, pH 7.2) was used to elute the columns.

Because of the fragility of the beads, a pump was not employed to maintain a constant flow rate, lest the beads be shattered and pack down, giving a progressively lower flow rate. Instead a reservoir with a constant pressure head was used. The reservoir consisted of a large separatory funnel. An unplugged Pasteur pipette was inserted through a one-hole stopper into the funnel so that the small opening of the pipette was about 1 cm above the stopcock. As the funnel was now open to the air only through the end of the pipette, there was constant pressure exerted by the buffer in the flask at all levels, provided that the buffer level was maintained above the end of the pipette. The distance from the opening of the pipette to the outlet from the column never exceeded 20 cm.

3. Ammonium sulfate/kieselguhr. Johns Manville Hyflo Supercel was the supporting medium for another type of chromatographic procedure (158), based on the salting out of proteins by high salt concentrations (159), and the reversal by lower salt concentrations. The kieselguhr was washed with 4 M HCl, and then washed extensively with distilled water. During the washing procedures about half the kieselguhr was removed as fines. The kieselguhr was then washed several times with 55% saturated ammonium sulfate and a suspension in 55% saturated ammonium sulfate was used to pour the column. There were occasional small aggregated particles in the slurry even after the extensive washing procedures; they did not, however, settle at significantly faster rates. With the aid of the pump the columns were washed with 55% saturated ammonium sulfate overnight. The material packed quite tightly; the flow rate was slow even with the pump.

The columns were charged with 0.55 ml (first experiment) or 0.6 ml (second experiment) of an enzyme preparation that had been precipitated by 55% ammonium sulfate and taken up in 0.1 M phosphate buffer, pH 7.0. The columns were eluted, after a preliminary wash with 55% saturated ammonium sulfate, with a linear gradient of decreasing ammonium sulfate concentration, from 55% to 35% (first experiment) or 55% to 20% (second experiment). For the first experiment the column was then washed with more 35% ammonium sulfate. In filling the cylinders for the gradient, equal hydrostatic pressure, rather than equal heights of fluid was used to achieve the linear gradient. While mixing experiments with dilute buffers would easily produce linear gradients, these concentrated ammonium solutions might be more of a problem due to differing

hydrostatic pressures and inadequate mixing. A procedure for determination of ammonium ions (160) was examined in order to test the linearity of the gradient; however it proved far too sensitive to be reliable in this case. The gradients were therefore in all cases assumed to be linear.

Protein concentrations were determined by analysis of absorption at 260 and 280 mμ, with a Beckman DU spectrophotometer. The concentration of protein was calculated from these values using a nomograph with protein and nucleic acid determinations (161). The high concentration of ammonium sulfate in these samples resulted in precipitation in the usual protein assay. On occasion this technique was used in other chromatographic experiments.

As a control to determine the denaturation of the enzyme due to long exposure at 4 C to ammonium sulfate, conditions present during the fractionation, a 0.1 ml sample of the same enzyme preparation applied to the column was diluted with 0.9 ml of 35% ammonium sulfate, and held in the cold room during the entire fractionation procedure. This control was assayed with the fractions eluted from the column to determine the extent of denaturation.

4. PABA Cellex

Cellex PABA (BioRad, Richmond, Calif.) is composed of celluose fibers charged with p-aminobenzyl side chains. These side chains may be diazotized and coupled to aromatic compounds. This new substituted cellulose may be a very specific selective agent in the fractionation of certain proteins. This procedure was used with some success in purifying antibodies (162,163), where a hapten was diazotized to the resin.

For formamidase fractionation, kynurenine, the product of the enzyme, was coupled to the column.

The diazotization procedure was adapted from the procedures described by Campbell (163) and Bio-Rad. Ten grams of Cellex PAB (0.14 meg/g) were added to a mixture of 20 ml 2N HCl and 40 ml distilled water in a beaker. The beaker was placed in an ice bath, and 75 ml 0.5 M sodium nitrate was added with constant stirring. After the mixture was stirred for another hour in the cold, the resin was collected on a Whatman #5 filter paper in a Buchner funnel. It was then washed with 100 ml of the following solutions in succession: 5% sodium acetate, 5% urea, and distilled water. The washed material was then suspended in 200 ml distilled water to which had been added 0.5 g DL-kynurenine sulfate, and sufficient KOH to bring the pH to 8.7. The reaction mixture was then stirred 40 hr in the cold room after the pH was adjusted to 7.0, the suspension was again filtered onto filter paper and washed with several volumes of distilled water.

The last step of the procedure involved blocking the remaining diazo groups with β-naphthol. One gram of β-naphthol was first dissolved in 5 - 10 ml 2N NaOH, diluted somewhat with distilled water and the pH adjusted to 8.0. It was brought to 1 liter with distilled water. The resin was stirred with 10 volumes of the β-naphthol solution for 30 min and then washed extensively with distilled water. A small column was poured with the resin, and charged with 0.2 ml of an enzyme fraction purified by ammonium sulfate. Fractions from the column were eluted with 0.01 M phosphate buffer pH 7.0. Unfortunately the results did not warrant further experimentation with this technique.

GENETIC STUDIES ON TRYPTOPHAN AND 5-HYDROXYTRYPTOPHAN DEGRADATION

A. Search for Mutants Lacking Tryptophan Oxygenase or Formamidase

Mutants lacking the tryptophan-induced formamidase or tryptophan oxygenase were to be isolated and examined to determine whether the 5-hydroxytryptophan-induced enzyme were present. This would demonstrate whether the two always occurred together or were independent. In addition the role of tryptophan as inducer would be examined, i.e. whether tryptophan or kynurenine would induce the remaining formamidase or oxygenase.

1. Mutagenic agents. The cells prepared for mutagenesis treatment were grown as described previously. A sample of the cells was diluted and plated on nutrient agar to determine the viable count before treatment with the mutagen. After treatment, the cells were washed if necessary and a sample used for estimation of the viable count. The assumption was that killing and mutagenic effect parallel one another. The bulk of the cells exposed to the mutagen was treated in one of three ways. 1) Cells were plated on either nutrient or basal-glutamate agar and examined for mutants by replica plating to basal-tryptophan agar. For this technique, plates with 30 - 100 colonies each were used. 2) In other experiments the cells were treated with a selective agent capable of killing dividing, but not resting cells. Under growth conditions with L-tryptophan as sole carbon source, the selective agent should kill only those bacteria able to use L-tryptophan as a carbon source, i.e. wild-type cells, thus permitting selective survival of mutants with defects in the degradative pathway. Experiments with penicillin and streptomycin as selective agents are described in a later

section. 3) For still other experiments the cells were plated on a differential medium, where the colonies of mutant and wild-type organisms could be distinguished from one another. Experiments were performed with basal medium containing a high concentration of L-tryptophan and a very low concentration of another growth substance, L-glutamate. Those cells able to use tryptophan should produce large colonies, the others, small ones.

All solutions used in these experiments were sterilized before use, if not otherwise stated, by autoclaving at 121 C for 10 min.

a) Ultraviolet light. Mutagenesis with ultraviolet light was used for most of the experiments, as it is a well-characterized mutagen, inducing a high frequence of deletions. The Mineralight lamp was allowed to warm up 15 - 30 min before irradiation. A petri dish with 10, or sometimes 20 ml of saline-suspended cells was placed directly under the lamp, at a distance of 16 - 20 cm. The lid was removed, and the dish gently swirled under the lamp for 15 to 20 sec. A 0.1 ml sample of cells was diluted and plated on nutrient agar to determine killing effect. Another sample of cells was either plated directly after irradiation or treated with penicillin or streptomycin and then plated. Plates were promptly placed in a dark 30 C incubator to reduce photoreactivation, and incubated for 2 days.

b) Ethyl methane sulfonate. Ethyl methane sulfonate or EMS (red label, Eastman Organic Chemicals, Rochester, N. Y.), was used as described by Hegeman (77). The 10 ml sample of cells in saline was centrifuged and taken up in 2 ml of saline. This sample of cells was

added to a solution of EMS for a final dilution of EMS from 1:80 to 1:200 in saline. The EMS solution was not sterilized before use. The cells were shaken for 1 hr at 30 C and either washed in saline or treated with thiosulfate. The treated cells were, in some cases, treated with a selection agent. After this treatment, or after treatment with EMS, they were plated for viability and mutagenesis.

Thiosulfate treatment consisted of adding a neutralized 5% solution of sodium thiosulfate sterilized by Millipore filtration to the EMS-treated cells to inactivate the remaining EMS. The cells were centrifuged down and suspended in saline.

c) Manganous chloride. The same procedure used to show that manganous ions are mutagenic for both E. coli (164) and pseudomonads (165) was followed in this experiment. The saline-suspended cells were centrifuged and resuspended in 10 ml of 0.3 M NaCl and shaken at 30 C for one hr. The cells were again centrifuged, resuspended in 10 ml of 0.04% $MnCl_2 \cdot 4H_2O$ and shaken at 30 C for 30 min. The cells were then centrifuged, washed, and diluted in saline. On occasion a sample was treated with a selection procedure. The cells were plated for viability and mutagenesis.

d) Nitrosoguanidine (MNNG). N-methyl-N-nitroso-N'-nitroguanidine (K & K, Hollywood, Calif.) was used as a mutagenic agent by a modification of the procedure used by Hollaway (166) on pseudomonads. The MNNG was prepared in a neutralized solution at 4 mg/ml, which was sterilized by Millipore filtration. The cells were suspended in 8 ml of 0.2 M acetate buffer, pH 5.0, and 2.0 ml of the MNNG solution

was added. The suspension was shaken for 30 min at 30 C. The cells were centrifuged, washed once, and suspended in saline, diluted, and plated for viability and mutagenesis.

e) 5-Bromouracil (5BU). A neutralized solution of 5BU (K & K Laboratories, Long Island, N. Y.) in L-glutamate was prepared by dissolving 12.5 mg 5BU in 250 ml of 1% L-glutamate. It was sterilized by Millipore filtration. Cells were resuspended in 8 ml of 4/5 basal medium. To this was added 1.0 ml of 1% glutamate and 1.0 ml of 5BU solution described above. The cell suspension was shaken at 30 C and samples removed at 1, 2, and 4 hr for viability and mutagenesis determinations. Although this reagent is not known to be mutagenic for bacteria, its incorporation into DNA might produce some mutagenic action. The concentration used will allow incorporation into DNA of thymine-requiring E. coli (167).

f) Hydroxylamine. Hydroxylamine has been shown to be mutagenic for E. coli (168), and various eukaryotic cells. The 10 ml sample of bacteria was suspended in 5 ml of saline. To 4.5 ml of bacterial suspension was added 0.5 ml of 0.1 M hydroxylamine HCl solution, which had been neutralized and Millipore filtered. The cells were incubated at 30 C for 60 min in this solution. The treated cells were then centrifuged and washed with saline. They were resuspended in saline, diluted, and plated in the usual manner.

2. Selection procedures. Two selective agents were used, penicillin (169) and streptomycin (170). If conditions could be adjusted so that these agents killed tryptophan-metabolizing cells, the proportion of mutants should increase several hundred fold.

The concentration of penicillin lethal for the isolate had to be determined as most of the published procedures using penicillin selection are for the more sensitive E. coli strains. The organism was grown for 48 hr in basal medium plus tryptophan with two-fold dilutions of penicillin (Potassium Penicillin G for injection, Pfizer, New York, N. Y.), and then diluted and plated for viability counts. It took a concentration of at least 50,000 units penicillin/ml to grossly interfere with growth.

The penicillin selection was usually done in three cycles. The culture was treated with a mutagenic agent, washed, suspended in basal medium with no added carbon source, and incubated for 6 hr. After this treatment it was washed and incubated in basal medium plus 0.1% tryptophan and 50,000 units/ml penicillin for 2 days. It was then washed and inoculated into basal 0.1% glucose until turbid. This culture was washed and resuspended in basal medium plus 0.1% tryptophan and 50,000 units per ml penicillin for 2 days more. After the second treatment the cells were washed and resuspended in basal medium plus 0.1% anthranilate. After 1-2 days the cells were washed and suspended in basal plus 0.1% tryptophan with 50,000 units/ml penicillin for the last two-day cycle. The cells were washed, grown briefly in basal medium with 0.1% glucose and plated. Plates were examined for mutants by replica plating or by the use of a differential medium.

A single experiment was carried out with streptomycin selection using the same conditions (with the substitution of streptomycin for dihydrostreptomycin) reported to successfully select against the wild-type growth response in another pseudomonad (170). A 5 ml suspension of

of cells in saline (5×10^9 cells/ml) was treated with hydroxylamine as described earlier. After treatment with the mutagen the cultures were washed and inoculated into 15 ml growth medium, either nutrient broth or basal medium with 0.1% L-glutamate. The cultures were shaken at 30 C for 6 hr, to allow segregation of nucleic acid and depletion of amino acid pools. Then 10 ml samples were centrifuged at 3,000 g for 15 min. After the cells were suspended in 10 ml sterile saline, a 0.5 ml of each sample of HA-treated cells was inoculated into 25 ml of basal 0.1% L-tryptophan medium. The cultures were shaken at 30 C for 2 hr. One mg streptomycin (Streptomycin sulfate, U.S.P., Squibb, New York, N. Y.) was added to each flask (400 µg/ml medium) and the cultures shaken for another 7 hr. At the end of this time the cultures were centrifuged, resuspended in saline, diluted, and plated for mutants.

B. Interstrain Genetic Studies

A differential medium (171) (see Table 2) was used to isolate pseudomonads. The medium contained L-arginine as main carbon source and the indicator Cresol Red which is red in acid and blue in base. The medium takes advantage of a characteristic alkaline reaction of pseudomonads on breakdown of L-arginine; the pseudomonad colonies are surrounded by a purple halo. The antibiotics select against many other microbes. Plates are poured with 15 ml apiece. Several soil samples were grown aerobically in nutrient broth, and then diluted and plated on the medium. From these a number of isolates were obtained. Other isolates had been obtained previously from soil samples which were inoculated into nutrient broth, and then plated on nutrient agar. Isolates were tested for failure to produce acid and gas from glucose, and for ability to produce

TABLE 2

Pseudomonas Isolation Medium (171)

Constituent	g/l	Constituent	g/l
NH_4NO_3	5.0	Cresol Red	0.020
$MgSO_4 \cdot 7H_2O$	1.0	yeast extract	0.025
$MnCl_2 \cdot 4H_2O$	0.2	Oxoid Ionagar 2	8.5
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.1	distilled water	1000
$CaCl_2 \cdot H_2O$	0.015	chloramphenicol	0.0025
$Na_2HPO_4 \cdot 7H_2O$	0.8	erythromycin	0.005
L-arginine HCl	10.0		

All components but the antibiotics are dissolved in distilled water, the pH adjusted to 7.2 with NaOH, and the volume brought to 1 liter. After the medium has been autoclaved and allowed to cool, the sterile antibiotics are added.

pigment on King B medium (172). The isolates were streaked out a number of times to insure that the cultures were pure.

1. Study to find a transducing phage. A transducing phage, carrying genetic information from isolate 3311 to either a mutant of 3311 or to another pseudomonad strain was sought. The joint transduction of genes for tryptophan-induced enzymes could then be tested. In order to find any phage, it is necessary to have an indicator strain, i.e. a bacterial strain senstive to the phage; the usual procedure for detecting phage is to mix a cell-free culture fluid with a culture of an indicator strain and plate the infected cells on a lawn of the indicator strain. After incubation, plaques are seen if there were phage in the culture fluid. The approach consisted of attempting to find an indicator strain for phage produced by isolate 3311. Isolate 3311 was also used as an indicator strain to assay supernatant fluid from the other isolates. To validate the assay procedure, all supernates were tested with all isolates; the presence of any phage plaques would indicate that the technique allowed visualization of plaques.

Phage tests were performed by the method described by Warner (173). It consisted of pouring agar plates, letting them dry, then pouring an overlay of 5 ml agar medium with 5 drops of an overnight culture of bacteria. In other samples of overnight cultures, the cells were centrifuged down, and the cell-free supernatant fluids, heated at 56 C for 30 min, were spread on the surface. Because some 33 isolates were to be tested one with another, a process which would require over a thousand plates, a modification was used in which the plates were divided into labeled sections, and one drop of supernatant solution applied to each

section. By this method 11 isolates were tested per plate.

The basic medium, termed PYG, used in this experiment consisted of Bactopeptone 1.5%, yeast extract 0.3%, and glycerol 2%. It afforded luxuriant growth of all cultures. The 33 isolates, including 3311, were each inoculated into about 20 ml PYG and shaken overnight at 30 C. The cells were removed by centrifugation, and the supernatants placed in labeled screw-capped tubes. The supernatant solutions were heated to destroy bacteria, and when they had cooled, a drop of chloroform was added to each tube. The treated supernatant solutions were stored under refrigeration until used.

For the overlay, each isolate was inoculated into 5 ml of PYG, and grown with shaking at 30 C overnight. For each isolate three plates of PYG hardened with 1.5% Bacto-Agar had been prepared and allowed to dry overnight. The day of the experiment, the overlay, comprised of PYG supplemented with 1% Bacto-Agar and the overnight culture, was poured over the surface of the PYG plates. Each isolate was represented by an overlay on three plates. The plates were dried at 37 C for 1 - 2 hr. Then the 33 supernatant solutions were applied to the surface of the plates so that each supernate was tested on the overlay of each isolate. The plates were allowed to remain undisturbed overnight at room temperature and were examined the following day for plaques. They were reexamined 1 day later.

2. Nutritional studies on Isolate 3311 and other pseudomonad strains. Nutritional studies were done for two reasons. Firstly, the results from the phage tests suggested that 3311 was not closely related to any of the other soil isolates. The nutritional study would serve to

confirm this. Secondly, if isolate 3311 could be classified as a result of such tests, e.g. as a P. putida strain, closely related pseudomonads could be obtained from other laboratories and transducing phages sought from these related strains.

An investigation was therefore made into the compounds - amino acids, tricarboxylic acid cycle intermediates and sugars - which could be used as sole energy source by these isolates. The compounds used were primarily those used in the classification scheme for pseudomonads proposed by Stanier, Palleroni and Doudoroff (174). The isolates were replica plated, testing about 7 isolates in each plate. Each isolate, except 3311 was tested one time with each carbon source; 3311 was present on all plates and was therefore tested in quintuplicate. Basal medium unsupplemented with yeast extract used as carbon sources 0.1% acids or DL-amino acids, and 0.1 - 0.15% sugars. As usual, carbon sources were autoclaved separately from the 4/5 volume basal medium.

Tests were done in two parts. The first test consisted of examining a series of "critical" compounds, those which more clearly differentiate the major groups. These included galactose, glycine, trehalose, inositol, glucose, lactose, sucrose, valine, p-aminobenzoate, betaine, maleate and tartrate. Also included were King B plates for demonstration of fluorescin production, and control plates in which no carbon source had been added to the basal medium for determination of residual growth. There were too many different plates for one set of replica plates. The technique works best if there are only 6 - 8 plates replicated. So the isolates were first patched (seven per plate including 3311) on 5 initial template plates of nutrient agar. After the

patches grew 24 hr, each plate was replica plated to four other nutrient agar plates each. Thus each isolate was represented in four plates. These four nutrient agar plates were used as the templates in the testing procedure, each plate being replicated on to about four different carbon sources. The plates were read daily for 4 days after growth at 30 C.

Some of the isolates were eliminated from the second round of tests mainly because the preliminary test served to group them. Although some isolates were selected for further study from these groups, most of the isolates were taken from the ungrouped organisms. The next set of compounds to be tested included citrulline, ornithine, arginine, glutamate, serine, alanine, threonine, leucine, isoleucine, aspartate, malate, and fumarate. These were done in a similar manner with four master replicas.

Several metabolites were eliminated before the final analysis. These were compounds unable to serve as carbon source for any of the pseudomonad isolates, and included norvaline, p-aminobenzoate, tartrate, lactose, maltose, and maleate. It is possible some of these compounds had decomposed or produced toxic substances during autoclaving. However, several of the compounds, including maltose, maleate and tartrate were used by only a few of the pseudomonads studied by Stanier and coworkers (174).

In addition to the replica plating experiments, the isolates were tested for the ability to liquify gelatin, by stabbing each isolate into a standard gelatin tube. Tubes were examined daily for 4 days. Isolate 3311 was tested for accumulation of fat granules by Burdon (175) by staining cells from a 24 hr nutrient broth culture.

ENZYME INDUCTION STUDIES

Induction studies were carried out to determine the degree of similarity in the kinetics of induction of the first three enzymes of the tryptophan and 5-hydroxytryptophan pathways, and to provide information as to whether the control of degradation was the same for both enzyme systems.

A. Effect of Inducer Concentration

In one set of experiments, the concentration of the inducer, DL-5-hydroxytryptophan or DL-kynurenine (Sigma Chemical Co.) was varied, with a constant induction time of 3 hr. As kynurenine was an effective inducer, it was used for tryptophan. Tryptophan is a less efficient inducer as it is withdrawn for protein synthesis. Studies on the properties of the kynurenine-induced formamidase show it is similar to the tryptophan enzyme.

B. Effect of Induction Time

In the second set of experiments, the inducer concentration in the medium was held constant at 1 mM for DL-5-hydroxytryptophan or kynurenine or 5 mM for L-tryptophan. The length of time of incubation with these inducers was varied from 1 to 24 hr.

Cells were induced during growth on plates of basal medium with 0.1% L-glutamate and the inducer. The inoculum had been grown overnight on basal-glutamate medium, as described previously, and plated on the induction medium. The cells were harvested, washed and sonicated. The cell-free supernatant solution was assayed for tryptophan oxygenase, formamidase, kynureninase, and protein content. The graphs presented in

a later section represent composite data from a number of experiments, as only four samples were generally run simultaneously.

COMPARATIVE STUDIES WITH DENATURING AGENTS

A comparative study was made on the effects of heat treatment and of a variety of enzyme inhibitors on the tryptophan- and 5-hydroxytryptophan-induced formamidases. These experiments were designed to demonstrate whether the responses of the formamidases were strikingly different from one another. Any such differences would be expected to provide information on the nature of these effects. Some of the inhibitors tested were used by Kotake (42) on rabbit liver formamidase activity. A comparison is presented of the bacterial enzymes with the published results of the mammalian enzyme.

The K_m values of the formamidase for formyl-L-kynurenine were determined to discover whether the enzymes have the same affinity for the substrate. A Lineweaver-Burk plot of inhibition by $HgCl_2$ for each enzyme was also determined to ascertain whether the same mechanism of inhibition occurred with both enzymes.

Several of the intermediates in the tryptophan degradative pathway, notably alanine and formyl-L-kynurenine, were studied to determine whether they exerted some control over the degradation of either tryptophan or 5-hydroxytryptophan through influence on the formamidase reaction. Alanine was tested as a repressor of the tryptophan-induced formamidase. Alanine and another important intermediate, anthranilate, were tested for ability to inhibit either formamidase activity. In addition, the ability of these three intermediates to protect either formamidase activity against inactivation was also tested.

All experiments unless expressly stated in the text were repeated one or more times with essentially the same results.

A. Reagents Used in Inhibition Studies

All reagents unless otherwise specified were used without further purification. Iodoacetate (which was recrystallized from benzene), N-ethylmaleimide (NEM. B grade), L-cysteine, anthranilic acid, DL-alanine, urea, dithiothreitol (DTT) and reduced glutathione (GSH) were purchased from Nutritional Biochemicals Corp. Other reagents used in these studies were $HgCl_2$ (reagent grade. Merck & Co., Rahway, N. J.); pCMB, 8-hydroxyquinoline, and 2,3-dimercaptopropanol (BAL) (K & K Laboratories Inc.); and (ethylenedinitrilo)-tetraacetic acid (EDTA. Eastman Organic Chemicals), L-alanine (pfs Sigma), potassium arsenite (C.P., Coleman & Bell Co., East Rutherford, N. J.), and potassium fluoride (reagent grade. Mallinckrodt, St. Louis, Mo.).

The NEM was purchased shortly before the experiments and stored in the freezer. All solutions, with the exception of $HgCl_2$, pCMB, and DTT were routinely prepared the day of the experiment. After the DTT solution was prepared, aliquots were frozen for later use. To facilitate solution of the pCMB, it was dissolved in about 500 ml of distilled water to which had been added several drops of 20% KOH. When the mercurial was dissolved, more water was added, the solution neutralized, and made to one liter. All solutions were neutralized before use.

