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# THE ACIDIC TRANSFORMATION PRODUCTS OF INDOLES FORMED BY BASIDIOMYCETES

BY

CHUNG-KE, CHANG -1935-

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A

THESIS

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## ABSTRACT

A survey of the ability of basidiomycetes to convert tryptophan, tryptamine and indole acetic acid to other acidic indoles has been made.

The product formed from tryptamine by Tricholoma nudum was identified as indole acetic acid. The product formed by Cantharellus cibarius from D-tryptophan was identified as N-acetyl-D-tryptophan. The products were purified and identified by means of paper chromatography, thin layer chromatography, infrared spectroscopy and melting points.

The conversion of tryptamine and D-tryptophan was also studied in cultures of Leucopaxillus paradoxus, Hygrophorus conicus and Naucoria confragosa. H. conicus, L. paradoxus and N. confragosa transformed tryptamine and indole acetic acid into oxindole acetic acid. L. paradoxus and N. confragosa also transformed D-tryptophan to oxindole acetic acid, but in poor yield. Two unidentified indole compounds were found in the culture media of L. paradoxus and N. confragosa grown on tryptamine.

Another product formed from tryptamine and indole acetic acid by T. nudum and N. confragosa was identified as 5-hydroxy-indole acetic acid. T. nudum also transformed N,N-dimethyl-tryptamine into indole acetic acid and 5-hydroxy-indole acetic acid, but no transformation was observed with either α-ethyl- or α-methyl-tryptamine.

An attempt to develop an assay method for the mono-amine oxidase of T. nudum was not notably successful.

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# I. INTRODUCTION

The catabolism of tryptophan in animals occurs by way of two major pathways. One involves oxidation of tryptophan to kynurenine which is then converted to 3-hydroxyanthranilic acid and finally into nicotinic acid. The other pathway involves hydroxylation of tryptophan to hydroxytryptophan and decarboxylation of the amino acid to 5-hydroxytryptamine (serotonin). Other minor pathways also exist in animals' tissue. In microorganisms, additional reactions occur including some that are similar to those catalyzed by enzymes occuring in animal tissues. In plants, the origin of indole acetic acid has been extensively studied because of its importance as a plant-growth hormone. The indole nucleus is also found in plant and microbial pigments, alkaloids and toxins. Some of these secondary metabolites have attracted considerable attention because of their possible relationship to mental disease.

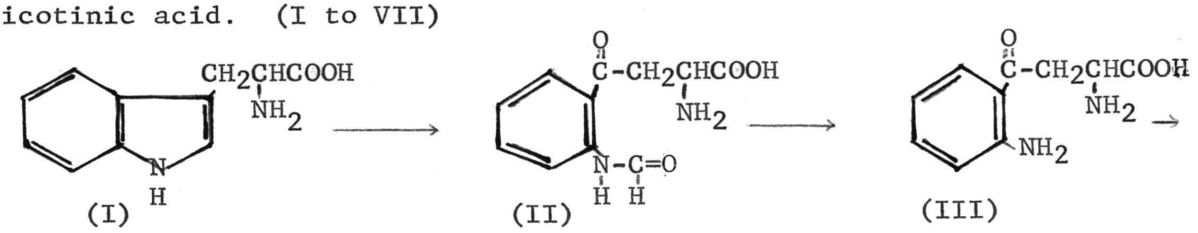
Many of the advances in the study of the metabolism of indole compounds have been possible through the use of paper chromatography and Ehrlich reagent which gives a distinctive color with the indole nucleus.

A survey, relying primarily on paper chromatography, was made of the ability of five species of basidiomycetes to transform tryptophan, tryptamine and indole acetic acid. This was done in an effort to discover pathways for the metabolism of indole compounds not as yet discovered in other organisms.

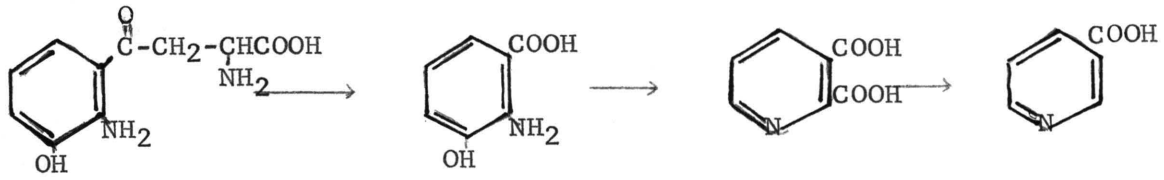
## II