B. Techniques Used in the Inactivation Studies

1. Heat inactivation. For the study of heat inactivation of the formamidases, the crude preparations were warmed in waterbaths set

at 36, 45 or 56 C for a defined period of time and then assayed. The tubes were first warmed in the waterbath, and if any solution was to be incubated with the enzyme preparation, it too was warmed. The enzyme preparation was gently added to the tubes, incubated for the set time, and then cooled. In the initial studies on heat inactivation where a small sample had been heated, the cooling was done by withdrawing a 0.1 - 0.2 ml sample of enzyme from the tube and diluting it into the cuvette which contained semicarbazide, buffer and water at ambient temperature. After the 5 min incubation period the enzyme was assayed in the usual manner. As the sample size in the later studies of protection by various compounds against heat inactivation reached 0.4 ml, this method of cooling was not used. Instead, the tube was withdrawn from the waterbath and plunged into an ice-water bath. Within a few minutes, sample was withdrawn and incubated with semicarbazide as above. Within any experiment, both formamidases were treated in the same manner.

A study of the effect of salts on the heat inactivation of the tryptophan- and 5-hydroxytryptophan-induced formamidases was performed. The tryptophan-induced enzyme was heated at 56 C for 60 min, while the 5-hydroxytryptophan enzyme was heated at 36 C for 8 min. This constituted the standard heating procedure for the enzymes. Each enzyme was heated after dilution in an equal part of one of the following solutions: distilled water; 0.25 M, 0.5 M or 1.0 M K_2HPO_4 pH 7; 1 M Na_2HPO_4 pH 7; 0.5 M K_2SO_4 pH 7; and 1.0 M NaCl and KCl. The enzymes were either "heated in salt" or "heated in water." The rates of the heated enzymes were compared to the unheated enzyme and an evaluation of the effect of the salt on heating was determined. There were two necessary controls. The

effect of the added salt in the assay mixture was tested with both the native (unheated enzyme) and the heated (denatured) enzyme. In order to do this the assay mixture was supplemented with the concentration of either 1.0 M K_2HPO_4 or NaCl maximally present in the assay mixture of the enzyme heated with salt.

2. Studies with inhibitors. A single experiment was done to determine whether the responses of the tryptophan- and 5-hydroxytryptophan-induced formamidases were similar to those reported by Kotake (42). The inhibitors at 2 mM were incubated with either enzyme for 5 min, the enzyme was assayed, and the amount of inhibition determined. The assays were done in duplicate and are reported as the percent inhibition, giving the mean and the standard deviation. The 8-hydroxyquinoline was dissolved in a small volume of alcohol, and water was added to bring the solution to volume. Each enzyme was tested for inactivation due to the presence of alcohol during the preincubation and assay procedure. The activity of the enzyme incubated with alcohol was used as the 100% value for the enzyme incubated with 8-hydroxyquinoline.

Experiments were performed with pCMB, $HgCl_2$, NEM, urea, and iodoacetate to determine the extent of inactivation of each enzyme with these reagents. For most of these studies, the enzyme was incubated 5 min with semicarbazide, buffer, and inhibitor and then assayed. Preliminary studies with urea, $HgCl_2$, pCMB and NEM revealed that for periods less than 5 min, the reaction rate varied greatly with time, but the difference between the rates of the reaction for enzyme incubated with inhibitor at 5 or 10 min was relatively small, 10 - 20%. The 5 min rate was also chosen to minimize thermal denaturation of the 5-hydroxytryptophan-

induced formamidases.

The reversability of mercurial treatment was examined for both formamidases by treatment with a mercurial for 5 min followed by the addition of a sulfhydryl reagent, such as GSH; after 5 or 10 min further incubation the enzyme was assayed and the activity compared to the mercury-treated enzyme. In a preliminary set of experiments, each of the formamidases was incubated with the sulfhydryl reagent, usually at 1m$\underline{M}$, for 5 or 10 min to determine the effect of the sulfhydryl reagent on the enzyme activity. The sulfhydryl reagents investigated were GSH, DTT, and BAL.

The K_m values of both the tryptophan- and 5-hydroxytryptophan-induced formamidases for formyl-L-kynurenine were determined by assaying the enzymes with substrate concentrations ranging from $5 \times 10^{-4}\underline{M}$ (standard concentration) to $2.5 \times 10^{-5}\underline{M}$. The usual preincubation period of 5 min was maintained.

The kinetics of the $HgCl_2$ inhibition of the tryptophan-induced formamidase were significant to warrant further studies with this reagent. The type of inhibition, i.e. competitive, noncompetitive or uncompetitive, of this agent for each formamidase was investigated by studying the activity of the enzyme assayed with different substrate concentrations in the presence of $HgCl_2$, usually at 10 $\mu\underline{M}$. The inhibition of the tryptophan-induced formamidase by NEM (0.2 m$\underline{M}$) was studied in a similar manner.

In order to determine whether $HgCl_2$ and NEM affected the same sites on the tryptophan-induced formamidase molecule, a binding study was done. The enzyme was incubated in a variety of $HgCl_2$ concentrations, ranging from 2 - 20 $\mu\underline{M}$, for 5 min. One set of samples was incubated 10 min

longer and assayed to determine the $HgCl_2$ inhibition curve. NEM, to a final concentration of 1 mM, was added to the second set of samples, and the incubation allowed to proceed for 10 min and assayed. Thus it was possible to determine the inactivation at any given concentration of $HgCl_2$ produced as a result of the $HgCl_2$ treatment, and the inactivation as a result of the added NEM. The amount of inhibition of NEM alone was determined for 1 mM, NEM in an incubation period of 10 min. The experiment was not done with the 5-hydroxytryptophan-induced enzyme as the high concentrations of NEM that would be needed to effect any substantial inhibition would interfer with the enzymatic assay.

The ability of alanine to repress formamidase synthesis was studied in two experiments. In the first experiment the inducers were either 10^{-3}M DL kynurenine or 8.6×10^{-4}M DL 5-hydroxytryptophan. Each inducer was used with either 10^{-3}M DL alanine or 10^{-3}M succinate in basal agar. There was, in addition, 0.1% L-glutamate in each sample to promote growth. The cells were harvested after 3 hr and the specific activities of formamidase, kynureninase and tryptophan oxygenase determined. In the second experiment, the effect of alanine on the tryptophan-induced formamidase was studied, using a variety of alanine concentrations. If alanine represses, there should be more repression the more alanine is added. Six sets of plates were used. All sets had 10 mg/100 ml basal agar-L-tryptophan. The first set had in addition 100 mg DL alanine; the second, 100 mg of L-glutamate; and the third, 100 mg L-glutamate and 100 mg DL alanine. The other plates had higher concentrations of alanine, up to 2,000 mg/100 ml. An overnight culture of isolate 3311 on basal medium with 0.1% L-glutamate was inoculated on the plates, allowed

to incubate 3 hr, and harvested. The specific activities of the formamidase enzymes were determined.

Both alanine and anthranilate were tested for inhibitory action on the formamidase reaction. Each formamidase enzyme was incubated 5 min with or without 1 mM concentration of either anthranilate or L-alanine, and the activities were determined.

Binding of these compounds to the formamidase enzymes was examined by testing their ability to protect the enzyme against inhibitory agents. Both were tested with pCMB inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases. The enzyme was incubated with 5 or 10 μM pCMB for 5 min with or without 1 mM L-alanine or anthranilate. Then it was assayed. A control was done by incubating the enzyme with the mercurial alone for 5 min, adding 1 mM alanine or anthranilate, and immediately adding the substrate. The activity of this latter reaction was compared to that where the compound was present during treatment with the mercurial to determine whether there was any protection. The ability of anthranilate and alanine to protect the 5-hydroxytryptophan-induced enzyme from $HgCl_2$ inhibition (4×10^{-6} M $HgCl_2$) was also examined in the same manner. The binding of formyl-L-kynurenine to both formamidases as a protective mechanism was also tested. These experiments involved comparing the activities of the enzyme which had been exposed to a known concentration of substrate during the inhibition treatment to the enzyme exposed only to inhibitor, with the extra substrate added together with the standard amount of substrate. In a study of the ability of the substrate to protect either enzyme to $HgCl_2$ inactivation, the enzyme was incubated with 10 μM (tryptophan-induced enzyme) or 3 or

4 μM (5-hydroxytryptophan-induced enzyme) $HgCl_2$ for 5 min with the addition of 0.1 ml of 0.005 M formyl-L-kynurenine. At the end of the incubation period, the standard substrate concentration was added, and the reaction followed.

A second set of protection experiments with the substrate involved heat inactivation. For tryptophan-induced enzyme the heat treatment was 20 min at 56 C, while for the 5-hydroxytryptophan enzyme this was 8 min at 36 C. The concentration of substrate used with either enzyme for protection was 2.5×10^{-4}M. The rate of reaction of the enzyme heated with substrate was compared to that heated with water, but with the same final concentration of buffer in the assay system. The controls were difficult to design, because of the enzymatic and possible thermal decomposition of the substrate both during the heat treatment and during the preincubation period. The controls consisted of assaying heated versus unheated substrate, the enzyme (either heated or unheated) with the concentration of substrate used in the protection studies added at the time of assay or before the 5 min preincubation period, and the unheated enzyme assayed with the standard plus the protective substrate.

RESULTS

GROWTH PROPERTIES OF THE ORGANISM

The growth rate of isolate 3311 in basal medium plus 5 x 10^{-3}M L-tryptophan, DL-5-hydroxytryptophan and L-glutamate has been followed by examining the turbidity of the media. The results are found in Table 3. The cells grow 1.3 times as slowly on 5-hydroxytryptophan and 3.5 times as slowly on tryptophan as glutamate. All compounds are capable of supporting logarithmic growth.

STUDIES ON PURIFICATION OF THE TRYPTOPHAN-INDUCED FORMAMIDASE

A. Preliminary Fractionation Steps

The results of six experiments on preliminary fractionation in the purification of the tryptophan-induced formamidase are presented in Table 4. The cells were harvested from different volumes of yeast extract-supplemented basal agar plus different inducer concentrations ranging from 0.1 to 0.2% L-tryptophan. These differences in consequent amount of cells and concentration of inducer likely account for the differences in mg amounts and specific activities of the starting material. The characteristic features of these preliminary steps are as follows: 1) About 20% enzyme activity is lost after treatment with protamine sulfate coupled with a similar loss in protein, often producing a slight increase in specific activity. 2) About 10% of this enzyme activity precipitates at 35% saturation of ammonium sulfate. 3) The protein is split about evenly between the 35 and 55% saturated ammonium sulfate with three times the enzyme activity precipitating in the 55% ammonium sulfate to produce three-fold purification. Only an insignificant enzymatic activity remained in the 55% saturated ammonium sulfate fluid.

TABLE 3

Growth of Isolate 3311 on Basal Medium and Tryptophan, 5-Hydroxy-tryptophan, or Glutamate

Time (hr)	Optical density of cells grown with		
	Glutamate	Tryptophan	5--Hydroxytryptophan
0	0.02	0.01	0.02
0.5	0.03	0.01	0.02
1.0	0.02	0.02	0.02
1.5	0.03	0.02	0.03
2.0	0.02	0.02	0.02
2.5	0.04	0.02	0.02
3.0	0.04	0.02	0.02
3.5	0.05	0.02	0.03
4.0	0.06	0.02	0.02
4.5	0.06	0.02	0.03
5.0	0.09	0.03	0.04
7.0	0.24	0.03	0.04
7.5	0.31	0.03	0.06
8.0	0.38	0.04	0.06
8.5	0.46	0.04	0.06
9.0	0.54	0.05	0.08
9.5	0.64	0.04	0.10
10.0	0.75	0.04	0.11
10.5	0.88	0.05	0.13
11.0	1.04	0.05	0.16
11.5	1.28	0.06	0.19
22.5	0.84	0.25	0.45

The cells were grown overnight on basal medium with 0.1% L-glutomate and then transfered to 250 ml of basal supplemented with 5×10^{-3} M L-glutamate, L-tryptophan, or DL-5-hydroxytryptophan. At half hour intervals 10 ml of culture fluid was withdrawn from each flask and the optical density (turbidity) at 520 mμ was measured.

TABLE 4

Preliminary Fractionation Steps of the Tryptophan-induced Formamidase

Fraction	Expt no.	Mg. protein/ml	Total ml	Total mg protein	Specific activity	Total activity	%Activity recovered
Crude	1	23.2	21.5	499	659	328,481	
	2	15.0	17.5	263	161	42,342	
	3	11.1	21.0	231	430	99,330	
	4	15.4	22.0	339	336	113,904	
	5	31.6	35.0	1092	361	394,212	
	6	11.2	49.8	558	1156	645,048	
Protamine supernate	1	10.6	22.0	234			
	2	10.4	17.8	185	154	28,490	67
	3	10.0	23.5	235	436	102,460	103
	4	11.8	22.0	260	323	83,980	74
	5	14.0	37.0	518	633	327,894	83
	6	10.2	52.0	530	1103	584,590	89
35% Ammonium sulfate precipitate	1	12.1	4.5	54	49	2,646	8
	2	19.8	2.0	40	26	1,041	2
	3	23.2	2.9	67	276	18,492	19
	4	25.6	2.0	51	256	13,056	11
	5	28.4	4.0	113	161	18,193	5
	6	16.0	14.5	232	439	101,848	16
55% Ammonium sulfate precipitate	1*	25.4	4.8	122	763	93,086	28
	2	12.2	3.0	37	257	9,406	22
	3	23.6	2.0	47	1276	59,737	60
	4	23.6	2.0	47	997	46,859	41
	5	24.0	4.0	96	1409	135,264	34
	6	18.3	2.4	44	2012	88,528	14

* A 50% saturated ammonium sulfate solution was employed in experiment 1.

B. Paper Electrophoresis of Bacterial and Guinea Pig Formamidases

The results of paper electrophoresis of the tryptophan-induced bacterial formamidase and a partially purified guinea pig liver formamidase are reported in Table 5. The enzyme from guinea pig has an isoelectric point between 8.0 and 8.6. The small migration at 8.0 suggests that the isoelectric point is nearer to 8.0 than 8.6, perhaps 8.2. The bacterial enzyme has an acid isoelectric point, near pH 5.6.

In some cases it was not possible to localize the enzymatic activity. This is probably due to the long duration of electrophoresis, although it might reflect a lessened activity of the enzyme in phosphate buffer. No determinations were made of the comparative activity of the enzymes in various buffers.

CHROMATOGRAPHIC BEHAVIOR OF THE TRYPTOPHAN-INDUCED FORMAMIDASE

The purification procedure was to be established with the tryptophan-induced formamidase, as the 5-hydroxytryptophan-induced formamidase was highly labile. The labile enzyme could then be examined under conditions optimal for purification of the tryptophan-induced enzyme.

The chromatography was to employ conventional approaches, consisting of a combination of an ion exchange resin, either DEAE cellulose or Amberlite, used in conjunction with Sephadex filtration. In addition an adsorbent like hydroxyapatite was considered for use. However, before these techniques were refined, two nonconventional approaches were tried. The first approach described in this section is an elution of an enzyme-charged kieselguhr column with a decreasing ammonium sulfate gradient, starting with the concentration precipitating the protein down to a concentration where the protein will dissolve, and presumably

TABLE 5

Migration of Bacterial and Guinea Pig Liver Formamidases on Paper Electrophoresis

Buffer	pH	Hours electro-phoresis	Migration* (cm)	
			Bacterial enzyme range	Guinea pig enzyme range
Tris HCl	8.6	6	-3.4 to -4.0 -3.0 to -4.0 -2.5 to -3.3 -3.4 to -4.0	-0.5 to -3.2
	8.0	6	-0.1 to -1.6 +0.6 to -1.0	+3.5 to -0.9 +1.5 to +0.4
	7.0	3	0 to -2.0 +0.3 to -1.7	+2.4 to -0.5
Phosphate	7.0	17	not detectable	+8.3 to +1.5
	6.7	17	not detectable	+8.5 to +3.4
Acetate	5.6	5.25	+0.5 to -0.6 +0.5 to -1.0	not determined
	5.6	6	+0.6 to -0.4 +0.3 to -0.3 +0.5 to -0.4	not determined

* The - indicates migration to the cathode side and the + indicates migration to the anode side.

be eluted off the column. The results are described below in section A.

The second approach was one of the earliest studies made of the enzyme. Kynurenine was diazotized to a cellulose resin by way of p-aminobenzyl groups on the cellulose. The formamidase and kynureninase enzymes alone should have affinity for these new groups and be retained on the column, to be eluted off with a high salt concentration in the eluent. The results of this experiment are described in Section B.

DEAE cellulose was chosen for ion exchange chromatography. The results of studies with this resin are contained in Section C.

Section D contains the results of experiments on filtration of the enzyme several sizes of Sephadex beads. A discussion of the behavior of the enzyme relative to the void volume is also contained in this section.

A. Kieselguhr-Ammonium Sulfate Columns

1. First fractionation. The column bed measured 1.9 x 17.5 cm with a flow rate of 6.5 ml/hr. The column was charged with 0.55 ml of an ammonium sulfate-fractionated enzyme with 18.3 mg protein/ml and a specific activity of 567. (In all cases the material which was applied to all the columns had been fractionated by precipitation with 50 or 55% ammonium sulfate first unless otherwise stated.) The gradient used 130 ml each of 35% and 55% saturated ammonium sulfate. Finally the column was washed with 40 ml of 35% saturated ammonium sulfate. Very little protein was eluted from the column; it was present only in tubes 26 - 30 and represented only 11% of the protein applied to the column. Any other protein eluted off was present in minute quantities.

There was no formamidase activity in any of the fractions collected from the column. The enzyme was not affected too adversely by the incubation at 4 C in an ammonium sulfate solution, as the control with enzyme diluted 1:10 in 35% saturated ammonium sulfate and held in the cold room for the duration of the elution retained 70% of the activity.

2. Second fractionation. In an attempt to elute the enzyme off the kieselguhr, the ammonium sulfate gradient was adjusted to a range of 55 to 20% saturation. The gradient was started with 210 ml of 55% saturated ammonium sulfate and 250 ml of 20%. The flow rate was 12.7 ml/hr, with 0.6 ml of the same enzyme preparation used in the previous study. Again there was only one protein peak in tubes 25 - 33, and no enzyme activity. A cold room control diluted in 55% saturated ammonium sulfate had 60% activity when it was diluted and assayed.

The results indicate that either the enzyme was bound irreversibly to the kieselguhr, or was denatured by it. This procedure was therefore changed for more gentle ones.

B. Kynurenine-PABA Cellex

The column bed measured 0.9 x 9.5 cm and the flow rate was 8.3 ml/hr. The column was charged with 0.2 ml of a preparation with 24 mg protein/ml and a specific activity of 318. The effluent was collected in volumes of 5 ml/tube. The elution was performed at room temperature, which would facilitate chemical interactions between the enzyme preparation and the resin. The results are outlined in Table 6. There was little increase in specific activity of the fractions eluted; the highest (tube 3) had a specific activity of 355. Recovery was 37% for

TABLE 6

Elution of the Tryptophan-Induced Formamidase from a Kynurenine PABA Cellex Column

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity
1	130		nil	
2	100		nil	
3	240		125	355
4	100		31	211
5	60		8	91
6-10		850	nil	
11-15		325	nil	
16-20		575	nil	
21-25		2100	nil	
26		90	nil	

The specific activity of the material applied to the column was 318. The rate is expressed as optical density $x10^{3}$ $minutes^{-1}$. Nil indicates that the rate was less than 2.

the enzyme activity and 159% for the protein (measured by absorption at 260 and 280 mμ). Apparently, 1) kynurenine is being eluted off the column absorbing at 280 to produce the high protein values and 2) the enzyme was not retained by the column, or if a fraction of it were retained, it had not been eluted off by tube 26. In view of the poor results with this preliminary run, attention was focused on more conventional methods of column chromatography.

C. DEAE Cellulose

1. Preliminary DEAE cellulose runs. Preliminary runs were done with stepwise additions of increasing concentrations of salt, either KCl or NaCl. In the first two experiments the enzyme preparation was that soluble in 55% but precipitated by 70% ammonium sulfate. In the first experiment with 0.01 M phosphate buffer pH 8, and linear salt increments of 0.048 M, the enzyme was eluted in to tubes 1 through 4 (0.048 M to 1.46 M KCl). There was no increase in specific activity since the input specific activity was 168, and that of tubes 1 and 3 was 108 and 119. A total of 19% of the activity and 52% of the protein were recovered from the column. The other preliminary KCl runs were done with additions of 0.070 M KCl in 0.01 M phosphate buffer. In one experiment the same enzyme preparation was passed through DEAE cellulose equilibrated at pH values 6.3, 8.0 and 9.0, and the proteins and enzymes assayed. The activity in all three cases was eluted in tubes 4 - 7 (0.21 - 0.35 M KCl) with maximum purifications of 1.5 fold (tube 4) at pH 6.3, 1.6 fold (tube 5) at pH 8.0 and 1.6 fold (tube 4) at pH 9.0. The purification is defined as the ratio of specific activity of the

fraction to that of the input. A 20 - 26% recovery of enzyme activity was achieved in all these columns. In a final experiment with phosphate buffer the input activity was quite low and there was no increase in specific activity. The enzyme was eluted in tubes 5 - 6 (pH 7) and 6 - 8 (pH 8). The recovery of enzyme activity was 27 and 26% in accord with previous experiments. All these preliminary experiments were done at room temperature with very small columns and fairly large samples of proteins, but they suggested that the enzyme could be eluted off a column with a salt gradient, that the gradient ought to be in the neighborhood of 0.1 to 1.0 M and that larger column beds would prove useful.

The last preliminary approach was to examine the behavior of the enzyme on a DEAE cellulose resin equilibrated with 0.01 M Tris-HCl buffer pH 8, with a three-step gradient of 100 ml of Tris-HCl without NaCl, 100 ml Tris-HCl with 0.1 M NaCl, and 100 ml of Tris-HCl with 1.0 M NaCl. Ten ml volumes were collected. The enzyme was found in tubes 21 - 23, with a 1.6 fold purification in tubes 21 and 23. The recovery of activity was 34% and the protein was 23% in those three tubes. Thus the enzyme was eluted off in the start of the 1 M NaCl step. A repeat experiment unaccountably showed a broader spread of activity from tubes 15 to 24, with maximum purification of 4.3 fold (tube 17), 3.8 fold (tube 18) and 2.1 fold (tube 21). The enzyme appeared to be eluted at the end of the 0.1 M step and the beginning of the 1 M as well.

The remaining experiments were done using linear salt gradients with Tris-HCl buffer 0.01 M pH 8 in the cold room with 1.9 X 20 cm (approximately) bed dimensions, and an input of 0.5 to 1.0 ml of

fractionated enzyme preparation.

2. DEAE cellulose with a gradient from 0.1 to 1.0 M NaCl. The first experiment with a linear gradient used 0.1 M NaCl to 1.0 M NaCl in Tris-HCl buffer, as the results of the preliminary experiments suggested that this range would elute the enzyme off the column. In an experiment using this gradient, the column bed measured 1.9 x 22 cm and the flow rate was 4.01 ± 0.03 ml/tube. Tubes were collected every 15 min. The column was charged with 1.0 ml of enzyme preparation with 27.6 mg protein/ml and a specific activity of 436. The column was first washed with 0.1 M NaCl in buffer and a gradient with 111 ml of 0.1 M and 140 ml of 0.1 M was then run (starting with tube 25). The results of this experiment are presented in Table 7.

The enzyme was eluted from the column in two peaks, one at tubes 16 - 19, in the 0.1 M wash solution, and the other in tubes 44 - 54, midway through the gradient. In both cases one of the tubes had a specific activity greater than the input, 2.5 fold higher in the early peak and 1.3 fold in the later peak. The majority of the protein eluted appeared in the first peak (41% of the protein applied to the column), although there were lower peaks at tubes 55, 65, and 71. In all 55% of the protein and 17% of the enzyme were eluted off the column.

The low yield of enzyme was not solely due to denaturation in the cold room buffer. A control diluted 1:10 in 0.01 M Tris-HCl buffer and 0.01 M NaCl and retained at 4 C for the duration of the run had a specific activity 75% that of the enzyme stored in the freezer.

As the bulk of the enzyme was eluted off the column in the 0.1 M NaCl wash, the next fractionation attempt used a gradient of 0.01 - 0.1

TABLE 7

Pattern of Elution of the Tryptophan-Induced Formamidase on DEAE Cellulose with a Linear Salt Gradient of 0.1M to 1.0M NaCl.

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity	Purification ratio
1-10		24	nil		
11	5		nil		
12	33		nil		
13	91		nil		
14	366		nil		
15	320		nil		
16	604		148	173	
17	256		258	686	
18	50		81	1091	2.5
19	75		45	409	
20	72		nil		
21-30		223	nil		
31-40		168	nil		
41	3		nil		
42	13		nil		
43	17		nil		
44	22		16	496	
45	25		17	450	
46	33		18	372	
47	16		14	596	1.3
48	42		16	260	
49	58		26	305	
50	98		32	223	
51	68		15	150	
52	77		24	208	
53	116		27	155	
54	98		19	132	
55	84		nil		
56-75		720	nil		

The specific activity of the material applied to the column was 436. The first eight tubes were not tested. The rate is expressed as optical density x 10^3 minutes^{-1} and the purification ratio is the ratio of specific activity of the sample to that of the preparation applied to the column. Nil indicates that the rate was less than 2.

M NaCl. The rationale for this was that as the enzyme lagged somewhat behind the protein peak, the two might be better separated on a dilute salt gradient.

3. DEAE cellulose with 0.01 to 0.1 M NaCl. The column bed measured 1.9 x 21.5 cm and the flow rate was 13.8 ml/hr; 3.45 ml were collected per tube. The input consisted of 1.0 ml with 27.6 mg protein/ml and a specific activity of 464. The column was washed with 158 ml of 0.01 M Tris-HCl with 0.01 M NaCl. Then a gradient was run with 140 ml each 0.01 M NaCl and 0.1 M NaCl in buffer (starting at tube 43) for 255 ml, and washed with 0.1 M NaCl (Tubes 132-152). The specific activity of the cold room control for this longer experiment was only 28% that of the input. The results of this experiment are presented in Table 8.

There was no increase in specific activity over the input in any of the samples. The enzyme was again eluted in two peaks, in tubes 15 - 17 (0.01 M wash) and tubes 151 - 152 (0.1 M) The recovery was 69% for protein and only 2.6% for enzyme activity.

In an attempt to free the enzyme from the other proteins, a double gradient was tried.

4. DEAE cellulose with 0.01 to 0.1 and 0.1 to 1.0 M NaCl gradients. The column bed measured 1.9 x 19.5 cm and the flow rate was 13.8 ml/hr; 3.45 ml were collected per tube. To the column was added 1.0 ml of enzyme preparation with 27 mg protein/ml and a specific activity of 207. The column was washed with 150 ml of 0.01 M NaCl in buffer, and a gradient started at tube 42 with 100 ml each of 0.01 M

TABLE 8

Pattern of Elution of the Tryptophan-induced Formamidase on DEAE Cellulose with a Linear Salt Gradient from 0.0M to 0.1M NaCl.

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity
1-10		604	nil	
11	30		nil	
12	25		nil	
13	97		nil	
14	227		nil	
15	624		8	8.9
16	534		35	45
17	448		15	22
18	72		nil	
19	30		nil	
20	51		nil	
21-40		1570	nil	
41-60		1270	nil	
61-80		640	nil	
81-100		700	nil	
101-120		1490	nil	
121-140		1100	nil	
141-149		69	nil	
150	108		nil	
151	336		55	112
152	782		29	25

The specific activity of the material applied to the column was 464. The rate is expressed as optical density x 10^3 minutes^{-1}. Nil indicates that the rate was less than 2.

and 0.1 M NaCl in Tris-HCl buffer; this ran for 180 ml. At tube 83 the second gradient with 80 ml of 0.1 M and 75 ml of 1.0 M NaCl in Tris buffer was initiated. The column was washed from tubes 123 - 155 with 1 M NaCl in Tris buffer. Tubes were screened for enzymatic activity by incubating 0.5 ml samples from each tube with 2 ml of buffer, 0.3 ml of semicarbazide and 0.2 ml of 0.005 M formyl-L-kynurenine for 60 - 90 min. Those tubes with any heightened absorption at 360 mμ were assayed for enzyme activity by the conventional manner. The results are described in Table 9.

There was no purification. Again, the enzyme was eluted early in the preliminary wash (tubes 14 and 15) and late in the gradient between 0.1 M and 1 M salt. The maximum specific activity was only about 25% of the input specific activity. The recoveries were 85% of protein and 2.8% of activity.

The gradients with low salt concentrations did not seem to elute much activity off the column. In addition the activity was found in two peaks. The consistent decrease in recovery of the enzyme with decreasing salt concentrations suggested that more success might be found with higher salt concentrations. An experiment was done with a gradient from 1 - 5 M NaCl in Tris-HCl.

5. DEAE cellulose with a 1.0 to 5.0 M NaCl gradient. The column bed measured 1.9 x 16 cm and the flow rate was 18.68 ± 0.04 ml/hr with 4.67 ml/tube. To the column was applied 0.5 ml of an enzyme preparation with 35.5 mg protein/ml and a specific activity of 79.8. The column was washed with 200 ml of 1.0 M NaCl and then a gradient with

TABLE 9

Pattern of Elution of the Tryptophan-Induced Formamidase on DEAE Cellulose with Linear Salt Gradients from 0.01-0.1 and 0.1-1.0 M NaCl.

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity
1-10		28	nil	
11	0		nil	
12	114		nil	
13	282		nil	
14	684		22.6	22
15	984		26	
16	110		nil	
17	19		nil	
18	18		nil	
19	0		nil	
20	18		nil	
21-40		176	nil	
41-60		552	nil	
61-80		462	nil	
81-100		932	nil	
101-110		538	nil	
111	24		nil	
112	60		nil	
113	127		nil	
114	145		nil	
115	236		16	
116	237		nil	
117	276		nil	
118	217		nil	
119	228		nil	
120	214		nil	
121-140		3560	nil	
141-154		3060	nil	

The specific activity of the material applied to the column was 207. The rate is expressed as optical density x 10^3 minutes^{-1}. Nil indicates that the rate was less than 2.

100 ml of 5 M and 115 ml of 1 M NaCl was run. The data are presented in Table 10.

There was only one protein peak which appeared in tubes 8 - 10 of the wash, and this had enzymatic activity. The purification ratios were 1.3, 3.2 and 5.5 (tube 10). The recovery of enzyme activity was 69% and of protein 106%. Thus this procedure was successful in increasing purification of the enzyme.

An examination of the data from the studies with DEAE cellulose chromatography reveals that the technique was successful when high salt concentrations were used to elute the enzyme off the column. Thus in one preliminary experiment with 3 steps of NaCl concentrations, the protein was eluted off early in the 1 M NaCl step with 36% yield of enzyme. Similarly in the early 1 M wash of the last experiment there was a 69% recovery of enzymatic action. The recovery is higher than the earlier experiment, probably because the enzyme was in contact with the resin and incubated at 4 C for a much shorter period of time.

Experiments with lower salt concentrations uniformly produced two peaks of enzyme activity, one always occurring from tubes 14 - 19 and the other variable, from tube 50 (mid-way between a 0.1 - 1.0 M salt gradient) to tube 150 (in a 0.1 M wash). This latter peak is obviously responding to changes in salt concentration; it is the early peak that must be explained. It is either due to a property of the enzyme or a characteristic of the experimental procedure.

Allosteric proteins are often unstable in low salt concentrations and dissociate into subunits. However, a decline of inactivity 25 - 75% in an enzyme preparation incubated in Tris HCl 0.01 M NaCl for 2 days at

TABLE 10

Elution Pattern of the Tryptophan-Induced Formamidase on DEAE Cellulose with a Linear Salt Gradient from 1M to 5M NaCl.

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity	Purification ratio
1-5		40	nil		
6	0		nil		
7	32		nil		
8	1040		156	103	1.3
9	980		350	256	3.2
10	168		109	440	5.5
11	25		n.d.		
12	14		n.d.		
13	16		n.d.		
14	44		n.d.		
15	0		n.d.		
16-25		322	n.d.		
26-35		327	n.d.		
36-80	32		n.d.		

The specific activity of the material applied to the column was 79.8. The rate is expressed as optical density x 10^3 $minute^{-1}$ and the purification ratio is the ratio of specific activity of the sample to that of the preparation applied to the column. Nil indicates that the rate was less than 2 and n.d. that the rate was not determined.

4 C is not an unusually high amount of denaturation. Thus the behavior of the enzyme in a low salt concentration does not suggest inactivation or dissociation.

Dilution of allosteric proteins also causes dissociation into subunits. It is possible that some protective protein may be diluted also. However such a phenomenon might more logically affect later peaks in the chromatographic procedure as the dilution of the enzyme would take time. Such a pattern would reveal "smearing" due to the progressive splitting of the protein into subunits. The characteristic considered here is a sharp peak, occurring quite early in the elution pattern.

This property might be an artifact related to the preparation of the sample used for fractionation. After the enzyme preparation was precipitated by 55% saturated ammonium sulfate and centrifuged, the supernatant fluid was carefully decanted and the tube inverted in the refrigerator to allow excess fluid to drain off. The inside of the tube was blotted with a Celluwipe and buffer was added to suspend the fraction. This fraction was used without dialysis or desalting treatment. It is possible that the ammonium sulfate was still present to a significant extent, altering the elution pattern of a fraction of the enzyme. That would explain the lack of effect of high salt concentrations in producing two peaks. In the preliminary fractions either the second peak was missing because the columns were somewhat overloaded, or perhaps the enzyme preparation had less ammonium sulfate remaining because of better blotting of the excess fluid.

For whatever reason, the protein is eluted in two peaks in low salt concentrations. It can be eluted in one peak with a significant

retention of activity and increase in specific activity in a 1.0 M solution of NaCl in buffer applied directly to the column.

D. Sephadex Chromatography

1. G-75 beads. The column was 1.9 x 28 cm, the flow rate was in the range of 7 - 10 ml/hr; 5 ml samples were collected. The input consisted of 1.0 ml of an enzyme preparation with 17.5 mg protein/ml and a specific activity of 373. The column was eluted with 0.01 M Tris buffer pH 7.2. The results of this fractionation procedure are presented in Table 11.

The enzyme was eluted in tubes 3 - 7 with a maximum increase of specific activity of 6.6 fold. The recovery of protein was 38% and of enzyme 66%. Since the enzyme activity appeared after the second tube, it started to appear in 10 - 15 ml of effluent.

2. G-100 beads. The column was 1.9 x 10.5 cm, and 5 ml samples were collected. The input consisted of 0.7 ml of a preparation with 23.6 mg protein/ml and a specific activity of 328. Due to an error in the collection procedure, only the first three tubes could be analyzed quantitatively. The enzyme was eluted starting with tube 2, 0.27 mg protein/ml and a specific activity of 52. Tube three had 0.53 mg protein/ml and a specific activity of 162. Later fractions apparently had specific activities higher than the input. The enzyme was initially eluted in 5 - 10 ml of effluent.

3. G-200 beads. This bead size was used for two experiments. The same column was used for both experiments, with a long wash of buffer

TABLE 11

Elution of the Tryptophan-Induced Formamidase from a Sephadex G-75 Column.

Tube	µg Protein per ml	Total µg protein	Rate per ml	Specific activity	Purification ratio
1	19		nil		
2	18		nil		
3	323		23	71	
4	310		504	1626	4.4
5	102		252	2471	6.6
6	80		2.7	34	
7	40		4.1	103	
8	20		nil		
9	16		nil		
10	0		nil		
11	15		nil		
12	8		nil		
13-37		4960			

The specific activity of the material applied to the column was 373. The rate is expressed as optical density x 10^3 minutes^{-1} and the purification ratio is the specific activity of the sample to that of the preparation applied to the column. Nil indicates that the rate was less than 2.

between experiments. The column had a bed size of 0.9 x 11 cm with a flow rate of 4.67 ml/hr.

In the first experiment, fractions were collected every 45 min (3.50 ml/tube). A 0.1 ml sample of enzyme with 24 mg protein/ml and a specific activity of 653 was applied to the column. The results (together with those of experiment 2) are to be found in Table 12. The enzyme was eluted from the column into tubes 3, 4, and 5 with no increase in specific activity. The recovery of enzyme activity was 38% and that of protein was 123%. The low amount of protein applied to the column made an estimate of total protein highly inaccurate. The enzyme began to be eluted off the column between 7.0 and 10.5 ml of eluent.

Experiment 2 was a repeat of the same experiment, except that samples were collected every 15 min, in order to refine the behavior of the enzyme relative to the void volume. The same input was used. The results are also found in Table 12. The enzyme was eluted in tubes 6 - 10 with no increase in specific activity. The enzyme was initially eluted in 5.9 to 7.0 ml of eluent. There was 43% recovery of enzyme activity.

4. Void volume calculations. According to the manufacturer, Sephadex beads have the following characteristics:

Bead Size	Fractionation Range, mol. wt.	Volume per gm bed	outer	inner
G-75	3,000 - 20,000	13 ml	5 ml	7 ml
G-100	4,000 - 150,000	17 ml	6 ml	10 ml
G-200	5,000 - 800,000	30 ml	9 ml	20 ml

By calculation of the volume of Sephadex by the dimensions of the bed

TABLE 12

Elution of the Tryptophan-Induced Formamidase from Sephadex G-200 Columns.

	Experiment 1			Experiment 2			
Tube	ug Protein per ml	Rate per ml	Specific activity	ug Protein per ml	Total ug protein	Rate per ml	Specific activity
1	1	nil		0		nil	
2	142	nil		30		nil	
3	485	195	274	10		nil	
4	215	143	453	150		nil	
5	33	11	232	290		nil	
6	0	n.d.		430		83	132
7	0	n.d.		390		233	408
8	0	n.d.		420		250	460
9	0	n.d.		360		127	242
10	0	n.d.		160		50	313
11	0	n.d.		0		29	high
12	0	n.d.		910		57	44
13	0	n.d.		100		nil	
14	0	n.d.		490		nil	
15	0	n.d.		0		nil	
16	0	n.d.		230			
17	0	n.d.		0			
18	0	n.d.		17			
19	0	n.d.		95			
20	0	n.d.		0			
21-30	0	n.d.			2900*		
31	0	n.d.		213			
32	0	n.d.		119			
33	0	n.d.		40			

The specific activity of the preparation applied to the column in both experiments was 650. The rates are expressed as optical density x 10^3 minutes^{-1}. Nil indicates that the rate was less than 2 and n.d. indicates that the rates were not determined. A zero value indicates that the protein measurement was 5 ug per ml or less.

* This figure reflects the inaccuracy of determining protein concentrations by measuring absorbance at 260 and 280 mu in samples containing little protein. For example the maximum absorption was at tube 26 with absorbancies at 260 and 280 mu of 0.085 and 0.066, respectively.

volume and multiplication by the ratio of the outer volume to bed volume, the outer or void volume of the beads may be determined. If the enzyme is eluted within this figure, it is not within the fractionation range of the beads.

The bed volume for the G-75 column was 64 ml. As the ratio of void volume to bed volume is 5/13, the void volume is 25 ml. The enzyme began to be eluted between 10 - 15 ml, indicating that it was within the void volume. Thus the molecular weight of the enzyme is in excess of 20,000.

A similar calculation for the G-100 column gives a bed volume of 24 ml and a void volume of 8.5 ml. The enzyme is initially eluted between 5 - 10 ml, suggesting that it might be retarded by the column somewhat. This might place the molecular weight toward the high end of the fractionation range.

The G-200 column had a void volume of 2.6 ml. The enzyme was eluted in 5.9-7.0 ml eluent, and was significantly retarded by the column. The enzyme has a molecular weight of 20,000 to perhaps 800,000; this range could be narrowed to 20,000 - 200,000, by the behavior on Sephadex G-100.

The results of the Sephadex chromatography indicate that a partial purification is achieved by passage through the G-75 column. Maximum purification achieved was 6.6 fold. This chromatographic separation could be coupled to the 5.5 fold purification in the 1 M NaCl-DEAE cellulose column and the 2 - 3 fold ammonium sulfate purification to achieve about 70 fold purification.

It would be possible to examine the behavior of the 5-hydroxytryptophan-induced enzyme in the same manner. However as most of the

protein in the tryptophan-induced formamidase preparation was eluted through the column with the enzyme, the chromatographic behavior of the 5-hydroxytryptophan-induced enzyme would not be significant unless this one protein behaved differently from the majority of the proteins. The G-75 column presents a similar picture. As the tryptophan-induced enzyme was not purified by any procedure involving separation from the bulk of the protein, a comparison of it and the 5-hydroxytryptophan-induced enzyme under the same conditions was not likely to prove fruitful.

On the other hand, a preparation purified 70 fold is certainly not pure enough to do any delicate physicochemical determinations, such as ultracentrifugation or fingerprinting. It could be used as an antigen for immunological studies, but as it is not pure, these studies would be subject to reasonable criticism.

One reasonable approach would be to investigate other means of purification, such as adsorbent gels, to aim for further purification. The purification studies were very time consuming, however, and once the enzyme was obtained in pure form it would take a fair amount of time to acquire skills for even simple measurements like ultracentrifugation and moving boundary electrophoresis. The more complicated fingerprinting probably could not be done in the time available. Thus the problem would be resolved to doing a limited number of measurements on one purified protein which would have no direct application to the properties of the 5-hydroxytryptophan-induced formamidase. Instead, a different approach was tried.

GENETIC STUDIES ON TRYPTOPHAN AND 5-HYDROXYTRYPTOPHAN DEGRADATION BY ISOLATE 3311

As the possibilities offered by the study on purification of the tryptophan-induced enzyme were so limited in scope, the problem was attacked on a different frontier. The difference between the formamidases was to be investigated by genetic techniques. The first approach was to obtain mutants of isolate 3311 unable to convert L-tryptophan into L-kynurenine. These mutants would be induced by mutagens known to be effective on pseudomonads and isolated either with the use of a selective agent such as penicillin or with a differential medium which would support only limited growth of the mutants. Mutants would be tested by replica plating for ability to grow on kynurenine but not tryptophan. They would then be examined to determine whether the block was in tryptophan oxygenase or formamidase. In either case the induction of the remaining enzyme with tryptophan and kynurenine would be tested to determine whether this system of tryptophan degradation was also induced only by kynurenine. In addition, these mutants would be tested for growth on 5-hydroxytryptophan. Thus it would be possible to determine whether the loss of the enzyme in the tryptophan-induced system obligatorily meant loss of the same enzyme in the 5-hydroxytryptophan system, which might be the case if the two activities were on the same protein. If the activities were on different proteins, the loss of one activity should not affect the other activity. With this distinction in mind, a study was initiated to produce and isolate mutants.

A. Mutagenesis Studies

1. Lethal effects of the mutagens. The lethal effect of treatment with various mutagens was determined, and used to quantitate treatment with ultraviolet light (UV) and some of the mutagens. The results of these experiments are found in Table 13. Treatment for 60 sec with UV usually resulted in about 99% lethality, while treatment with a 1:100 dilution of ethyl methane sulfonate (EMS) resulted in about 80%, if the action of the mutagen was stopped with thiosulfate. Extensive killing occurred if the thiosulfate was not added. Nitrosoguanidine, $MnCl_2$, and hydroxylamine did not produce much lethality, randing from 20 - 50% depending on the conditions used.

B. Selection Procedures

After either penicillin and streptomycin treatment, the bacteria were washed in sterile saline, diluted, and plated on nutrient agar. After 2 days incubation at 30 C, these plates were replica plated onto plates of 1) basal agar plus 0.1% L-tryptophan, 2) basal agar plus 0.1% L-glutamate, and sometimes 3) basal agar plus 0.08% DL-5-hydroxytryptophan. Yeast extract was in most cases not added to these media, to minimize growth due to utilization of it as carbon source. Any colony which did not replicate onto tryptophan or 5-hydroxytryptophan medium was saved for further testing.

The streptomycin selection procedure with hydroxylamine mutagenesis was not very successful. Out of 1206 colonies screened by replica plating, only one was saved for further testing. In contrast, in one experiment with penicillin selection of mutants generated by UV treatment, 25 out of 2361 colonies screened were saved for further testing.

TABLE 13

Viability of Isolate 3311 after Treatment with Various Mutagenic Agents

Mutagen	Initial count (viable cells/ml)	Final count (viable cells/ml)
Ultraviolet light		
15 sec	5.9×10^8	4.8×10^7
30 sec	5.9×10^8	1.1×10^7
45 sec	5.9×10^8	5.0×10^6
60 sec	5.9×10^8	4.5×10^5
Ethyl Methane Sulfonate (1:100)		
With Thiosulfate	3.3×10^9	6.2×10^8
Without Thiosulfate	3.3×10^9	$< 10^6$
Manganous Chloride	5.0×10^7	6.9×10^6
Nitrosoguanidine	4.9×10^8	4.0×10^8
Hydroxylamine	3.5×10^7	2.6×10^7
5-Bromouridine		
1 hr	2.1×10^9	3.2×10^9
2 hr	2.1×10^9	2.0×10^9
4 hr	2.1×10^9	1.8×10^8

The differential medium, with a high tryptophan and low glutamate content, was used for all later studies. It avoided the many changes of medium used in the penicillin technique, thus reducing chances of contamination. It also allowed preliminary screening on plates too crowded with colonies for replica plating (e.g. over 200 colonies/plate), and thus more colonies could be examined in a single experiment. Both concentrations of tryptophan (0.1 and 0.2%) appeared to work equally well, although the results with this technique were never quantitated. There was one slight disadvantage of the differential medium. Some of the small colonies picked for further testing proved to grow poorly on either tryptophan or glutamate. Apparently 'leaky' auxotrophic mutants are also selected.

C. Results of Preliminary Tests with the Mutagens

It is evident from Table 14 that certain mutagens are more effective than others. Manganous chloride apparently had an undetectable level of mutagenesis, while UV, EMS and hydroxylamine were effective, at least in these preliminary tests. Nitrosoguanidine is not listed in the table as it was used solely with differential medium, where the apparent mutagenesis rate was not determined.

D. Further Tests of Mutants

Mutants were tested and evaluated about every 10 days during this experimental section. The first test consisted of replica plating of colonies of eight presumed mutants isolated from UV and hydroxylamine treatment to tryptophan and glutamate basal agar. All isolates grew satisfactorily on tryptophan and were discarded. The second test was on

TABLE 14

Results of Penicillin Selection with Certain Mutagens

Mutagen tested	Colonies Examined	Colonies saved for further tests
UV 45 sec	2361	4
50 sec	204	3
60 sec	339	0
	58	1
Hydroxylamine	629	2
	384	0
$MnCl_2$	142	0
	932	0
EMS (1:100)	2054	7

19 mutants isolated from UV treatment and 3 from EMS. On testing, five of the UV isolates gave mixed growth on tryptophan, with some colonies growing and some not. Further experiments with one of these isolates showed that even when a colony which would not grow on tryptophan was grown and replated, it preserved its mixed growth. Six of the UV isolates did not grow well on tryptophan and were preserved for further study. The remainder of the isolates grew on tryptophan and were discarded. Four more tests were done on accumulated isolates, until a number of isolates which behaved like mutants were obtained. The behavior of this final set of mutants to further testing is presented in Table 15.

The results seen in Table 15 were not very reproducible. This may be due to changes in the bacterial population between the tests, or uncontrolled environmental conditions. The latter did occur, as replica plating of duplicate plates often demonstrated different growth responses to tryptophan. The most obvious variable is the amount of hydration in the agar of each plate, which is influenced by the temperature of the hot agar poured into the plate and the depth of the agar. This might account for the serious discrepancies between the second plating experiment on the mutants and the other two. However, this variability made the replica plating technique inadequate for these tests.

The mutants were tested in other ways as well. A special analysis in the region of 200 - 400 mμ was done of the supernatant fluid of these mutants incubated in basal broth with 0.1% L-tryptophan to see if the absorption spectrum of any intermediates could be recognized. No

TABLE 15

Behavior of the Final Group of Mutants to Several Replica Plating Experiments

Isolate	Experiment 1 Growth on:		Experiment 2 Growth on:		Experiment 3 Growth on:	
	Try	5Htry	Try	5Htry	Try	5Htry
1 (UV)	-	-	+	-	+	-
2 (EMS)	-	-	+	1t	+	-
3 (MNNG)	-	-	+	1t	+	-
4 (MNNG)	-	-	+	-	+,-	-
5 (EMS)	-	+	1t	+	-	+
6 (MNNG)	-	+	-	1t	-	-
7 (MNNG)	-	+	-	-	-	-
8 (MNNG)	-	+	-	+	-	+
9 (5BU)	-	-	-	+	-	-
10 (5BU)	-	-	-	+	+	+
11 (5BU)	-	-	1t	1t	-	-
15 (5BU)	-	-	-	+	+	+

+ signifies normal growth, - little if any growth, 1t an intermediate amount of growth, and two present signifies two types of colony growth. Try is tryptophan and 5Htry, 5-hydroxytryptophan.

characteristic spectrum was found. Several of the mutants (nos. 5 and 15) were examined for formamidase activity. The specific activity of this enzyme was within normal limits.

Although UV treatment was used for most of the studies as it was most likely to induce non-revertible mutations, it is evident from Table 15 that, in addition to nonreproducible effects seen mostly in replica plating 2, there is a reversion to some of the mutants to wild-type constitution. In fact the majority of nonstable isolates, which continually produce both mutant and wild-type colonies, were generated from UV treatment. The results seem to indicate that an organism can lose the ability to grow on 5-hydroxytryptophan but retain or regain ability to grow on tryptophan. Thus the abilities are at least partially distinct.

The revertability of the mutants as well as the problems encountered with the replica plating techniques made this particular approach not very successful. However at the same time a second approach was under way.

E. Soil Isolate Experiments

While the mutagenesis studies were under way, a study on the isolation of a transducing phage was in progress. If both formamidase activities are on one protein, then one gene might code for both formamidases. If they are on two proteins, then the genes would be distinct. These two possibilities could be distinguished by an examination of cotransduction of the two characteristics. If the two activities are invariably transduced together, it is likely that one gene codes for

both. If the two activities are separated on transduction, two genes are involved. Consequently a search was started for phage active on isolate 3311. This was done by isolating other soil pseudomonads and testing them for phage active on 3311.

1. Phage studies. The technique used for demonstration of phage showed that all of the other isolates did produce phage. However no phage were found active on isolate 3311, nor did 3311 release phage active on any other isolate (Table 16). In addition to the ability of all of the new isolates to produce phage, all but 11 were sensitive to at least one phage.

As these tests were done but once with a single concentration of phage preparation, there were probably undetected phage produced. However, considering all the negative results with 3311, and the fact that the "phage" preparation of 3311 was tested in triplicate for each isolate, it is quite likely that this organism neither produced phage infective for the soil isolates, nor was sensitive to the phage produced by them. It is not too likely that isolate 3311 produced a heat-labile phage. Heat treatment of phage preparations is standard technique in studies of pseudomonad phages (176,177). In one study where heat treatment was tested versus chloroform treatment, both revealed the same spectrum of phages (177).

Most pseudomonads are lysogenic for one or more phage (176,177, 178), and thus show cross reactions with other strains of the same species. Therefore, it is likely that isolate 3311 is unrelated to the other soil isolates. In order to test this hypothesis and further

TABLE 16

A Summary of the Phage-Producing Pseudomonad Isolates and their Indicator Strains.

I Phage-producing strains which have a single indicator strain	Indicator strain	II Phage-producing strains which have a variety of indicator strains	Common indicator strains used by most isolates	III Strains in II with additional indicator strains	Indicator strains
11-7	12-7	11-1, 11-2, 11-9	15-7	11-17	11-14, 11-21, 13-6, 16-2, 104
11-14, 15-5	13-5	11-17, 11-21 12-2	15-8		
11-19, 15-8, 15-11	15-12	12-7, 13-1, 13-2	15-10	15-2	11-7, 13-6, 16-2, 15-11, 104
15-7	15-14	13-5, 13-6, 13-7	15-12		
16-3	15-10	13-8, 13-9, 13-10	15-14	15-16	13-2, 13-5, 13-9, 15-1
16-12	15-1	15-1, 15-2, 15-10	16-3		
63	15-11	15-12, 15-15, 16-1, 16-2, 24, 104	15-16		

characterize 3311, the metabolic properties of 3311 and the other isolates were investigated. The knowledge of certain metabolic properties would aid in the classification of 3311, and allow other related strains to be obtained, which could be examined for phage. Consequently 3311 and the isolates were examined for growth on a number of different carbon sources.

2. Nutritional studies. There is a myriad of classification procedures for pseudomonads (174, 179, 180), ranging from the complex to very simple. The method chosen (174) employed primarily an examination of the carbon sources which could be used for growth. The reported classification is unusual in that it not only lists the carbon sources used by the various taxa, but also the proportion of positive strains to total strains for each carbon source. Thus the effectiveness of the carbon sources can be evaluated. A comparison of isolate 3311 to those isolates maintained in culture in Dr. Stanier's collection would be particularly useful in obtaining similar organisms.

The isolates were divided into pigmented or nonpigmented groups on the basis of King B medium (172), and are compiled in Tables 17 - 20. It may be seen that in contrast to the fluorescent pseudomonads, the nonpigmented pseudomonads are a heterogeneous group. No isolate resembles isolate 3311, nor does any isolate reported by Stanier. The properties of isolate 3311 are as follows. It grows well on glutamate, isoleucine, histidine, phenylalanine, tyrosine, tryptophan, and fumarate. It grows poorly on glucose, valine, alanine, leucine, and proline. It does not grow on inositol, trehalose, galactose, citrulline, ornithine,

TABLE 17

Some Nutritional Abilities of Fluorescent Pseudomonad Soil Isolates.

Isolate	Gelatin Liquefaction	Growth on: Glucose	Glycine	Galactose	Inositol	Trehalose	Sucrose	Betaine	Valine
15-5 15-12 15-8 11-21	-	+	+	-	-	-	-	+	+
16-2	-	+	+	-	-	-	-	-	-
15-1 15-16	-	+	-	-	-	-	-	+	+
104 63 15-10	-	+	+	+	-	-	-	+	+
11-19	-	+	-	+	-	+	+	+	+
13-9 13-10 13-2 13-5	+	s1	-	+	-	-	-	-	-
15-7 15-14 11-14	+	+	+	-	+	+	-	+	+
11-1	-	+	-	+	-	+	-	s1	+
11-17	-	+	+	-	-	-	-	+	+
16-3	-	s1	-	-	-	-	-	-	+
P. aeruginosa	+	+	+	+	-	-	-	+	+
P.fluorescens	+	+	+	-	+	+	+	+	+
P. putida	-	+	+	+	-	-	-	+	+

All isolates produced pigment on King B medium.

TABLE 18

Further Nutritional Abilities of Fluorescent Pseudomonad Soil Isolates.

Isolate	Pigmentation on King A	Growth on: Succinate	Ser	Phe	Asp	Ileu	Leu	Arg	Ornithine	Citrulline	His	Try
11-9	+	+	-	+	+	+	+	+	+	-	-	-
13-9	+	+	-	+	+	+	+	+	-	-	-	-
11-1	+	+	-	+	+	+	+	+	+	+	+	+
16-3	+	sl	-	-	-	-	-	+	-	-	-	-
13-1	sl	+	-	+	+	+	+	+	+	-	+	-
13-10	sl	+	-	-	sl	+	+	+	+	-	-	-
15-5	sl	+	-	+	-	+	+	+	+	+	-	-
15-4	sl	+	-	+	-	+	+	+	+	+	-	-
11-17	sl	+	+	+	-	+	-	+	+	+	+	-
11-21	sl	+	+	+	-	+	-	+	+	+	+	-
13-2	sl	+	-	-	-	+	sl	-	-	-	-	-
12-7	sl	-	+	+	+	+	+	+	-	+	+	+
63	sl	-	+	+	+	+	+	+	+	-	+	-
15-7	sl	-	+	-	-	+	+	+	+	-	+	-
104	sl	-	-	+	+	+	+	+	-	-	-	-
13-7	-	+	-	+	+	+	+	+	+	+	-	-
15-7	-	+	+	+	sl	+	+	+	+	+	-	-

All isolates grew on fumarate, all (but 13-2) grew somewhat on acetate, glutamate, proline, and alanine. No isolate grew on malate and threonine. Only 11-1 and 13-2 grew on citrate. Sl indicates that the growth was slight.

TABLE 19

Some Nutritional Abilities of Nonfluorescent Pseudomonad Soil Isolates.

Isolate	Gelatin Liquefaction	Growth on: Glucose	Glycine	Galactose	Inositol	Trehalose	Sucrose	Betaine	Valine
24	+	-	-	-	+	+	-	-	-
12-8	-	+	-	-	+	+	-	sl	sl
16-1	-	-	+	-	-	-	-	-	-
13-8	-	-	-	-	-	+	-	-	-
12-2	-	+	-	+	+	-	-	-	sl
15-11	-	-	-	-	+	+	-	-	-
12-7	-	+	+	+	+	-	-	-	-
15-1	-	sl	-	+	-	+	-	-	-
13-6	-	+	-	-	-	-	-	-	-
3311	-	sl	-	-	-	-	-	-	+

No isolate produced pigment on King B medium. Sl indicates slight growth.

TABLE 20

Further Nutritional Abilities of Nonfluorescent Pseudomonad Soil Isolates.

Isolate	Growth on:										
	Succinate	Acetate	Phe	His	Try	Asp	Ileu	Leu	Arg	Ornithine	Citrulline
12-8	+	+	+	+	+	sl	+	+	+	+	+
13-6	sl	sl	-	-	-	-	-	-	-	-	-
15-11	+	+	+	+	-	+	-	+	+	+	+
13-8	+	+	-	+	-	sl	-	-	-	-	-
16-1	-	+	-	sl	-	-	sl	+	-	-	-
24	-	-	sl	-	-	sl	+	+	+	+	+
3311	-	sl	+	+	+	-	+	sl	-	-	-

All isolates grew on fumarate and glutamate. In addition, 13-6 did not grow on proline, alanine (16-1 also negative), or tyrosine. The only isolate to grow on malate or citrate was 12-8; the only isolate to grow on serine was 13-8.

arginine, glycine, serine, threonine, aspartate, acetate, citrate, succinate, or malate. It does not hydrolyze gelatin or produce pigment. It is unable to reduce nitrate, and electron microscopy (181) shows that the cells, which characteristically occur in pairs, have polar flagella. Cells grown in nutrient broth display sudanophilic granules, suggestive of poly β-hydroxybutyrate accumulation.

The closest resemblance between isolate 3311 and any group reported by Stanier is to Pseudomonas acetovorans (Table 21). However 3311 is entirely deficient in metabolizing those acids characteristically metabolized by that organism. Eight of 28 characters differ. The resemblance is not at all close.

Since 3311 was not very similar to the other soil isolates or to the reported taxa, it was not possible to obtain similar strains for phage testing. The alternative was to isolate new classes of soil isolates by growth on some of the carbon sources metabolized by isolate 3311 to obtain similar strains. Thus tryptophan, phenylalanine or histidine enrichment of soil could be done. These new isolates could be tested for phage. But as the replica plating technique necessary to score transformants for tryptophan and 5-hydroxytryptophan utilization was not reliable, the problem might remain unresolved. In order to obtain significant answers to the differences between the enzymes in a reasonable length of time, a third, successful approach was initiated and completed as described below.

KINETIC ANALYSIS OF TRYPTOPHAN AND 5-HYDROXYTRYPTOPHAN DEGRADATIVE ENZYMES

TABLE 21

A Comparison of the Metabolic Properties of Isolate 3311 and *Pseudomonas acetovorans*.

Character	*P. acetovorans*	3311	Character	*P. acetovorans*	3311
Growth on:			Growth on:		
Galactose	-	-	D-tryptophan	+(v)	-
Inositol	v	-	Histidine	+	+
Trehalose	-	-	Proline	+	+
Glucose	-	+	Phenylalanine	+	+
Betaine	-	-	Tyrosine	+	+
Glycine	+	-	Acetate	+	+
Valine	-	+	Citrate	+	-
Arginine, Ornithine	-	-	Succinate	+	-
Glutamate	+	+	Malate	+	-
Serine	-	-	Fumarate	+	+
Alanine	+	+	Ability to:		
Leucine, Ileucine	+	+	Liquify gelatin	-	-
Aspartate	+	-	Denitrify	-	-
L-tryptophan	-	+			

Characters marked v vary from strain to strain. $\pm$ signifies poor growth.

An analysis was made of the kinetics of induction of the first three enzymes on the pathway induced by either tryptophan, 5-hydroxytryptophan or kynurenine; an analysis was made of the tryptophan- and 5-hydroxytryptophan-induced formamidases when exposed to heat or inactivating agents. A study was made also of the role of other metabolites in controlling the pathway of degradation with either compound as inducer.

A. Studies on Induction of Tryptophan Oxygenase, Formamidase and Kynureninase

1. Effect of length of induction time on the specific activity of the enzymes. The effect of the incubation time on the specific activities of the first three enzymes on the pathway induced by DL kynurenine, L-tryptophan or DL-5-hydroxytryptophan is seen graphically in Figures 7 through 9. Although these experiments were done but once, the wide variety of conditions (time or inducer concentrations) used validates the results. The evidence that the kynurenine-induced formamidase has the properties of the tryptophan-induced enzyme is as follows. The kynurenine enzyme is fairly stable to heat, as is the tryptophan-induced enzyme, with 25% of the enzymatic activity remaining after 60 min at 56 C. Furthermore it has the same sensitivity to NEM, where there is but 19% of the activity left when incubated with 1 mM NEM for 5 min. Thus, the kynurenine-induced enzyme is similar in these key properties to the tryptophan-induced enzyme.

The salient features in Figures 7 through 9 are 1) the similarity in induction of formamidase activity by all three inducers, 2) the low level of induction of tryptophan oxygenase and kynureninase activity by 5-hydroxytryptophan, and 3) the similarity in the shape of the induction

Figure 7

Effect of time on induction of formamidase activity by tryptophan, 5-hydroxytryptophan and kynurenine. The concentration of L-tryptophan (triangles) in the medium was 5 mM, and the concentration of DL-kynurenine (open circles) and DL-5-hydroxytryptophan (closed circles) was 1 mM. After the induction period had elapsed, the bacteria were harvested from the solid medium, washed, and sonicated. Enzyme assays were performed on the cell-free preparation. The activity is expressed as mumoles kynurenine produced min^{-1} mg^{-1} protein.

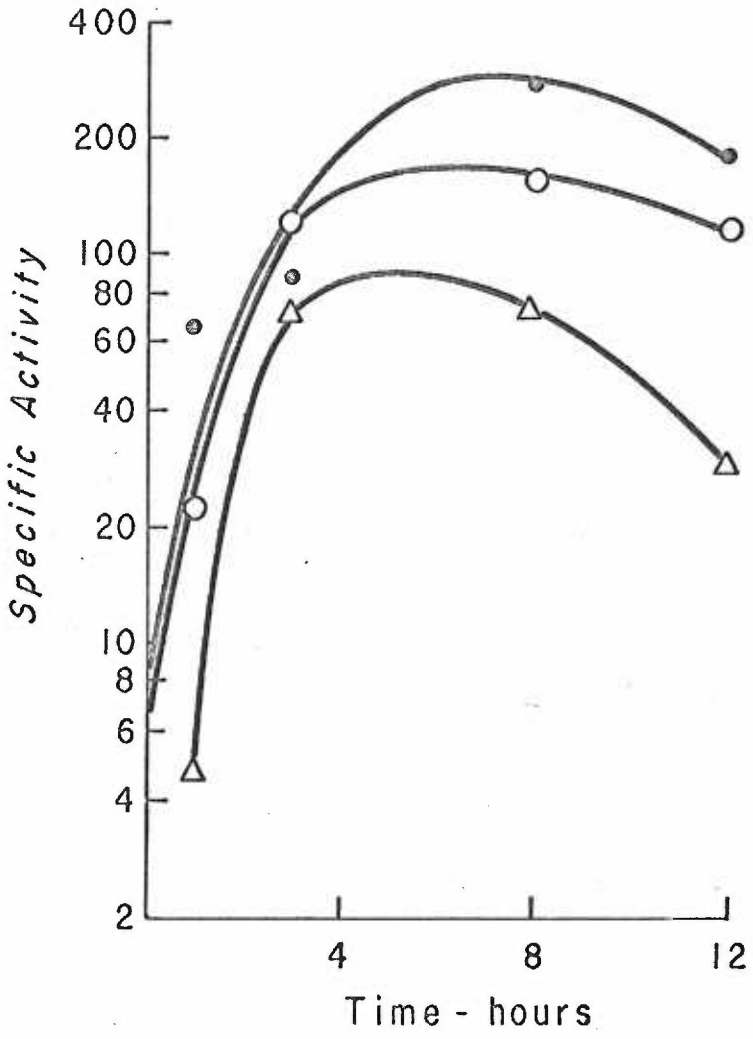

400
200
100
80
60
40
20
10
8
6
4
2
Specific Activity
4
8
12
Time - hours

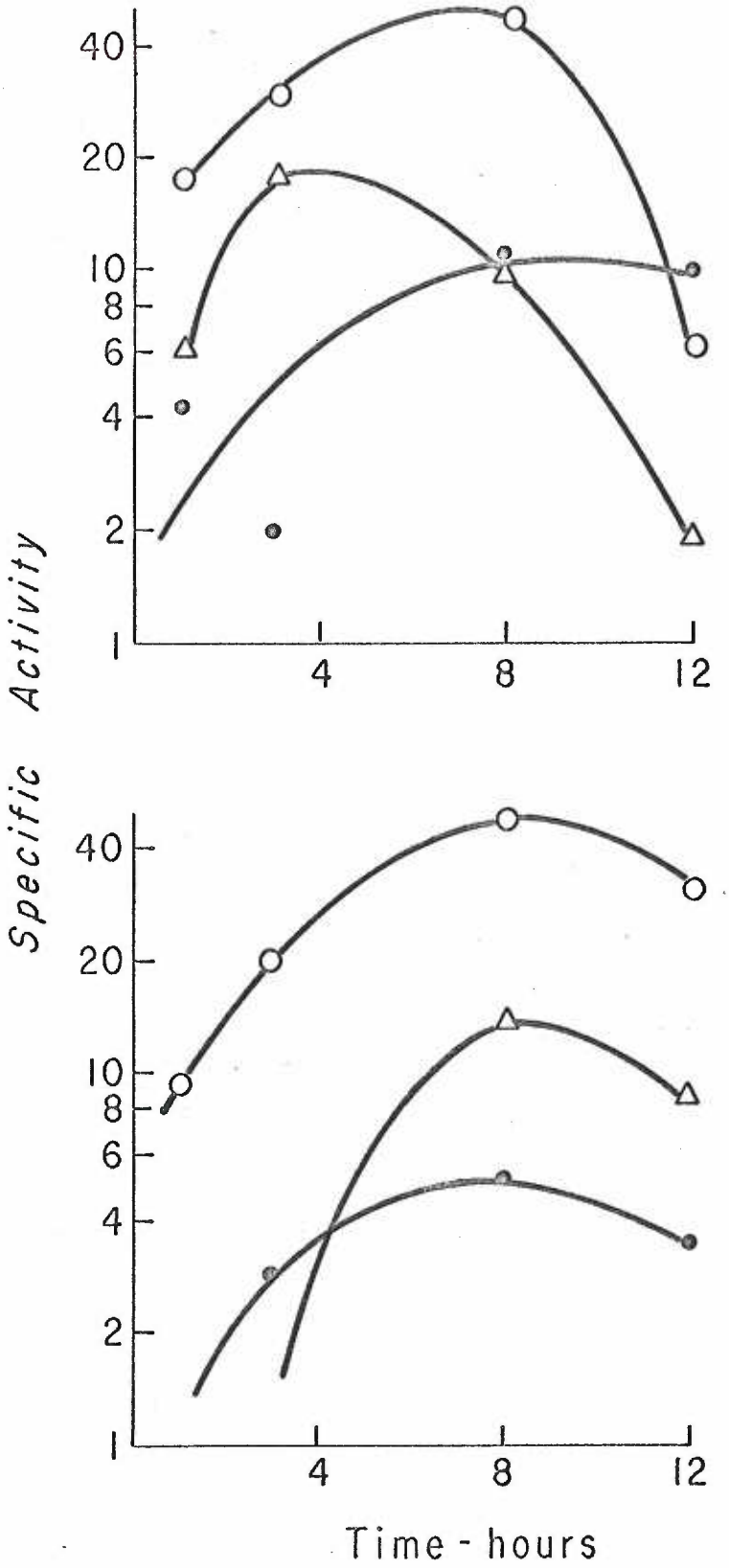
Figure 8

Effect of time on induction of kynureninase activity by tryptophan, 5-hydroxytryptophan or kynurenine.

Figure 9

Effect of time on induction of tryptophan oxygenase activity by tryptophan, 5-hydroxytryptophan or kynurenine. Two of the values for the tryptophan-induced enzyme fall below the values on the chart and are therefore omitted.

The concentration of L-tryptophan (triangles) in the medium was 5 m$\underline{M}$, and the concentration of DL-kynurenine (open circles) and DL-5-hydroxytryptophan (closed circles) was 1 m$\underline{M}$. After the induction period had elapsed, the bacteria were harvested from the solid medium, washed, and sonicated. Enzyme assays were performed on the cell-free preparation. The activity is expressed as mμmoles kynurenine hydrolyzed (Fig. 9) or produced (Fig. 8) min^{-1} mg^{-1} protein.


Specific Activity
40
20
10
8
6
4
2
1
4
8
12
Time - hours

curves of kynureninase by tryptophan and 5-hydroxytryptophan. Thus the kinetics of induction by 5-hydroxytryptophan resemble those of induction by tryptophan. The levels of one of the enzymes (formamidase) is normal, while the other two (oxygenase and kynureninase) are depressed. As the level of the tryptophan oxygenase is depressed relative to that seen with the tryptophan-induced system, it would appear that the oxygenase is not synthesized coordinately with the formamidase, unlike Stanier's TR23 strain. However the synthesis might be coordinate, and the enzyme with tryptophan oxygenase activity might either be less active when induced by 5-hydroxytryptophan or might be less stable and be degraded more rapidly. The latter hypothesis is somewhat unlikely as although the 5-hydroxytryptophan-induced formamidase is much more labile than that induced by tryptophan or kynurenine (see section B. 1. below), the specific activities are still comparable.

2. Effect of concentration of inducer on the specific activity of the enzymes. The results from studies where the concentration of the inducer, DL kynurenine or DL 5-hydroxytryptophan, was varied are presented in Figures 10 through 12. As noted in part 1, above, the induction level of kynureninase and tryptophan oxygenase is low, even at high inducer concentrations. Induction does occur however, as the basal specific activity of tryptophan oxygenase is about 0.13 mumoles kynurenine min^{-1} mg^{-1} protein, and that of kynureninase is about 0.24 (formamidase specific activity is higher, about 5.5). The levels are thus increased about 23 fold for tryptophan oxygenase and 13 fold for kynureninase; the levels of both enzymes are about 8 fold higher with induction by kynurenine. In other respects, the curves are similar.

Figure 10

Effect of inducer concentration on induction of formamidase activity by 5-hydroxytryptophan or kynurenine. The cells were induced for a 3 hr period with varying concentrations of DL-5-hydroxy-tryptophan (triangles) or DL-kynurenine (circles). After the induction period had elapsed, the bacteria were harvested from the solid medium, washed, and sonicated. Enzyme assays were performed on the cell-free preparation. The activity is expressed as mμmoles kynurenine produced min^{-1} mg^{-1} protein.

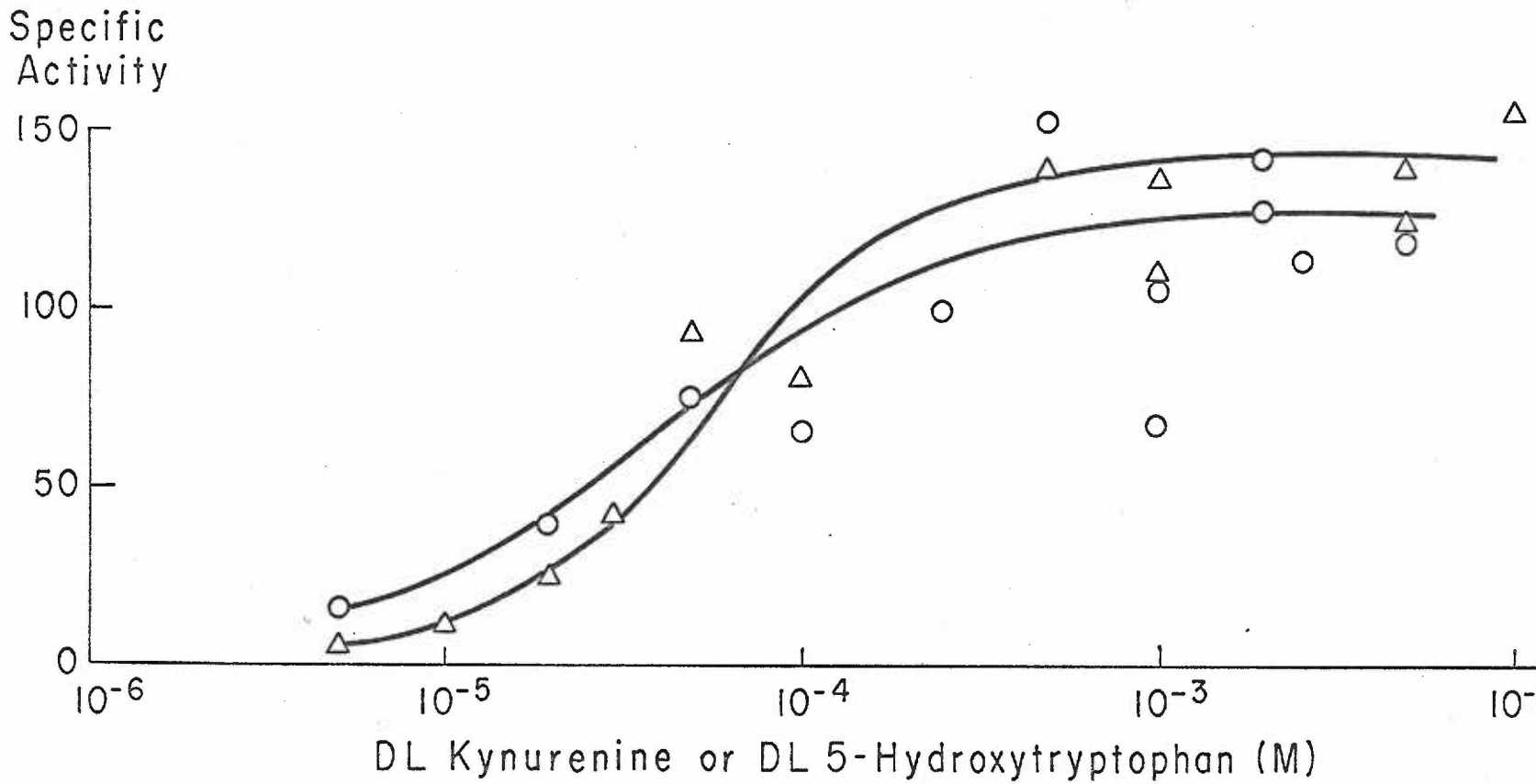

Specific
Activity
150
100
50
0
10-6
10-5
10-4
10-3
10-2
DL Kynurenine or DL 5-Hydroxytryptophan (M)

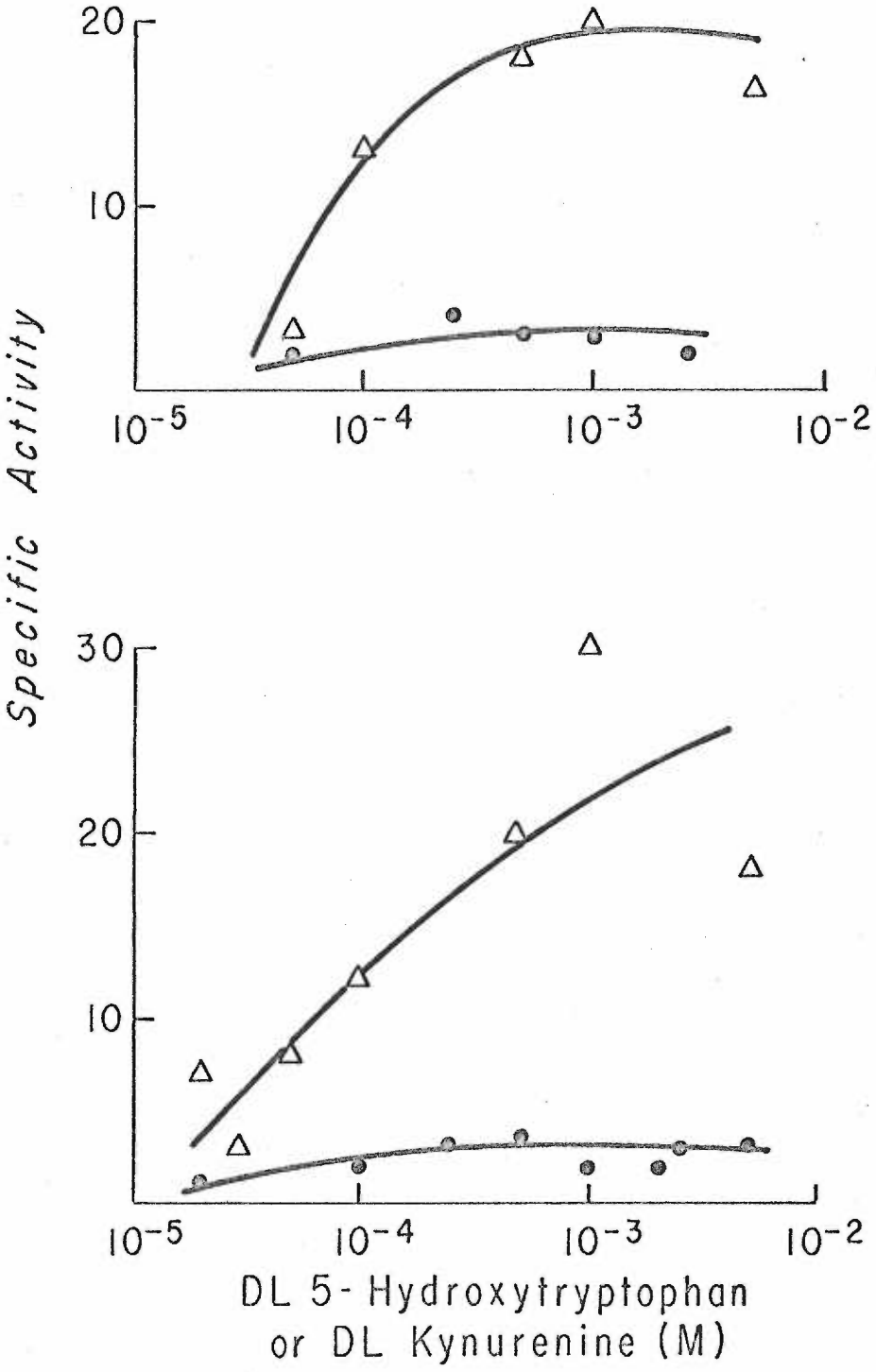
Figure 11

Effect of inducer concentration on induction of tryptophan oxygenase activity by 5-hydroxytryptophan or kynurenine.

Figure 12

Effect of inducer concentration on kynureninase activity by 5-hydroxytryptophan or kynurenine.

The cells were induced for a 3 hr period with varying concentrations of DL-5-hydroxytryptophan (circles) or DL-kynurenine (triangles). After the induction period had elapsed, the bacteria were harvested from the solid medium, washed, and sonicated. Enzyme assays were performed on the cell-free preparation. The activity is expressed as mμmoles kynurenine produced (Fig. 11) or hydrolyzed (Fig. 12) min^{-1} mg^{-1} protein.


20
10
30
20
10
Specific Activity
10^{-5}
10^{-4}
10^{-3}
10^{-2}
DL 5-Hydroxytryptophan
or DL Kynurenine (M)

Thus it would appear that the control of induction of the first three enzymes of the pathway by 5-hydroxytryptophan is the same. However the levels of two of the enzymes are markedly depressed.

B. Comparative Studies with Denaturing Agents

1. Heat inactivation. Table 22 presents the profile of heat inactivation of both the tryptophan and 5-hydroxytryptophan-induced formamidases. The 5-hydroxytryptophan-induced formamidase is quite heat labile, in contrast to that induced by tryptophan. The half life at 56 C is about 13 sec for the 5-hydroxytryptophan-induced enzyme, and 45 min for that induced by tryptophan.

The responses of the enzymes heated in the presence of salts were different (Table 23). The tryptophan-induced enzyme assayed in the presence of additional phosphate had 25% less activity than the control. However, the diluted heated enzyme was unaffected by the addition of this phosphate to the assay mixture. There was a 65 - 70% reduction in activity for the enzyme "heated in salt" relative to the enzyme "heated in water." The 5-hydroxytryptophan enzyme was affected the same way with the phosphate added to the reaction mixture; the unheated enzyme had 10% less activity while the heated enzyme was unaffected by the addition. However the enzyme heated in salt had twice the activity of the enzyme heated in water (82% control vs 47%). The results with other salts (NaCl, KCl, and K_2SO_4) were not very different for either enzyme. Both chlorides caused an increase in heat lability for both enzymes.

These data suggest that the denaturation process, a process of unfolding, produces differences between the two molecules, reflected by their interaction with phosphate.

TABLE 22

Heat Inactivation of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases

Minutes heating	Per cent activity remaining					
	Tryptophan-induced formamidase		5-Hydroxytryptophan-induced formamidase			
	56 C		56 C	45 C	36 C	
	Exp. 1	Exp. 2			Exp. 1	Exp. 2
0.12				110		
0.16			96 ± 19	94 ± 10		
0.25			81 ± 8	76 ± 2		
0.33			38 ± 22	87		
0.50			8 ± 3	72 ± 6		
0.75			12 ± 9	33 ± 3		
1.0			7 ± 2	26 ± 2		
2.0					93 ± 3	92 ± 3
5.0					61 ± 4	66 ± 5
10	70 ± 5	92 ± 6			63 ± 4	60 ± 6
15	55 ± 5				68 ± 0	
20	70 ± 12	76 ± 2			32 ± 4	
30	64 ± 4	61 ± 2				31 ± 4
40	61 ± 3	55 ± 2				
50	62 ± 1	38				17 ± 4
60	45 ± 11	15 ± 1				

The values represent in most cases the mean of two determinations ± SD.

TABLE 23

The Effect of Salts on Heat Inactivation of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases.

Enzyme heated with an equal volume of	Per cent activity: 5-Hydroxytryptophan-induced formamidase			Tryptophan-induced formamidase	
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2
Water	27 ± 1	47 ± 1	57 ± 11	65 ± 6	58 ± 9
1.0 M K_2HPO_4	68 ± 25	82 ± 8	58 ± 2	20 ± 1	20 ± 3
0.5 M K_2HPO_4	42 ± 4	87 ± 10	51 ± 3	18 ± 2	
0.25M K_2HPO_4		79 ± 2		29 ± 6	25 ± 3
1.0 M Na_2HPO_4			49 ± 6	11 ± 1	
0.5 M K_2SO_4			41 ± 1	32 ± 5	55 ± 4
1.0 M NaCl		19 ± 1	31 ± 5	24 ± 5	41 ± 1
1.0 M KCl			28 ± 4	19 ± 3	
Controls:					
Enzyme which was heated in water preincubated with an aliquot of:					
1.0 M K_2HPO_4	26 ± 1		60 ± 7		58 ± 4
1.0 M NaCl		51 ± 4	54 ± 6		69 ± 5
Unheated enzyme preincubated with an aliquot of:					
1.0 M K_2HPO_4			89 ± 2		76 ± 4
1.0 M NaCl		114 ± 9	98 ± 11		80 ± 3

The tryptophan-induced enzyme was heated at 60 C for 1 hr; the 5-hydroxytryptophan, at 36 C for 8 min. For the controls, the amount of salt added was equal to the amount found in the incubation mixture of the enzymes heated in 1.0 M salt.

2. Comparison of the bacterial enzymes with rabbit liver enzymes. The response of the bacterial formamidases to these agents reported (42) to inhibit rabbit liver formamidase was studied. The concentrations used were those used by Kotake (42), for a valid comparison. The results of this single experiment are displayed in Table 24.

The responses of the tryptophan- and 5-hydroxytryptophan-induced enzyme differ from one another and from the rabbit liver enzyme. The striking difference between the bacterial enzymes and the rabbit enzyme is the lack of specificity in the effect of chelating agents. The rabbit enzyme is inhibited by 8-hydroxyquinoline but not by EDTA, whereas the bacterial enzymes are inhibited by both. The tryptophan- and 5-hydroxytryptophan-induced enzymes differ markedly from one another in the response to arsenite. This reagent, known to react with sulfhydryl groups, inhibits the 5-hydroxytryptophan-induced enzyme strikingly, but produces only moderate inhibition of the tryptophan enzyme. This suggests a structural difference between the enzymes.

3. Urea inhibition. As shown in Figure 13, the response of both enzymes to urea inhibition is similar, although the slopes of the curves are different. The 5-hydroxytryptophan-induced enzyme is more rapidly inactivated by the reagent. The data suggest possible hydrophobic differences between the two molecules.

The majority of the reagents studied react with sulfhydryl groups. Their differences in size, rates of reactions, and substrate specificities produce different results.

TABLE 24

Inhibition of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases by Agents Inhibitory to Rabbit Liver Formamidase.

Inhibitor	Concentration	Per cent inhibition		
		Tryptophan-induced formamidase	5-Hydroxytryptophan-induced formamidase	Rabbit liver formamidase (42)
8-Hydroxyquinoline	2 mM	75 ± 12	23 ± 2	40
EDTA	2 mM	18 ± 2	31 ± 5	0
Fluoride	2 mM	35 ± 2	45 ± 1	40
Arsenite	2 mM	19 ± 0	95 ± 1	50

The bacterial enzyme preparations were incubated in the standard incubation mixture 5 min. with the inhibitor and then assayed. The per cent inhibition for those determinations is expressed as the mean of two determinations ± SD. The values for 8-hydroxyquinoline were corrected for the 6.65% alcohol present by an independent determination of the effect of the alcohol on the enzyme activities.

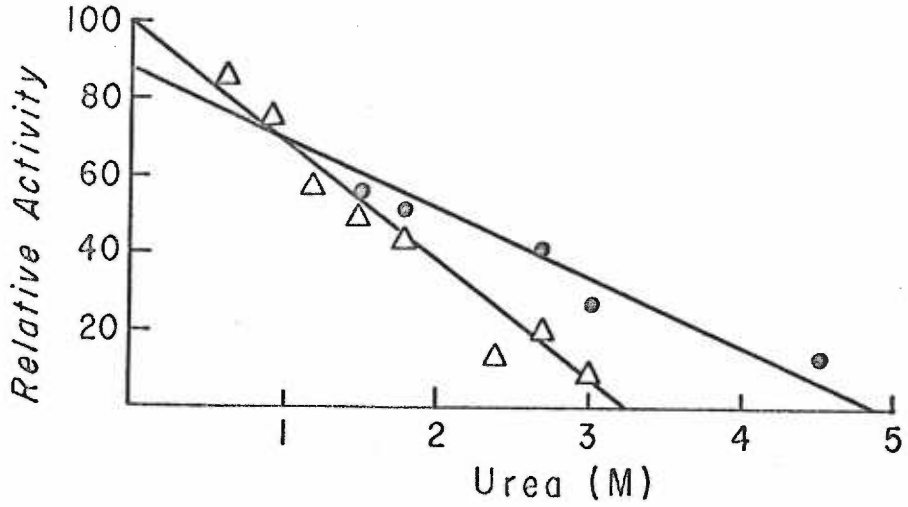
Figure 13

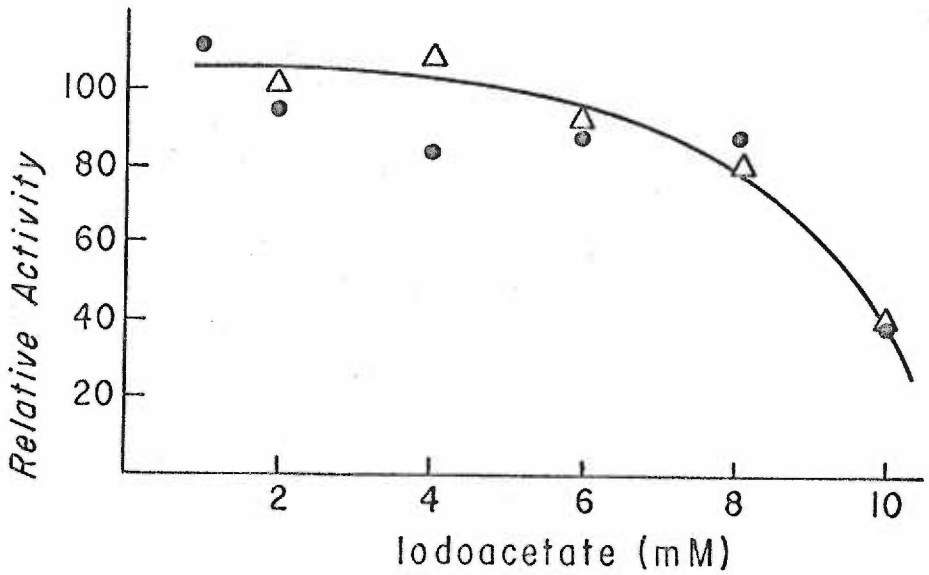
Inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases by urea.

Figure 14

Inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases by iodoacetate.

The tryptophan- (circles) and 5-hydroxytryptophan-induced (triangles) formamidase preparations were incubated with semicarbazide (final concentration 10 mM, phosphate buffer and inhibitor for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction. The value for the uninhibited enzyme has been set at 100.


Relative Activity
100
80
60
40
20
1
2
3
4
5
Urea (M)


Relative Activity
100
80
60
40
20
2
4
6
8
10
Iodoacetate (mM)

4. Iodoacetate inhibition. This alkylating agent affects both enzymes to the same extent. The data are presented graphically in Figure 14. Neither enzyme is very sensitive to this reagent.

5. pCMB inhibition. This reagent produces similar inhibition of both enzymes at lower concentrations (Figure 15). When the concentration is raised, a difference appears between the two enzymes. While the 5-hydroxytryptophan-induced enzyme is completely inhibited by this reagent, there is a fraction of the tryptophan-induced enzyme activity remaining, even at high pCMB concentrations. This suggests a difference in structure between the enzymes, or a heterogeneity in the tryptophan-induced enzyme preparation.

6. $HgCl_2$ inhibition. The inhibition curves of both enzymes are given in Figure 16, and the data for inhibition of the tryptophan enzyme in Table 25. The inhibition of the 5-hydroxytryptophan-induced enzyme resembles that seen with pCMB, with a steady decrease in enzymatic rate with increasing concentrations of the mercural. The inhibition curve of the tryptophan-induced formamidase is anomalous. The activity drops with increasing $HgCl_2$ concentration to a value about 15 - 25% the control value. Then with increasing concentrations of $HgCl_2$ the activity abruptly increases to about 40% the control value. The activity remains at this plateau, only slowly declining with further increases of $HgCl_2$ concentrations. This anomalous curve is reproducible, as shown in Table 25, although as the trough is defined within a narrow concentration of $HgCl_2$ concentrations, care must be taken to find those concentrations encompassing it. Over a period of a year the concentration

Figure 15

Inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases by pCMB. The tryptophan- (open circles) or 5-hydroxytryptophan-induced (closed circles) formamidase preparations were incubated with semicarbazide (final concentration 10 mM), phosphate buffer and pCMB for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction. The value for the uninhibited enzyme has been set at 100.

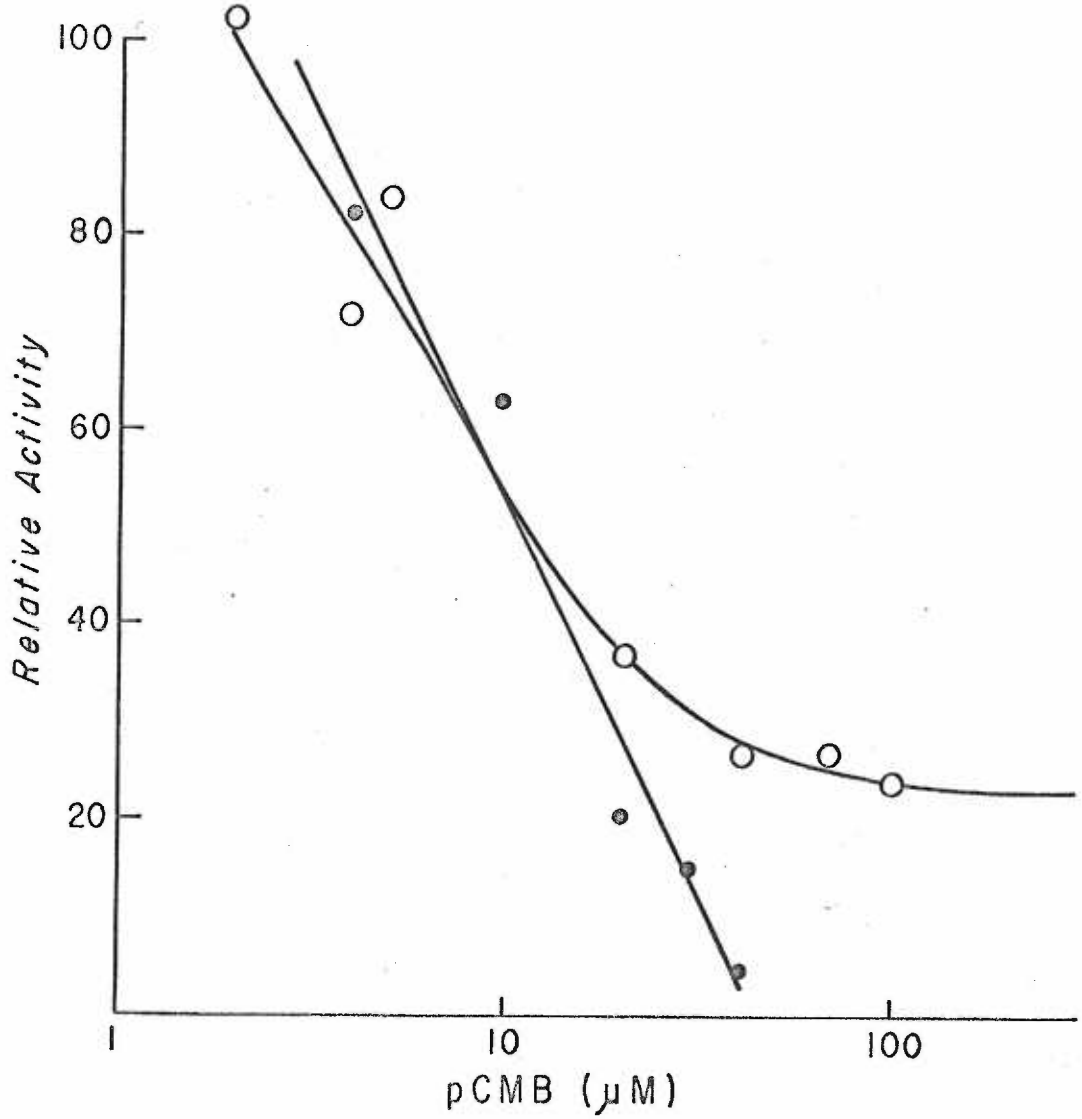

100
80
60
40
20
Relative Activity
1
10
100
pCMB (μM)

Figure 16

Inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases by $HgCl_2$. The tryptophan- (open figures) or 5-hydroxytryptophan-induced (closed figures) formamidase preparations were incubated with semicarbazide (final concentration 10 mM), phosphate buffer and $HgCl_2$ for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction. The value for the uninhibited enzyme has been set a 100.

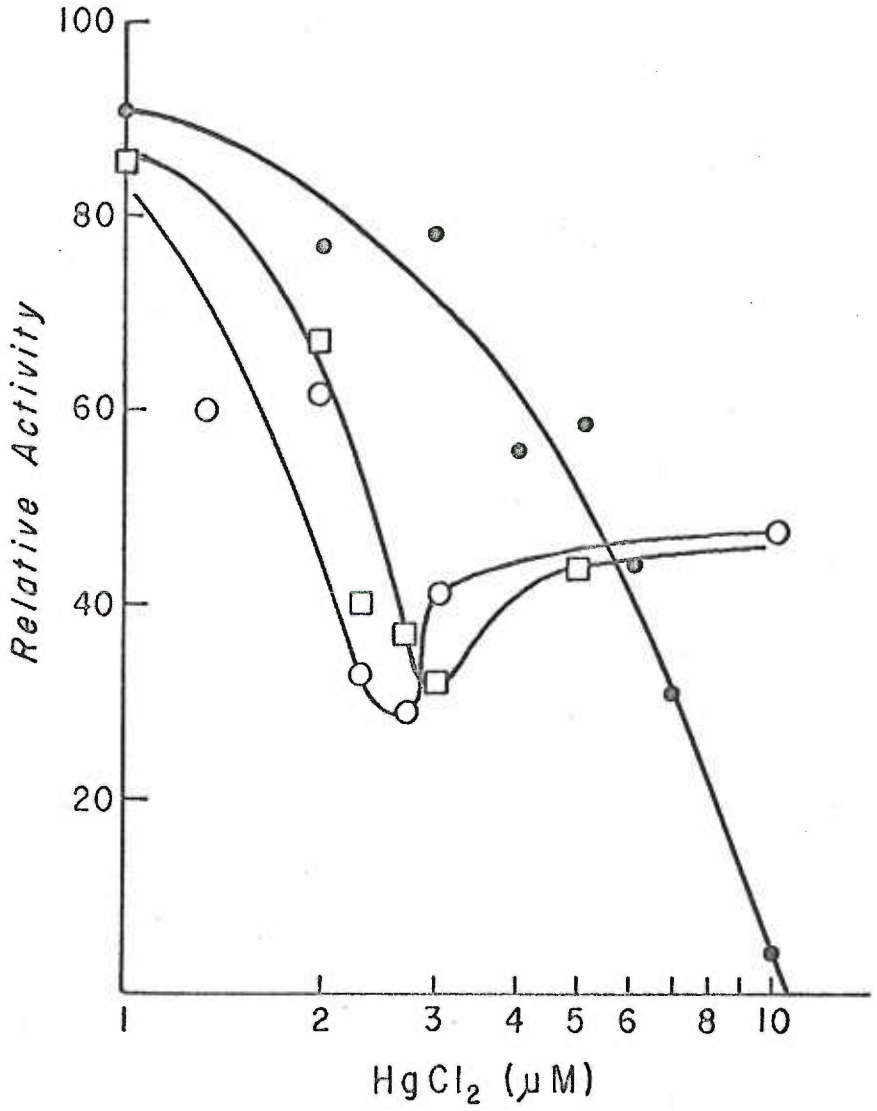

100
80
60
40
20
Relative Activity
1
2
3
4
5
6
8
10
HgCl2 (μM)

TABLE 25

$HgCl_2$ Inhibition of the Tryptophan-Induced Formamidase

$HgCl_2$ (μM)	Rate per minute			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
1.0	40 ± 2			
2.0	29 ± 3	111 ± 6	81 ± 3	
2.3	16 ± 3			
2.67	16 ± 1			
3.0	20 ± 1			
4.0			36 ± 1	
5.0			16 ± 1	
6.0			29 ± 2	44 ± 3
7.0			33 ± 3	
8.0		20 ± 0	35 ± 3	14 ± 1
10.0	22 ± 1	31 ± 2	37 ± 4	22 ± 1
20.0	22 ± 1	37 ± 1	31 ± 1	22 ± 1
50.0		35 ± 1		
100.0	12 ± 2			

The enzyme preparations were incubated in the standard incubation mixture 5 min. with $HgCl_2$ and then assayed. The rate is expressed as optical density x 10^3 per enzyme sample. The sample size is constant within any experiment. The values are the mean of two or three determinations ± SD.

of $HgCl_2$ at the trough rose gradually from 1.3 μM to 5 (as seen in Figure 22) and 8 μM. This may simply be due to a population shift in the bacteria involving a protein with additional sulfhydryl groups, which would bind a significant fraction of the small amount of $HgCl_2$ present. The shift is not due to the loss of potency of $HgCl_2$ solutions, as freshly prepared solutions produce the same results.

The peculiar kinetics strongly suggest that the tryptophan-induced enzyme undergoes a conformational shift during $HgCl_2$ inhibition. The altered conformation either stabilizes the active site, or exposes new sulfhydryl groups to bind $HgCl_2$ and reduce the effective concentration. The 5-hydroxytryptophan-induced enzyme does not show this phenomenon: either its conformation does not change radically with increasing $HgCl_2$ concentration, or if such a change does occur, its activity is not affected. Either, then, no new sulfhydryl groups are exposed, or the enzymatic site is not stabilized by mercury. A highly purified preparation of each formamidase would be necessary to distinguish between the mechanism of action on the tryptophan- and 5-hydroxytryptophan-induced enzymes.

Furthermore, there must be a difference in action between $HgCl_2$ and pCMB, because only the former produces the anomalous curve. The difference could be related either to the smaller size of $HgCl_2$, allowing it to reach areas inaccessible to larger mercurials, or it could be due to its ability to attach to two sulfhydryl groups, forming a bridge. These possibilities are discussed in greater detail in the Discussion section.

7. NEM inhibition. This reagent also has different activity on the tryptophan- and 5-hydroxytryptophan-induced formamidases (Table 26). The 5-hydroxytryptophan-induced enzyme is strikingly more resistant than the tryptophan-induced enzyme. There was little or no inhibition of the 5-hydroxytryptophan-induced enzyme up to 10 mM NEM, while the tryptophan-induced enzyme was inhibited 75% at 1 mM. Higher concentrations were not tried with the 5-hydroxytryptophan-induced enzyme preparations as the inhibitor absorbs in the spectral region used in the enzymatic assay. Also it was felt that the specificities generally ascribed to any reagent used in one concentration would likely be different at 10 or 15 fold higher concentrations. The difference between the enzyme preparations here, with the 5-hydroxytryptophan-induced formamidase more resistant to a reagent known to react with sulfhydryl groups, is in contrast to the effects seen with arsenite, a second reagent with similar properties, where the tryptophan-induced enzyme was strikingly more resistant.

8. Reversal of pCMB and $HgCl_2$ inhibition by sulfhydryl-containing reagents. The reversibility of pCMB and $HgCl_2$ inhibition was studied by incubating the enzymes with the mercury compound for 5 min, adding a sulfhydryl-containing compound such as reduced glutathione, and incubating for 5 or 10 min longer before adding the substrate to initiate the reaction.

First the effect of various sulfhydryl reagents on the formamidase reaction was studied. If 1 mM concentrations of the various sulfhydryl reagents are incubated with either formamidase for 5 min before substrate

TABLE 26

Inhibition of the Tryptophan- and 5-Hydroxy-tryptophan-Induced Formamidases by NEM.

NEM (mM)	Per cent activity	
	Tryptophan-induced formamidase	5-Hydroxytryptophan-induced formamidase
0.067	69 ± 5	
0.10	69 ± 8	
0.20	43 ± 2	
0.40	38 ± 2	
0.50	21 ± 0	
1.00	28 ± 4	
2.00		108 ± 5
4.00		114 ± 6
8.00		97 ± 7
10.00		102 ± 2

The enzyme preparations were incubated in the standard incubation mixture 5 min. with NEM and then assayed. The values are expressed as the per cent activity of the untreated enzyme preparation. The values are the mean of two or three determinations ± SD.

addition, the following rates, relative to 100 for each enzyme incubated without any SH reagent, are as follows: the tryptophan-induced formamidase incubated with GSH (84 ± 1), DTT (46 ± 0) and BAL (38 ± 4) did have different rates than the 5-hydroxytryptophan-induced enzyme with GSH (87 ± 8) and DTT (95 ± 8); the rates in the presence of DTT differed. Reduced glutathione was used for the majority of the studies as it was least inhibitory and was readily dissolved.

The effects of these reagents on reversal of inhibition by pCMB and $HgCl_2$ are presented in Tables 27 and 28. The results indicate that the sulfhydryl reagents can reverse the effect of mercury treatment of either formamidase. Thus the inhibitory reaction produced by the mercury compounds on either enzyme is reversible.

9. Kinetics of the reaction. The Michaelis constants, or K_m values, for the tryptophan and 5-hydroxytryptophan formamidases for the substrate formyl-L-kynurenine were determined to establish whether the enzymes varied in this property as well. The results may be seen in Figures 17 and 18. The results are plotted as s/v versus s, a so-called Wolff plot, as this treatment is reported to yield a better estimate of the K_m (182). The lines were fitted by a least squares determination in all these plots. The K_m values of the two enzymes are similar, 2.4×10^{-4}M for the tryptophan-induced and 2.0×10^{-4}M for the 5-hydroxytryptophan-induced enzyme. Thus, the enzymes cannot be firmly distinguished on the basis of substrate affinity.

The K_i and class of inhibition was determined for two of the inhibitors. The results with NEM are presented in Figure 19. The inhibition

TABLE 27

The Effect of Sulfhydryl Reagents on the Activity of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases Incubated with pCMB.

Sample added to incubation mixture	Per cent activity	
	Tryptophan-induced formamidase 33 uM pCMB	5-Hydroxytryptophan-induced formamidase 10 uM pCMB
pCMB	34 ± 2	35 ± 3
pCMB + DTT	38 ± 0	66 ± 3
pCMB + GSH	74 ± 0	58 ± 0
pCMB + BAL	39 ± 7	

The enzyme preparations were incubated in the standard incubation mixture 5 min. with pCMB and the sulfhydryl reagent was added at 1 mM. The mixture was incubated 5 min. longer then assayed. The values are the mean of two determinations ± SD.

TABLE 28

The Effect of Sulfhydryl Reagents on the Activity of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases Incubated with $HgCl_2$.

Sample added to incubation mixture	Per cent activity			
	Tryptophan-induced formamidase		5-Hydroxytryptophan-induced formamidase	
	10 uM $HgCl_2$	50 uM $HgCl_2$	5 uM $HgCl_2$	6 uM $HgCl_2$
$HgCl_2$	30 ± 2	38 ± 8	32 ± 1	20 ± 5
$HgCl_2$ + DTT	41 ± 4	13 ± 2	56 ± 1	58 ± 2
$HgCl_2$ + GSH	78 ± 2	55 ± 4	42 ± 3	52 ± 6
$HgCl_2$ + BAL	38 ± 1	28 ± 4		

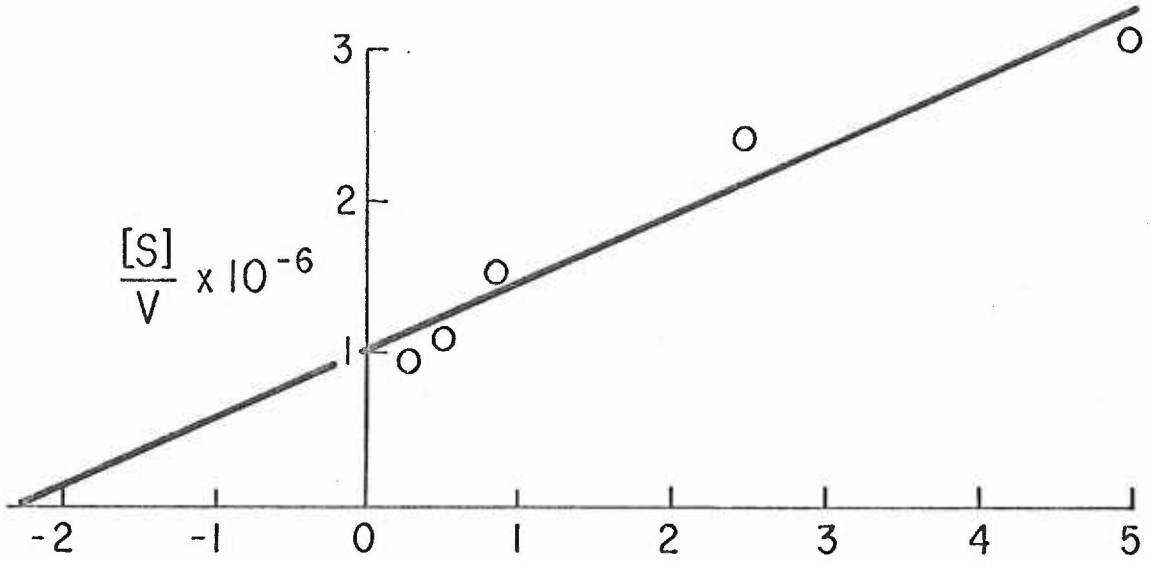
The conditions employed were the same as those in Table 27.

Figure 17

A Wolff plot for the tryptophan-induced formamidase.

Figure 18

A Wolff plot for the 5-hydroxytryptophan-induced formamidase


3
2
1
[S]/V x 10^-6
-2
-1
0
1
2
3
4
5

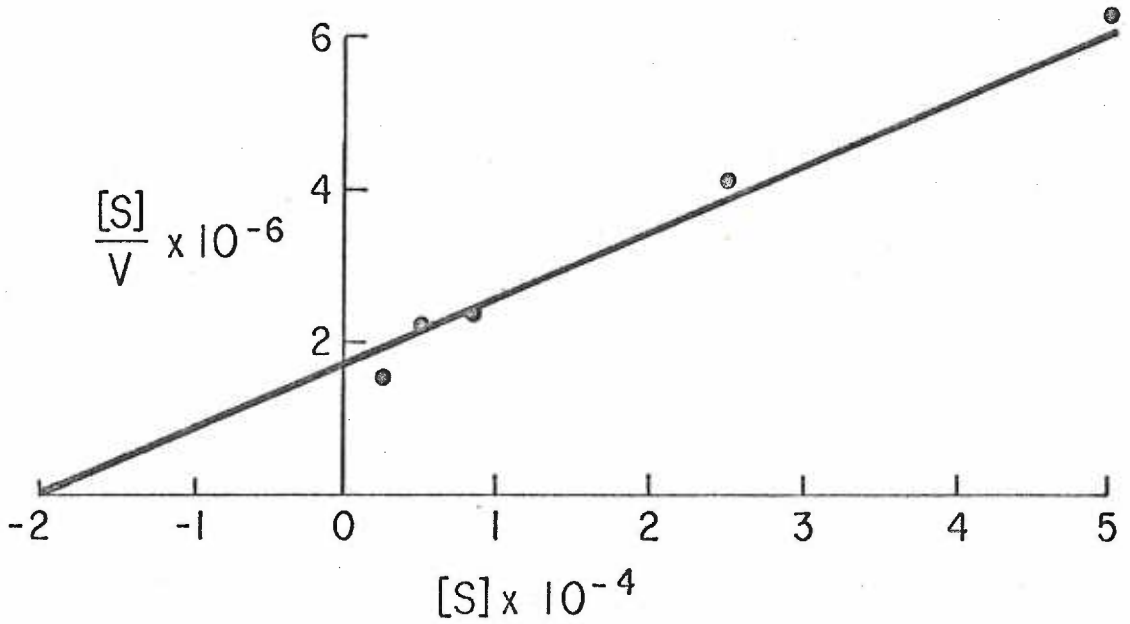
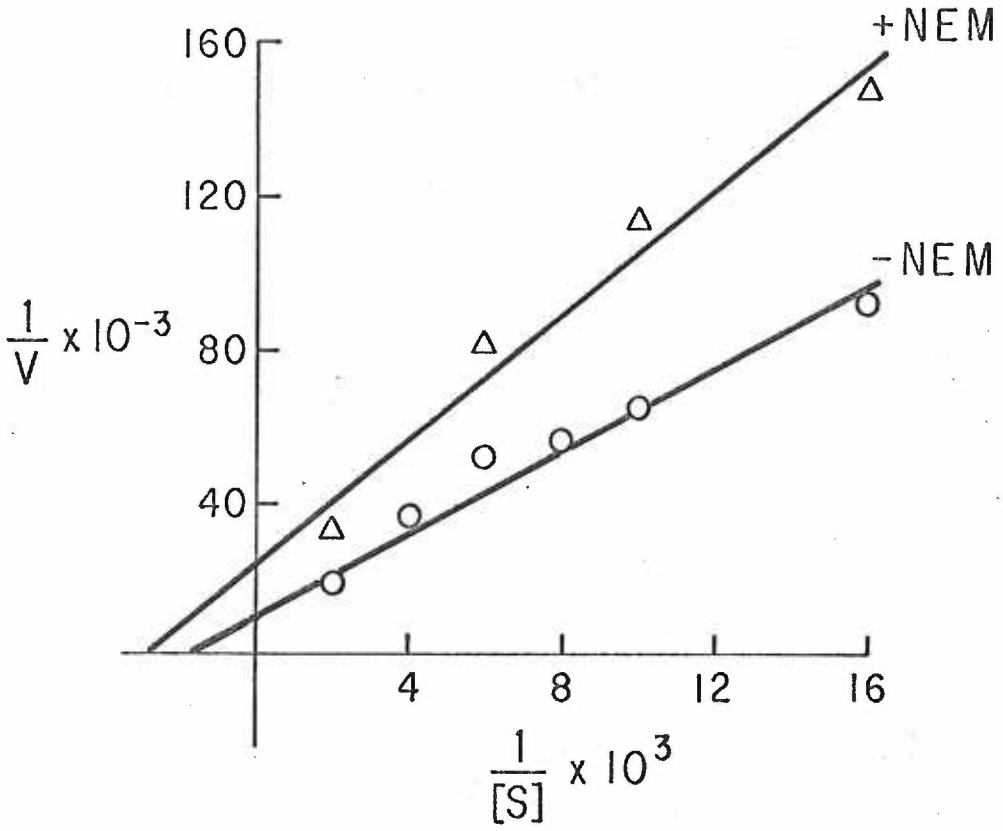

6
4
2
[S]/V x 10^-6
-2
-1
0
1
2
3
4
5
[S] x 10^-4

Figure 19

Lineweaver-Burk plots for the tryptophan-induced formamidase in the presence or absence of NEM. The enzyme preparation was incubated in the standard incubation mixture with (triangles) or without (circles) 0.2 mM NEM for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction.


+NEM
-NEM
160
120
80
40
$\frac{1}{V} \times 10^{-3}$
4
8
12
16
$\frac{1}{[S]} \times 10^{3}$

of this reagent with the tryptophan-induced formamidase is mixed, and the K_i of the inhibitor is 3.4×10^{-4}M, for a concentration of 0.2 mM NEM.

A similar experiment was done a number of times for $HgCl_2$ inhibition of the tryptophan- and 5-hydroxytryptophan-induced formamidases. The results of two of these determinations are presented in Figures 20 and 21. The plots are characteristically nonlinear; the plots chosen for representation showed the smallest deviation from linearity. In all 12 determinations, the points do not fit a straight line well. Assuming that the data do fit a straight line, the inhibition is strictly noncompetitive.

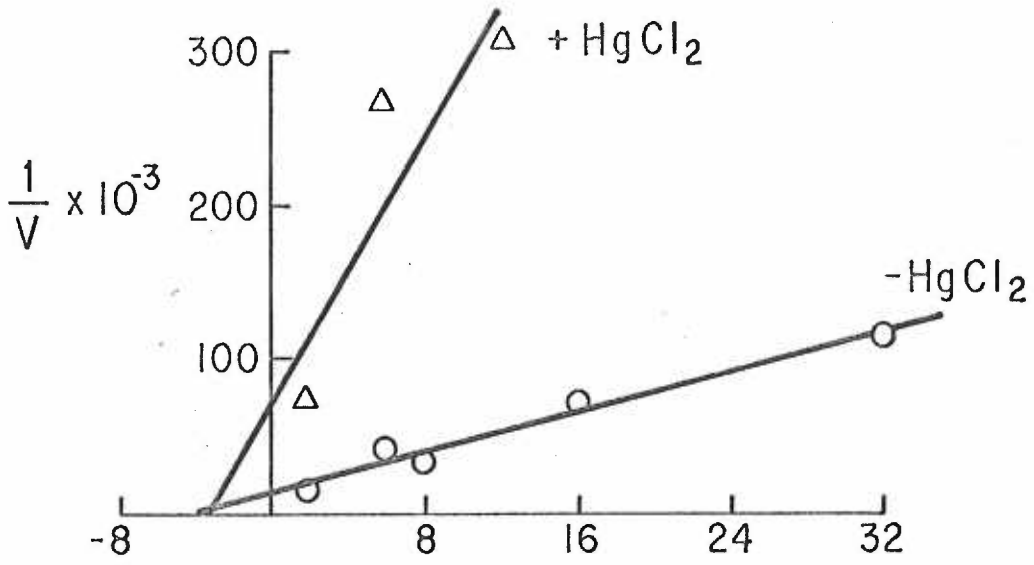
10. The effect of $HgCl_2$ on NEM inhibition of the tryptophan-induced formamidase. Both NEM and $HgCl_2$ bind to sulfhydryl groups, yet each yields a distinct pattern of inhibition when used with the tryptophan- or 5-hydroxytryptophan-induced formamidase. To see whether the same set of sites, presumably sulfhydryl groups, were attacked by both inhibitors, samples of the tryptophan-induced enzyme which had been incubated for 5 min in varying concentrations of $HgCl_2$ were exposed to 1 mM NEM for an additional 10 min and then assayed. A second set of assays was performed on the enzyme which had been incubated with the same concentrations of $HgCl_2$ without NEM for 15 min. The extent of inhibition produced by exposure of the enzyme to 1 mM NEM for 10 min was also determined. The results of a representative experiment are presented in Figure 22. The results indicate that $HgCl_2$ can occupy sites on the enzyme which would be occupied by NEM, thus desensitizing the

Figure 20

Lineweaver-Burk plots for the tryptophan-induced formamidase in the presence and absence of $HgCl_2$. The enzyme preparation was incubated in the standard incubation mixture with (open triangles) or without (open circles) 5×10^{-5} M $HgCl_2$ for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction.

Figure 21

Lineweaver-Burk plots for the 5-hydroxytryptophan-induced formamidase in the presence and absence of $HgCl_2$. The enzyme preparation was incubated in the standard incubation mixture with (closed triangles) or without (closed circles) 1×10^{-5}M $HgCl_2$ for 5 min, when formyl-L-kynurenine was added to initiate the enzymatic reaction.


300
200
100
$\frac{1}{V} \times 10^{-3}$
+HgCl2
-HgCl2
-8
8
16
24
32

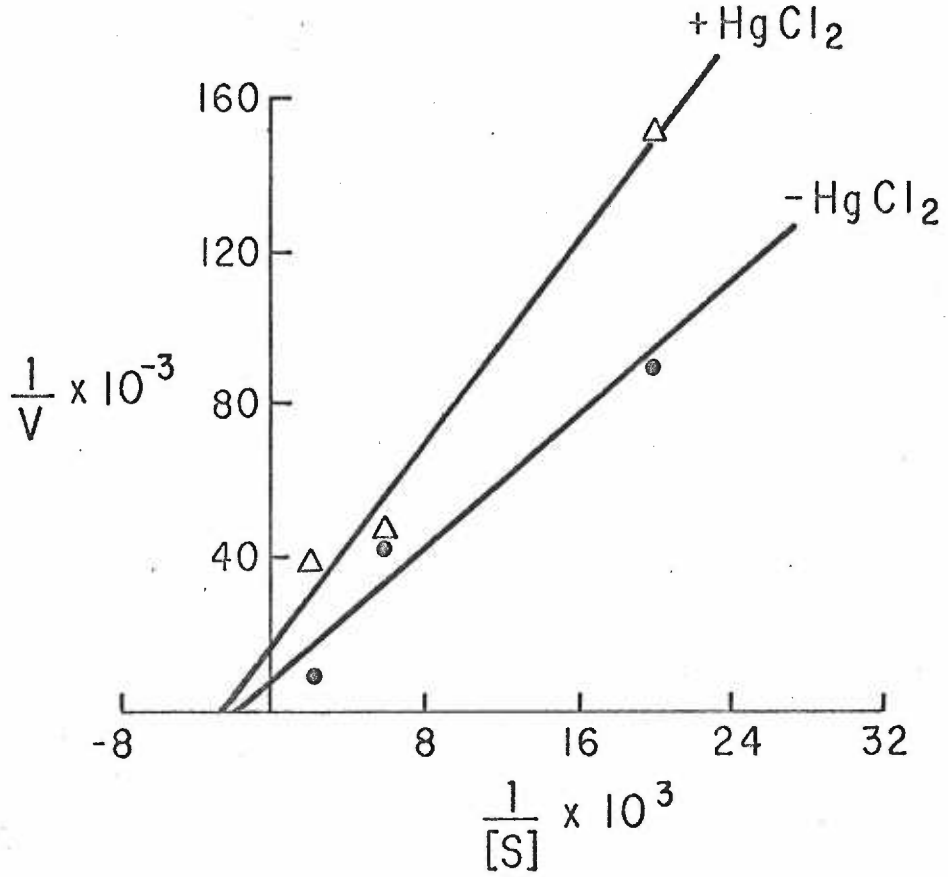
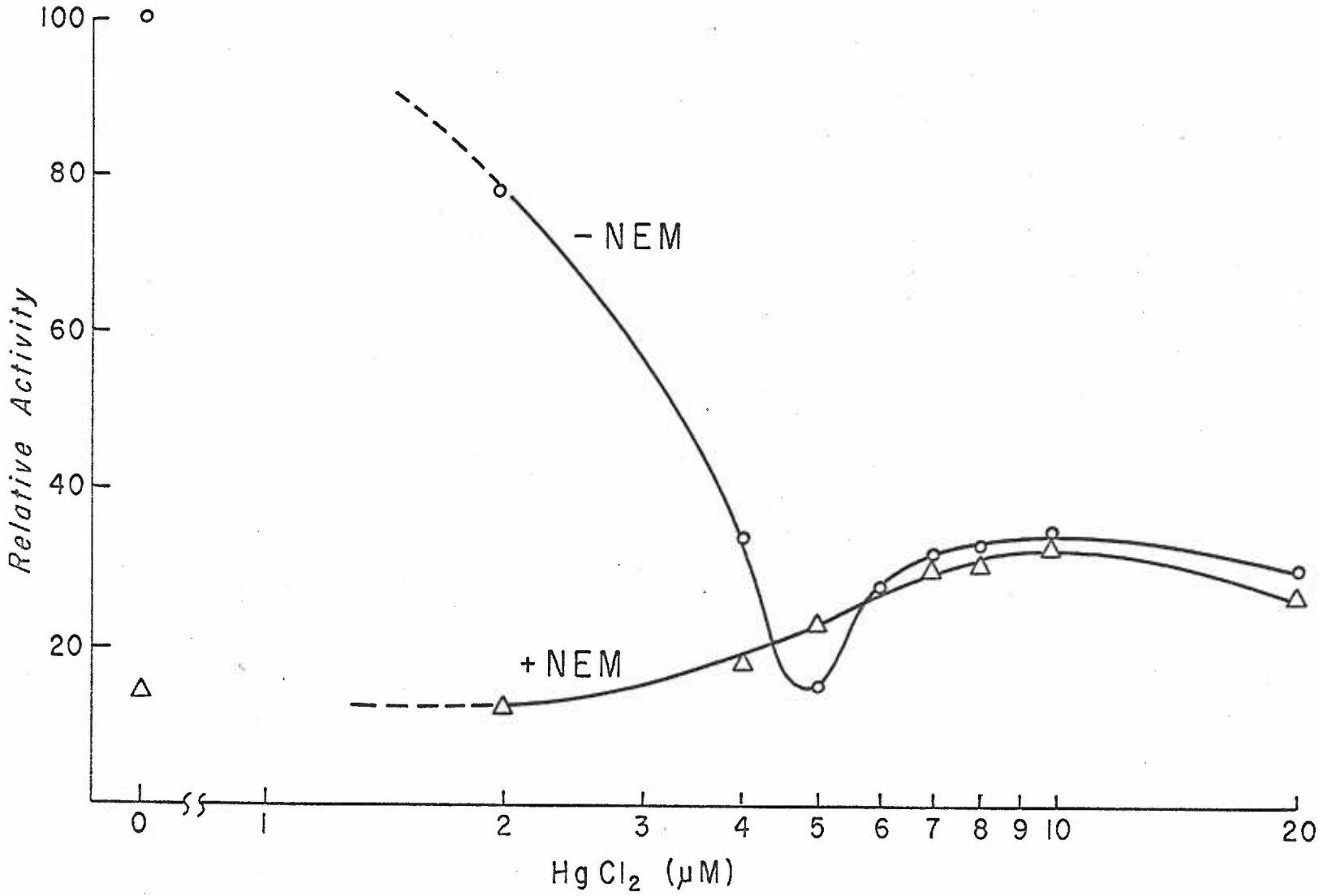

160
120
80
40
$\frac{1}{V} \times 10^{-3}$
+HgCl2
-HgCl2
-8
8
16
24
32
$\frac{1}{[S]} \times 10^{3}$

Figure 22

The effect of $HgCl_2$ on NEM inhibition of the tryptophan-induced formamidase. The enzyme was incubated in the standard incubation mixture 5 min with varying concentrations of $HgCl_2$. At this point the incubation was allowed to proceed 10 min longer with or without the addition of NEM (1 mM). The uninhibited enzyme activity has been set at 100.


100
80
60
40
20
Relative Activity
- NEM
+ NEM
0
1
2
3
4
5
6
7
8
9
10
20
Hg Cl2 (μM)

molecule to NEM. This matter will be discussed more thoroughly in the Discussion section.

11. Action of metabolic intermediates on formamidase activity. Control of degradative pathways is generally exerted by end products. End product repression plays a role in many of these pathways, such as the mandelate pathway, described in the Introduction. In that pathway, Stevenson and Mandelstam (75) found simple end products such as succinate and citrate repressed, as did end products which were terminal in the short sequence before another pathway converged into the mandelate pathway. Attention was directed to L-alanine and succinate. Alanine is actually the initial compound of a new pathway, while succinate is a terminal end product. Consequently the ability of these compounds to repress the synthesis of the tryptophan- and 5-hydroxytryptophan-induced formamidases was tested.

A repression experiment was done with 1×10^{-3}M DL kynurenine or 8.6×10^{-4}M DL 5-hydroxytryptophan as inducers. Each inducer was used with 10^{-3}M DL alanine or 10^{-3}M succinate. The induction time was 3 hr. The value of the specific activities of the enzymes were all within normal ranges. The kynurenine-induced formamidase specific activity was 103 (alanine) and 77 (succinate) while the 5-hydroxytryptophan-induced enzyme was 117 (alanine) or 131 (succinate). The kynureninases were about the same as usual, 26.9 (alanine) or 31 (succinate) for kynurenine induction versus 3 and 4. Tryptophan oxygenase was 45 (alanine) and 54 (succinate) for kynurenine versus 2 and 3 for 5-hydroxytryptophan induction. The enzyme activities may have dropped somewhat, but the levels

were not strikingly low.

In a more refined experiment, the key characteristic of a repressor, the more repressor is present, the more repression is shown, was utilized. The experiment consisted of varying the ratio of DL-alanine/tryptophan from 1:10 up to 1:200. The specific activity of the formamidase was not found to vary in any consistent manner with the ratio of repressor. The specific activity of the glutamate control was 72. For 100 mg alanine this dropped to 43, however for 400 mg it was 73. With 800 mg alanine the specific activity increased to 123, but dropped to 86 with 2000 mg alanine. Thus it is unlikely that alanine controls the level of the formamidase enzyme.

Alanine and anthranilate were tested for the ability to inhibit the activity of the formamidase enzymes. There was a somewhat variable response of the tryptophan-induced formamidase, where in one experiment there appeared to be activation of the enzyme when incubated with 1 mM concentrations of these compounds, with 132% activity with alanine and 135% with anthranilate. Later experiments with this formamidase as well as the 5-hydroxytryptophan-induced one show activities of 90 - 100% when incubated 5 min with the metabolite. Thus, there was certainly little if any inhibition shown with these end products. It is unlikely then that these compounds regulate the pathways of tryptophan and 5-hydroxytryptophan degradation.

The third approach used with these reagents was to attempt to show their binding to the formamidase molecule, which would lead to protection against denaturing or inhibiting treatments. This approach was tried because tryptophan and 5-hydroxytryptophan were known to inhibit the

formamidases (105). Many enzymes which are inhibited by key compounds on metabolic pathways have been determined to be allosteric proteins, and generally have a large number of effectors, some of which protect the protein to denaturing agents or inhibitors. Consequently, the ability of anthranilate or alanine to protect either formamidase to pCMB was studied. The results are reported in Table 29. The enzyme was incubated with pCMB either with or without the added metabolite (at 1 mM) and assayed. The rate of the enzyme incubated with pCMB and the metabolite is compared to the rate of reaction of the enzyme preincubated with pCMB where the metabolite was added immediately before the substrate. As seen in the table, there was no protective effect of either metabolite with either formamidase when tested with pCMB. The results of a similar experiment with alanine and anthranilate on $HgCl_2$ inhibition of the 5-hydroxytryptophan-induced formamidase were also negative.

The substrate was also tested for its ability to protect the formamidases against $HgCl_2$ and heat inactivation. For the protection study with $HgCl_2$, 0.1 ml of 0.005 M formyl-L-kynurenine was preincubated with the $HgCl_2$. This rate was compared to the rate where the additional 0.1 ml of substrate was added with the substrate to initiate the reaction. The results are presented in Table 30. Addition of the substrate during the $HgCl_2$ treatment did not result in a higher activity relative to the control.

Several experiments were performed to test substrate protection of the formamidase enzymes against heat inactivation. If a comparison is

TABLE 29

The Effect of Anthranilate and Alanine on pCMB Inhibition of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases

Sample incubated with enzyme	Sample added at assay time	Per cent activity: 5-Hydroxytryptophan-induced formamidase, 10 μM pCMB	5-Hydroxytryptophan-induced formamidase, 20 μM pCMB	Tryptophan-induced formamidase, 10 μM pCMB	Tryptophan-induced formamidase, 10 μM pCMB
pCMB		64 ± 9	21 ± 0	40 ± 1	65 ± 0
pCMB + Anth.		44 ± 3	39 ± 9	52 ± 4	63 ± 5
pCMB	Anth.			70 ± 16	72 ± 11
Anth.		92 ± 6	92 ± 6	85 ± 28	95 ± 2
pCMB + Ala		53 ± 5	33 ± 1	39 ± 5	51 ± 7
pCMB	Ala			41 ± 6	52 ± 10
Ala		105 ± 17	105 ± 17	96	

The enzyme preparations were incubated 5 min. in the standard incubation mixture with the addition of the compounds indicated; anth. is 1 mM anthranilate and ala is 1 mM L-alanine. Then the enzyme was assayed and compared to the noninhibited controls. The values are expressed as means of two determinations ± SD, except in the one case where only one determination was made. In some cases indicated, a metabolite was added to the mixture immediately before the substrate was added.

TABLE 30

The Effect of Formyl-L-Kynurenine on $HgCl_2$ Inhibition of the Tryptophan- and 5-Hydroxytryptophan-Induced Formamidases.

Sample incubated with enzyme	Sample added before substrate	Per cent activity			
		Tryptophan-induced formamidase		5-Hydroxytryptophan-induced formamidase	
		10 μM $HgCl_2$	10 μM $HgCl_2$	3 μM $HgCl_2$	3 μM $HgCl_2$
$HgCl_2$		25 ± 2	22 ± 7	62 ± 4	66 ± 5
$HgCl_2$ + S		29 ± 10	17 ± 3	65 ± 3	88 ± 3
$HgCl_2$	S	33 ± 10	33 ± 1	76 ± 13	91 ± 10

The concentration of S is formyl-L-kynurenine, 1.67×10^{-4}M. The enzyme preparations were incubated in the standard incubation mixture 5 min. with $HgCl_2$ and sometimes S, then assayed. Other samples of $HgCl_2$-treated enzyme received the extra substrate just before the normal concentration of substrate was added. The values represent the mean of two determinations ± SD.

made between the rates for either enzyme heated with 2.5×10^{-3} M formyl-kynurenine versus the rates for the enzyme heated in water and assayed with the same final concentration of substrate, the rates are higher in the latter case for 3 out of 4 duplicate experiments. In the fourth case the range of values is high for each assay, and the means are quite close. Thus it is unlikely that the substrate, at least at this concentration, significantly protects the enzymes against heat inactivation. Higher concentrations of substrate could not be tested unless the assay system were modified, as the presence of additional substrate would result in higher slit widths and less accurate assays.

DISCUSSION

The problem of this thesis research, as reported in the Introduction section, was to determine whether the two degradative pathways induced by growth on tryptophan or 5-hydroxytryptophan are catalyzed by distinct enzymes. In a number of approaches the research has been focused on the formamidase activities, to answer the question of whether these enzymes are distinct. The properties of the tryptophan-induced enzyme as determined from the purification procedures will be discussed, as will the results from the studies with mutants. The major part of this discussion will examine the results of the *in vitro* kinetic studies with inhibitors in the light of the hypotheses proposed for the characters of the enzymes, i.e. whether there are single, duplicate, or dicephalic enzymes.

Purification procedures on the tryptophan-induced formamidase. The kynurenine-PABA-cellex procedure was unsuccessful in achieving purification of the enzyme. This was not probably due to improper diazotization of the cellulose, as the DL-kynurenine sulfate was in excess, and the procedure was a relatively standard one. The diazotization reaction with kynurenine should couple the compound at the 3 position, ortho to the amino group. Unless this sterically deforms the molecule, it should allow it to complex with the protein molecule. It may be that the original assumption, that formamidase would have an affinity for its product, was in error.

Apparently another problem was non-specific retention of the bulk of the protein by the resin. The column bed was 1.9 ml, providing a

void volume of about 0.8 ml (The void volume is stated by the manufacturer to be 40% of the bed volume.) The nonspecific binding of the protein to the resin, resulting in its retention by the resin, might have been eliminated with the use of a more acidic buffer, at least in the preliminary elution step. The technique of using an acidic buffer to limit nonspecific binding followed by a more basic buffer has been used with success by Lerman (183) in purification of tyrosinase activity. Recently a diazotized column has been used successfully for purification of a flavokinase (184).

The kieselguhr technique (158) was unsuccessful because of irreversible adsorption or denaturation of the enzyme. A survey of the current literature revealed that this technique had not met with much popularity. The only paper found to cite the technique (185), reported an unsuccessful attempt to purify phosphofructokinase with this method. The enzyme was eluted from the column with no increase in specific activity. The method is probably limited in applicability to certain proteins resistant to denaturation.

Fractionation of the enzyme preparation with DEAE cellulose produced two peaks when gradients of NaCl less than 1.0 M were employed. This is unlikely to have been due to channeling of the resin as there were no air bubbles present in the resin, and the bed was formed very evenly. Furthermore, the two peak effect occurred as a function of the salt concentration. The early peak may be an artifact, resulting from an interaction between residual ammonium sulfate and a fraction of the preparation. It may, however, reflect some second molecular form of the enzyme possessing enzymatic activity. The characteristics of this form must differ from the parent compound enought to allow

chromatographic separation. Multiple peaks on elution from DEAE cellulose with 0.01 and 0.08 M Tris-HCl were seen when carbonic anhydrase (186) was applied to such a column; such differences reflect the isozymic nature of the enzyme. Three peaks were eluted off the column; they comprised 100% of the enzymatic activity.

The behavior of the enzyme on Sephadex is suggestive of a large protein. There was no retention of activity by Sephadex G-75, only slight retention by G-100, and significant retention by G-200. From the chromatographic behavior, the molecular weight can be estimated to be in the range of 20,000 to 200,000. This range could have been narrowed considerably if one or more proteins of known molecular weight were chromatographed with the enzyme in a Sephadex G-200 column (187). This has become a popular means of estimating molecular weights.

The comparative chromatography was not done mainly because it would not provide information on the differences between the tryptophan- and 5-hydroxytryptophan-induced formamidases. However, there is a crude way of estimating the molecular weight of the enzyme. The enzyme is precipitated by a 35 - 55% saturated ammonium sulfate. A survey of the recent biochemical literature was done to compile Table 31, which lists fractionation ranges of ammonium sulfate and the molecular weight determination of the proteins precipitated within these ranges. Those studies were selected which employed an ammonium sulfate precipitation immediately after a protamine or streptomycin treatment of a crude preparation, so the studies were comparable with those on the tryptophan-induced formamidase. An examination of the table suggests that

TABLE 31

A Comparison of Ammonium Sulfate Precipitation Ranges and Molecular Weights of Proteins

Ammonium sulfate range	Molecular weight	Enzyme	Reference
0-35%	250,000	δ-aminolevulinic acid dehydratase	(188)
0-40%	540,000	β-galactosidase	(189)
30-40%	220,000	cis,cis muconate lacto nizing enzyme	(190)
37-53%	29,000	isomerase (histidine biosynthesis)	(191)
40-50%	190,000	β-carboxylactonizing enzyme	(192)
40-55%	200,000	threonine deaminase	(193)
47-53%	160,000	threonine dehydratase	(194)
40-65%	370,000	lactate oxygenase	(195)
40-60%	93,000	β-carboxymuconolactone-decarboxylase	(192)
45-60%	124,000	glutathione reductase	(196)
50-60%	93,000	muconolactone isomerase	(190)
	420,000	protocatechuate oxygenase	(192)
	33,000	muconate enol-lactone hydrolase	(192)

an enzyme precipitating at 35% saturation has a molecular weight about 240,000; at 55% the molecular weight would be about 130,000. Thus the molecular weight range of this formamidase would be from 130,000 - 200,000. This suggests a large protein comprised of a number of sub-units, some of which might be involved in control of the activity of the enzyme.

Mutant studies. The mutant studies were characterized by the instability of the mutants obtained. Some apparent reversion is due in part to the assay procedure which selects for organisms capable of metabolizing tryptophan or 5-hydroxytryptophan. The tryptophan plates were incubated for 2 - 4 days for the results and the 5-hydroxytryptophan plates for 1 - 2 days. The colonies which grew papillated on tryptophan or 5-hydroxytryptophan are a sign of mutation to the wild-type phenotype (197).

Some isolates were obtained which continued to segregate mutant and wild type cells, even on recloning. Such an isolate (labeled 2-reisolated) even though cloned 3 times from colonies failing to replicate onto tryptophan, continued to contain a mixture of cells with varying nutritional abilities on tryptophan. There are reports of such highly unstable strains (198). Such a strain may contain a mutator gene, if the instability is a property of many genes. If the effect is limited to one gene, it may reflect a break in the DNA. This break would usually be maintained on daughter strands. On occasion, the break would be closed by insertion of a base, which might produce a wild-type cell, which would be stable.

The penicillin procedure had one possible drawback. The use of broth cultures with penicillin might allow genetic recombination to be expressed. The recombination might be due to either phage or transforming DNA. Genetic recombination between two different mutants in the same gene could produce a wild type, thus removing one of the mutants (199). However as the mutational rate in any gene is small even with the best mutagens, the likelihood of two mutants with mutations in the same gene exchanging that genetic material is remote. If the mutational rate were so high as to make it possible, then penicillin selection would not be necessary. This did not seem to have been the case.

The replica plating technique posed another problem. The results were not very reproducible even on duplicate plates, and the decision of whether an organism had grown was quite subjective. Thus it was not possible to say with certainty after testing an organism for growth on tryptophan and 5-hydroxytryptophan what the growth response of the organism was on these substances.

The problem of the uniqueness of isolate 3311 has been raised in the Results section. Because of its limited growth response on most metabolites, it is not identifiable with any strain of pseudomonad. For example, it differs from P. acetovorans, a species of Pseudomonas generally isolated from the soil by enrichment techniques, by not growing on citric acid cycle intermediates. The loss of ability to utilize a wide spectrum of citric cycle intermediates could result from the loss of a single type of permease protein. The isolate has many properties of organisms in another genus, Moraxella. Members of this genus characteristically occur in pairs, the striking characteristic

of isolate 3311, and do not produce pigment. The members of one subtype of this group are nutritionally unexacting, do not grow on sugars (except for glucose) or liquefy gelatin, accumulate poly-β-hydroxybutrate, and do not reduce nitrate (200). However they do grow on acetate and do not grow on many of the amino acids utilized by isolate 3311, including tryptophan, phenylalanine, and leucine. Furthermore they are sensitive to low concentrations of penicillin. A final characteristic is a lack of flagella (220), whereas isolate 3311 is flagellated.

Isolate 3311 might be thought to be intermediate between these two genera. There is certainly a way of testing this idea as the base ratio (mole % G+C) of pseudomonads is from 60 - 67 (179) and for Moraxella it is 40 - 46% (200). If isolate 3311 is intermediate between the two, it implies that the G+C ratio of its DNA should also be intermediate.

Induction experiments. The induction of formamidase by 5-hydroxytryptophan appears entirely similar to the induction by tryptophan or by kynurenine. The enzyme is induced to significant levels by the end of a 3 hr period, although at 1 hr the specific activity is somewhat lower than that of the enzyme induced by the other inducers. The concentration of both 5-hydroxytryptophan and kynurenine for maximal induction is approximately 1×10^{-3} M.

However the induction of the other two enzymes is not entirely similar to the pattern of induction by tryptophan or kynurenine. The maximal level of the 5-hydroxytryptophan-induced tryptophan oxygenase

is not attained for 8 hr, while that induced by tryptophan or kynurenine is maximal by 3 hr. The kynureninase, on the other hand, is induced to maximum levels at 3 hr by both 5-hydroxytryptophan and kynurenine, whereas it takes 8 hr for tryptophan to induce maximally. Furthermore induction of the maximum specific activity of either enzyme requires $1 - 2 \times 10^{-4}$ M 5-hydroxytryptophan, while the concentration for induction by kynurenine in both cases is 1×10^{-3} M. A third point is that the level of the enzymatic activity, even when maximally induced is much lower (1/8 to 1/10) than that induced by kynurenine.

The differences in the induction time required to attain maximal specific activities between the enzymes induced by tryptophan and 5-hydroxytryptophan suggest that tryptophan oxygenase and formamidase are not synthesized coordinately in this organism, in contrast to the induction pattern of P. fluorescens TR 23 (49). The difference in the relative levels of formamidase and tryptophan oxygenase induced by the two compounds would substantiate this, unless the 5-hydroxytryptophan-induced tryptophan oxygenase has less activity per molecule than the tryptophan-induced enzyme.

As the amounts of the inducer 5-hydroxytryptophan required for saturation of the induction mechanism of formamidase on the one hand, and of tryptophan oxygenase and kynureninase on the other, are different, it is unlikely that the formamidase is synthesized under the same control mechanism as either of the other two. It is likely then that 5-hydroxytryptophan (or a metabolic derivative) induces formamidase and the set tryptophan oxygenase/kynureninase separately, as

seen in the case of P. fluorescens where formamidase and tryptophan oxygenase are in a set and kynurenine is not, yet they are both induced by kynurenine.

Another possibility is that the mechanism of induction is different in the two cases. One of the mechanisms could be the induction of the tryptophan-induced-type of enzymes. For reasons discussed later in this section, that is not the case for formamidase. Thus under this hypothesis, the 5-hydroxytryptophan would induce one special enzyme, formamidase, and by its resemblance to tryptophan, induce two tryptophan-induced type of enzymes. The induction of these two enzymes occurs at lower inducer concentrations than with kynurenine, a somewhat surprising conclusion. However in many cases unnatural inducers have been shown to be more efficient than the natural inducers. This then is another possibility suggested by the kinetic data.

A third similar possibility could be advanced. Since incubation with tryptophan does result in induction of all three enzymes, this could occur because tryptophan itself can induce or because it was metabolized to kynurenine, which induces. There are cases where more than one early intermediate can induce (for example, in the mandelate pathway). If both tryptophan and kynurenine were inducers, then tryptophan accumulation would induce the degradative enzymes even in the absence of kynurenine. The analog 5-hydroxytryptophan is known to inhibit greatly the tryptophan-induced formamidase, even at low concentrations (105). Thus when 5-hydroxytryptophan is added to the medium, it is likely that tryptophan would accumulate and induce. The disadvantage with this theory is that such tryptophan would induce a

formamidase as well, and this formamidase, being heat stable, would be detectable. Such an enzyme was not detected. Thus either tryptophan does not induce, or at that it only induces tryptophan oxygenase and kynureninase.

Other possibilities are advanced in a later section of this Discussion.

Studies on in vitro inhibition. In the step catalyzed by formamidase, there are three possibilities. The first suggests one enzyme, induced by either metabolite, catalyzes the step. The second possibility is one enzyme with two distinct enzymatic sites is induced by either metabolite; only one site is expressed and the decision of which site is expressed is influenced by the inducer. The third possibility suggests two distinct enzymes catalyze the reaction, and each is induced by only one metabolite. Evidence of the occurrence of each of these possibilities in other systems has been presented in the Introduction.

The molecular nature of distinctness of enzymes may be difficult to decide at times. Two totally unrelated activities will usually (with the exception of dicephalic proteins) reside on two totally unrelated proteins. For example the activities of succinic dehydrogenase and tryptophan oxygenase will respond differently to inhibiting agents, as a reflection of their dissimilar protein structures. However, enzymes with the same type of function will usually have similar properties particularly if they are subject to the same kind of control. For example most dehydrogenases now appear to be sulfhydryl enzymes (201, 202, 203) occurring as aggregates of several polypeptide chains (204,

205). This is the result of both homology at the active site, necessary for enzymes catalyzing similar functions, and a similarity in control manifested by changes in the tertiary and quartenary structures of the protein. The problem is even more difficult when the protein or enzyme evolved from another or both evolved from a common ancestor. Thus in the case of the homoglobins, chains of the protein evolved from one another and from common ancestors (206). When comparative analysis is made of binding to reagents, the proteins, even though distinct, would behave in a very similar manner. Thus if no differences are found with a limited number of agents, it is not safe to conclude that the proteins are the same, merely that they are very similar.

The empirical definition for distinctness used here is a significant difference in response to a number of inhibitors or inactivating agents. The results of these studies on the tryptophan- and 5-hydroxytryptophan-induced formamidases are summarized below:

Agent	Tryptophan formamidase	5-hydroxytryptophan formamidase
Heat	stable (biphasic)	labile (monophasic)
Phosphate on heat denaturation	increases denaturation	protects against denaturation
Urea, 3 M	34% activity	8% activity
NEM, 1.0 mM	28% activity	approx. 100% activity
$HgCl_2$	triphasic inhibition	monophasic inhibition
pCMB	diphasic inhibition	monophasic inhibition
Arsenite, 2 mM	81% activity	5% activity
Iodoacetate, 10 mM	40% activity	40% activity

For such comparative studies with inhibitors, it is preferable to work with pure preparations of the enzymes, to eliminate the possibility that the inhibitors are acting on molecules other than the enzyme, for example, activators. But this sort of work is useful even with crude enzyme preparations in obtaining relative data on differences between proteins. This is particularly the case when the preparations are obtained from the same organism under similar growth conditions. There will be the same spectrum of extraneous proteins present in each case.

Even in crude preparations of enzyme, if an enzyme is sensitive to an agent present in micromolar quantities, it is possible to say that the enzyme is acutely sensitive to that reagent, particularly as the concentration of the reagent available for inhibition is further reduced by its binding to other proteins present. If a reagent is inhibitory at millimolar concentrations, a comparison of behavior of the two enzymes should be valid as the concentration of unbound inhibitor in the reaction mixture is still likely to be the same; the concentration is high enough so that the presence of a minority protein species in one preparation strongly binding the reagent should not affect the results to any significant extent.

The similarities between the tryptophan- and 5-hydroxytryptophan-induced formamidases are many. Both appear to be proteins with essential sulfhydryl groups, as both are inhibited by micromolar concentrations of mercurials. It is possible that they each have a requirement for a metal ion, as 8-hydroxyquinoline, a complexing agent, inhibits. However, this compound can bind sulfhydryl groups (207). The inhibition of the rabbit enzyme by this reagent was said to be reversed by Zn^{++}

and Co^{++} (42), suggesting that a metal ion was involved. The concentration of chelator (2 mM) was extremely high though, and many nonspcific reactions might be involved. Similarly, EDTA, which caused some inhibition of the bacterial enzymes, was also used in millimolar amounts in order to compare the results with those of Kotake (42).

Both enzymes are sensitive to a concentration of urea considered low (208). Both have catalytic activity on formyl-L-kynurenine and 5-hydroxyformyl-L-kynurenine (105), and similar K_m values for the former substrate. However, there are also many profound differences.

The enzyme induced by 5-hydroxytryptophan is quite heat labile, while that induced by tryptophan is relatively stable, and thus quite distinct from the other enzyme. This alone precludes a single protein with a single active site. Thus there are only two possibilities to be seriously considered, one protein with two sites or two proteins. In the former case the enzymatic site expressed in the presence of 5-hydroxytryptophan is assumed to be heat labile.

The difference in behavior of the enzymes when exposed to an increase in phosphate ions during heat denaturation may suggest structural differences between the two proteins, although the mechanism of ion protection against inactivation is unknown. The two most likely explanations (209) suggest binding to amides or affecting the internal pressure of the protein. Both hypotheses appear to invoke broad structural properties of the enzyme, and thus it would seem that if a salt protected one protein and speeded denaturation in another, that the two proteins were different in the number or type of amide bonds or differed in internal pressure.

The slight difference in sensitivity to urea by the two formamidases may reflect differences in unfolding or disaggregation of these enzymes exposed to urea. In other proteins, the addition of urea exposes some additional sulfhydryl groups (210, 211), suggesting that the protein in the presence of urea has unfolded somewhat. Other structural changes are suggested by the desensitization by urea of certain proteins to their effectors (212). In one case a metal ion bound to the protein became easily removed after urea treatment (213). If the difference in urea sensitivity were due to differences in disaggregation, then the hypothesis of one protein with two sites is less tenable. However, there is no evidence to support this mechanism of urea action in this case, and the difference between the proteins is not profound.

The behavior of the enzymes toward $HgCl_2$ and pCMB, agents which can disaggregate or unfold proteins (203, 205) is similar up to about 70% inhibition of enzymatic activity. Thereafter differences appear. With both mercurials the 5-hydroxytryptophan-induced formamidase is increasingly inhibited with increasing concentrations of the reagent, the so-called monophasic curve.

The tryptophan-induced formamidase with increasing concentrations of $HgCl_2$ increases abruptly in activity, from 15% activity at the low point to 40% activity. The level of activity of the enzyme falls only slowly thereafter with increasing $HgCl_2$ concentrations, with the production of a broad, gently sloping plateau. This is termed a triphasic inhibition curve. This strongly suggests that a conformational change has occurred to the protein with 15% activity, usually at $2 - 8 \times 10^{-6}$ M $HgCl_2$,

leading to an increase in activity. The possible mechanisms of these changes will be discussed in more detail later.

If pCMB inhibition of the tryptophan-induced formamidase is studied, there is found a biphasic inhibition curve. The activity of the enzyme drops steadily with increasing concentration of the mercurial to some point, at about 20 mM pCMB; at higher concentrations, the rate of decay is even slower. A number of possibilities can explain this two-step curve. There may be a heterogeneity in the affinity of the binding sites on the enzyme for pCMB, such that certain sites are filled rapidly and then others are filled more slowly. The molecules themselves may be heterogenous, with one sub-population relatively resistant to this agent. The same diphasic inhibition curve is seen with heat inactivation, but if denaturation in this case is due to unfolding, either hypothesis will serve to explain this curve as well. Heterogeneity in the population is not due to 5-hydroxytryptophan-like formamidases, as these are extremely heat labile and would be inactivated completely before any inactivation of the tryptophan-induced enzyme would be apparent. A third possibility to be considered is that pCMB is doing, in a less efficient manner, what the $HgCl_2$ does. It does open the molecule up, but then cannot stabilize it. If this were the case, the inflection point on the pCMB curve should correspond to the concentration of $HgCl_2$ where the enzyme is 15% active, i.e. down in the trough. It does not; the concentration of pCMB is 5 to 10 fold higher. As pCMB is bulky and would be expected to bind to fewer groups than $HgCl_2$, a lower pCMB concentration than $HgCl_2$ could be explained. It is more difficult to rationalize a higher concentration. One could always

suggest that the affinity of pCMB for this special site (or sites) was lower.

The presence of two formamidases is indicated by their different response to NEM, which at least for the tryptophan-induced formamidase, is an uncompetitive inhibitor and therefore in addition to binding at the active site also binds to sites distinct from this. These latter sites apparently do not occur in the 5-hydroxytryptophan-induced formamidase, and thus they differ in more than the active site. The behavior of the enzymes is also different with arsenite. One formamidase is sensitive to arsenite and the other relatively insensitive. However, the enzyme sensitive to arsenite is insensitive to NEM. The different behavior of these sulfhydryl reagents for two sulfhydryl-dependent proteins must be explained.

Arsenite is a quite specific reagent for sulfhydryl groups (214). In some cases it may bind two vicinal sulfhydryl groups to produce inhibition. Thus it is possible that the 5-hydroxytryptophan-induced enzyme, which is more sensitive to this reagent, contains two adjacent sulfhydryl groups which the tryptophan-induced enzyme lacks. Another possibility is that the smaller size of the arsenite molecule allows it to reach an area inaccessible to NEM. However, the 5-hydroxytryptophan-induced enzyme is not grossly more inhibited by $HgCl_2$, which is small and may bind adjacent sulfhydryl groups. If the site were surrounded by an area of positive charge, for example protonated histidines, the negatively charged arsenite molecule could bind to produce inhibition while the positively charged Hg^{++} ion would be electrostatically repelled. Thus the difference in arsenite behavior of

the two molecules may either be due to vicinal sulfhydryl groups present in the 5-hydroxytryptophan-induced protein, or a difference in the charge in a restricted area of the molecule containing a sulfhydryl group, or both.

The behavior of NEM with the 5-hydroxytryptophan-induced enzyme suggests that the enzyme lacks a NEM binding site present on the tryptophan-induced formamidase. One would presume that this group is a sulfhydryl group and that this would also bind arsenite. Then the tryptophan-induced formamidase ought to be more sensitive to arsenite than the 5-hydroxytryptophan-induced enzyme, whereas the reverse is true. There are a variety of hypotheses which might be invoked to explain this difference in effect of inhibitors. 1) Perhaps the NEM is not binding a sulfhydryl group at all on the tryptophan-induced formamidase, but rather some other group. Although it has been used as a specific reagent for determination of sulfhydryl groups (215), it is known to add on to the N terminus of hemoglobin (216) and certain amino groups (217) under mild conditions. If $HgCl_2$ were known to bind only to sulfhydryl groups, the protection experiment where $HgCl_2$ increasingly occupied NEM sites until the protein was insensitive to this reagent would seem to preclude binding of groups other than sulfhydryl groups. However, the specificity of $HgCl_2$ is merely assumed. It remains possible that NEM binds to some site and causes inhibition by a consequent conformational change; $HgCl_2$ may protect the protein from such a change. 2) NEM binds a sulfhydryl group in both molecules, but in only the tryptophan-induced enzyme is there enough flexibility to allow conformational changes which are

inhibitory. This explanation is reasonble and is consistent with other hypotheses about the proteins. 3) NEM binds to one of the vicinal sulfhydryl groups postulated for the 5-hydroxytryptophan enzyme. The bulkiness of the group prevents binding to the other group. Only when both (or the second) groups are blocked is the molecule devoid of activity. As the tryptophan-induced molecule has only one such group by the same hypothesis, and because the NEM group is very bulky, the enzyme is inhibited.

What phenomena may account for the activation of the tryptophan-induced formamidase by $HgCl_2$ and not by pCMB? Allosteric proteins are desensitized toward their effector molecules by $HgCl_2$ (218,219), presumably by a structural change (205). However pCMB and other aromatic mercurials will also cause desensitization (204, 220, 221). Thus the action of $HgCl_2$ in producing activation of the enzymatic activity is not by a manner analogous to its desensitization ability, as pCMB has a similar ability to desensitize but lacks the ability to activate.

Similarly, the phenomenon is not due to $HgCl_2$-induced disaggregation, as pCMB is also reported to unfold proteins (203, 213, 222, 223).

Perhaps the small size of $HgCl_2$ is responsible for the increase in activity. As the protein is unfolded by treatment with the mercurial, at one stage in the unfolding the Hg^{++} can reach a sterically hindered sulfhydryl group and bind to it, to produce activation, either by a refolding to support the active site or to expose new sulfhydryl groups which would compete effectively for and remove mercury already bound to critical positions. Thus the effect is either a refolding to a stable

position, or an effective lowering of the free $HgCl_2$ concentration. The hypothetical reason for the inactivity of pCMB is its larger size. The sulfhydryl group that when bound induces conformational changes is not accessible to this larger reagent. The 5-hydroxytryptophan-induced enzyme, which shows no activation with either reagent either lacks the sterically blocked sulfhydryl group, or is unable to undergo the conformational changes. This hypothesis suggests that if an array of mercurials were ordered by size, the smaller ones such as methylmercuric chloride would allow an increase in activity in the tryptophan-induced enzyme, while the larger ones would not.

Another basic difference between $HgCl_2$ and pCMB is that the former is divalent and may form bridges between sulfhydryl groups, while the latter is monovalent and cannot. This hypothesis suggests that these bridges are essential to stabilize the conformational change. Thus, pCMB is ineffective because it cannot form bridges. Again with the 5-hydroxytryptophan-induced molecule either 1) there is no way for these bridges to form, due to a lack of one of the groups or to a rigid structure, or 2) even when bridges are formed the conformation is not the same as the active one. This hypothesis suggests that methylmercuric chloride and all monosubstituted mercurials would behave like pCMB, and the resulting curve for the tryptophan-induced formamidase should be diphasic. One might further predict that, barring problems with charged groups, arsenite would behave in the same manner as $HgCl_2$, as it is divalent. Indeed, at a certain arsenite concentration, the activity of the tryptophan-induced enzyme is higher than the

5-hydroxytryptophan-induced enzyme, but the behavior of the tryptophan-induced enzyme in varying arsenite concentrations has not been studied in any detail.

One last possibility remains. The lack of function of pCMB may not be due to a lack of $HgCl_2$ character (e.g. size or valence), but to the aromatic character of pCMB. For example, aromatic mercurials will dissociate glutamic dehydrogenase into subunits, while methylmercuric chloride is ineffective (205). Thus it is possible that pCMB dissociates the molecule, not allowing for interactions of the subunits. In this case, the bulky aromatic mercurials would act like pCMB, while methylmercuric chloride might act like $HgCl_2$. To distinguish this possibility from the first (that size is the key factor), a bulky, nonaromatic mercurial would need to be employed. According to hypothesis 1, it would behave like pCMB, according to hypothesis 3 it might behave like $HgCl_2$.

There is at least one other case in the literature of a difference in action of $HgCl_2$ and pCMB. Inhibition of hemoglobin with $HgCl_2$ increases the affinity of the molecule for oxygen, while pCMB has no such effect (224).

The peculiar kinetics with $HgCl_2$, when plotted to give the type of inhibition, were at first perplexing. However, if the agent is capable of inducing structural changes in the molecule, then the type of inhibition is apt to appear complex. When plotted in a Wolff plot, the points appear scattered, and do not appear a good fit, to the least-squares straight line fitted to the data. When the data are plotted in a Lineweaver-Burk plot, however, at high and low substrate values

the velocity values are too low. Thus the curve is concave upward. A similar case has been reported, where if the incubation period with the mercurial was short, the curves were concave upward (225). However if low concentrations of mercury or long incubation times were used, the kinetics were linear on such plots. This suggests time-dependent conformational changes are occurring, and that the velocity of such changes is perhaps dependent on the substrate concentration. This is peculiar as the inhibition in the formamidase case appears entirely noncompetitive.

On the basis of the inhibition studies, the enzymes are established to be different. This is not in conflict with the similar K_m values; the enzymes are merely equally efficient in binding substrate. The efficient binding by the 5-hydroxytryptophan-induced enzyme and the high levels to which it may be induced suggests that it is not merely a nonspecific hydrolase which could weakly catalyze the conversion of formylkynurenine to kynurenine. The other two enzymes may be a different case as they are not induced to high levels; their K_m values were not determined.

How might this second formamidase have arisen? One likely possibility is that it is an enzyme from the quinolinic pathway. Some pseudomonads with a functional aromatic pathway possess enzymes from the quinolinic pathway (4,45,226). These enzymes are physiologically nonfunctional, as often the precursor is not synthesized, yet are induced by growth on tryptophan. It might be that isolate 3311 has a formamidase that arose from the quinolinic pathway. The presence of two enzymes which catalyze a single step is physiologically reasonable

if they are each subject to different control mechanisms, as they lead to different products. As discussed in the Introduction, the presence of two steps each catalyzed by two enzymes has been found in a degradative pathway in Moraxella (81). Thus it might be that this second formamidase lost the ability to be induced by tryptophan but could be induced by 5-hydroxytryptophan, or a product thereof. If the specificities of the oxygenase and kynureninase were broad, and if they could be induced somewhat by growth on 5-hydroxytryptophan, it would be possible for the organism to grow on 5-hydroxytryptophan. The specificities of kynureninase are broad, as documented before. Tryptophan oxygenase of other pseudomonads, for example TR 23 (34) will not act on and is inhibited by 5-hydroxytryptophan, but it is easy to envision a loss of such inhibition by mutation. This hypothesis accounts for the difference in control of formamidase and the oxygenase and kynureninase by the 5-hydroxytryptophan-induced organism. In one case a new enzyme is formed, and in the other the tryptophan enzymes are formed. The induction curves (concentration of inducer vs. specific activity) are quite shallow; although the enzymes are maximally induced at lower concentrations of 5-hydroxytryptophan than of kynurenine, the specific activities of the enzymes when maximally induced are lower. This would suggest that 5-hydroxytryptophan cannot completely prevent a repressor from binding to the DNA, but can efficiently bind to the repressor.

This suggested mechanism would allow the organism to utilize a new compound without synthesizing any new enzymes, just by substitution of a formamidase less inhibited by 5-hydroxytryptophan. Because even though the tryptophan-induced formamidase is active on 5-hydroxyformyl-

kynurenine, it is so inhibited by 5-hydroxytryptophan that the degradation of 5-hydroxytryptophan is much too slow to serve as a reasonable pathway for the organism. Once this step is bypassed, the other broadly functional enzymes will carry out the necessary interconversions. This must also be the case for the catechol-degrading enzymes, as when 5-hydroxytryptophan-induced cells are incubated with 5-hydroxytryptophan, there is extensive degradation of the ring; no aromatic substances appear to accumulate (105).

One factor difficult to explain by this hypothesis is the ability of the tryptophan-induced tryptophan oxygenase and formamidase to hydrolyze 5-hydroxylated intermediates. There would be a selective advantage immediately apparent to an organism possessing the nonspecific kynureninase and the 'quinolinic' formamidase if the oxygenase could metabolize 5-hydroxytryptophan, but why should the formamidase, which physiologically cannot act on the 5-hydroxylated compound, have this specificity? The only explanation advanced here for this is that the two enzymes are related to one another, and this affinity for the 5-hydroxy derivatives is a property of a common intermediate.

There is a second major explanation for the ability of the organism to elaborate the second set of enzymes. The analog, 5-hydroxytryptophan, may induce the same set of enzymes as tryptophan, but it may be incorporated into protein preferentially instead of tryptophan, and alter their properties. Incorporation into formamidase would, according to this hypothesis, produce a protein with altered behavior, namely, heat labile, susceptible to arsenite, and insensitive to NEM.

Incorporation into tryptophan oxygenase or kynureninase would produce less functional proteins, or the ability for maximal induction might be impaired. Amino acid analogs have been known to enter protein synthesis (230). There are a number of altered proteins produced as a result of incorporation of these analogs, for example β-galactosidase synthesized in the presence of either β-thienylalanine (227) or selenomethionine (228) is more labile to heat, urea and proteolytic agents than is the parent enzyme. The incorporation of 5-hydroxytryptophan into the proteins of the other enzymes would explain the difference in behavior observed; the enzymes induced by tryptophan more rapidly hydrolyze nonhydroxylated intermediates, while those induced by 5-hydroxylated intermediates (105); this may result from a slight alteration in protein structure.

If 5-hydroxytryptophan is incorporated into proteins, the activating enzyme of the organism must be relatively nonspecific, accepting either tryptophan or 5-hydroxytryptophan. This incorporation may explain why certain pseudomonads, like strain TR 23 (34) are inhibited by 5-hydroxytryptophan. It may either compete for the activating enzyme or sites on the s-RNA yet not be incorporated into protein, or if it is incorporated into proteins, some key protein is not functional.

The presence of an analog in the protein of formamidase could well explain both the similarities and differences in the behavior of the molecule. It explains the similar induction of this enzyme by either tryptophan or 5-hydroxytryptophan, and explains the similar K_m

values. Many of the properties of interaction with inhibitors might be explained by a rigidity of the molecule caused by a difference in configuration because of the bulk of the hydroxyl group; or the hydroxyl group may participate in hydrogen bonding to stabilize the structure.

There are objections to this model, although some of them can be simply answered. 1) When the organism is induced by a mixture of tryptophan and 5-hydroxytryptophan, the heat lability of the formamidase appears to be a mixture of labile and stable entities, instead of an intermediate heat stability (229). However, there is only a small number of tryptophan residues in any protein, and the results may be due to a competition of two compounds to fill a very limited number of sites. 2) One of the characteristic effects of an amino acid analog which is incorporated into proteins is 'linear growth' of bacterial cultures; when the metabolite is added to the growth medium the growth of the organism becomes quite slow and does not approach logarithmic growth (230). This is thought to be due to the limitation of essential metabolites due to nonfunctional proteins. The isolate 3311 grows logarithmically, not linearly, on 5-hydroxytryptophan. However like most generalizations, exceptions have been found. In a Bacillus species, there is logarithmic growth with m-tyrosine, even though the analog is incorporated into proteins (231). This is thought to be due to either the limited number of proteins containing tyrosine, or the nonparticipation of tyrosine at the active site of proteins. The same argument could be made for tryptophan. 3) The 5-hydroxytryptophan-induced

formamidase begins to precipitate at lower ammonium sulfate concentrations than the tryptophan-induced enzyme, which suggests the protein is larger. Perhaps the hydroxyl groups change the shape so that the radius is larger, or induce a tendency for aggregation. Since there is a large amount of extraneous protein present, also presumably enriched with hydroxyl groups, this extraneous protein results in increased interactions with the formamidase protein. 4) The final objection is that the results with the mutants seemed to indicate that mutants could be obtained which would only grow on tryptophan, or 5-hydroxytryptophan. This could be explained by either of two hypotheses. There is likely a permease for the tryptophan molecule which also transports the 5-hydroxytryptophan molecule. The mutations might have affected the properties of the permease, so that only one compound was efficiently transported into the cells. If the metabolite was entering the cell extremely slowly by diffusion only this would explain the slow growth of the mutants on the compounds. The other site of mutation could be the activating enzyme. If the enzyme no longer activated 5-hydroxytryptophan, the organism could not grow on 5-hydroxytryptophan at all efficiently, as the formamidase would be inhibited. If it only activated 5-hydroxytryptophan and not tryptophan, the organism could not grow unless this compound were supplied. Thus the presence of organisms growing on 5-hydroxytryptophan and glutamate, but not tryptophan cannot be explained by this latter mechanism.

Is there a third possibility, namely that 5-hydroxytryptophan induces the tryptophan formamidase and then alters the preformed

protein? The alteration of a formed enzyme has been documented for glutamine synthetase (232,233), leading to an enzyme with altered behavior. However, this would imply an efficient conversion by 5-hydroxytryptophan, as heat-stable molecules are not formed. If a little tryptophan is added to the medium, however, this conversion would have to be inefficient, as now heat stable molecules are formed. This inefficiency could result from a slower rate of alteration, fewer sites altered or fewer proteins altered. If the process included aggregation, it would explain the ammonium sulfate behavior. This does not explain the alteration of induction of tryptophan oxygenase or kynureninase, nor the lower specific activities attained. Thus the theory is not very satisfactory in explaining the data.

The idea of physiologically distinct enzymes metabolizing tryptophan and 5-hydroxytryptophan is in agreement with the degradation of mandelate and p-hydroxymandelate discussed earlier. There too are some distinct enzymes, as well as some common enzymes. The other cases of metabolism of analogs cited in the Introduction appeared to be catalyzed by single enzymes. Either the situations are different for mammalian and bacterial enzymes, or the conclusions that the same enzyme was involved in the metabolism of both compounds were based on no serious examination of the alternate possibility. Few of the authors based their conclusions on more than substrate specificity and K_m values. This could lead to erroneous conclusions.

Control mechanisms. Perhaps it is not surprising that no repressors of the degradative pathways were found. Repression by exogenous

succinate would be unlikely as the absence of growth on this compound suggests that the organism has a deficiency in transporting this compound into the cell. The absence of repression by alanine is reasonable, as a fairly large pool size of alanine must normally be present within the cell, and an overabundance of that compound can be eliminated by a small number of degradative enzymes. Anthranilate would have been a logical candidate as a repressor, but as the compound does not support good growth of the organism, it might have toxic side effects which would obscure any role as repressor, or have been poorly transported into the cell.

The amount of repression demonstrated in the mandelate pathway is a reduction to 5% enzymic activity with the good repressors like benzoate, and to 44% by acetate (234).

The key question might not concern the type of control mechanism for the degradation of tryptophan, but rather the lack of control. The organism grows very slowly on tryptophan, even more slowly than on 5-hydroxytryptophan. This tends to suggest an imbalance in growth when excess tryptophan is present. If there is no ready way to shut off tryptophan degradation except by decreased induction and a dilution of the tryptophan-degrading enzymes, the organism might deplete its tryptophan pool, and have difficulty in maintaining protein synthesis. For if the induction had occurred with a saturating concentration of tryptophan, the levels of the degradative enzymes would increase 10 - 20 fold. This increase would compensate for the difference in K_m values for the s-RNA synthetase enzyme and tryptophan oxygenase. The

inhibition by tryptophan on the formamidase would not help, as the inhibition is competitive. This means, in a sense that the substrate and inhibitor are always present in the same ratio, unless there is a control exerted on the tryptophan oxygenase activity independent from this control on the formamidase activity. Thus, no special technique for turning the pathway off when the pool size of tryptophan has been reduced or when intermediates build up has been shown. Furthermore, the organism grows very slowly on the metabolite.

In this degradative pathway, both kynurenine and tryptophan were found to induce. With no mutants available with metabolic blocks between these two compounds, it was impossible to determine whether tryptophan could induce, or induced only through the formation of kynurenine. In the mandelate system, Hegeman (77) showed with blocked mutants that more than one metabolite could induce the degradative system, however Palleroni and Stanier (49) demonstrated that for tryptophan degradation in P. fluorescens TR 23, the sole inducer was kynurenine. One can certainly speculate that kynurenine is the sole inducer in the degradation of tryptophan in this organism. When the organism is grown in the presence of 5-hydroxytryptophan, the tryptophan pool size should increase as 5-hydroxytryptophan interferes with tryptophan degradation. Yet no heat stable formamidase, which would be induced by the greater concentration of tryptophan if tryptophan were an inducer, is formed. Thus, it is likely that kynurenine is the inducer in this system as well.

SUMMARY AND CONCLUSIONS

It was possible to effect partial purification of the tryptophan-induced formamidase molecule. Estimates made during the fractionation procedure suggest a molecular weight of 100,000 - 200,000.

Studies with mutants lacking the ability to degrade tryptophan and/or 5-hydroxytryptophan suggested the two abilities could be independently lost or regained.

Studies on the kinetics of induction of the first three enzymes on the pathway indicated that the control of formamidase production by 5-hydroxytryptophan was different from the control of kynureninase and tryptophan oxygenase production. The control of formamidase production was similar to those seen for tryptophan or kynurenine induction.

Studies with inhibitors of the formamidase preparation revealed that the 5-hydroxytryptophan-induced enzyme differed from that induced by tryptophan by its 1) extreme heat lability, 2) insensitivity to NEM and 3) sensitivity to arsenite. The behavior of the enzyme to $HgCl_2$ and pCMB when studied at a variety of inhibitor concentrations revealed significant differences from the tryptophan-induced formamidase.

The 5-hydroxytryptophan-induced formamidase appears distinct from the tryptophan-induced formamidase. The other two enzymes may also be distinct.

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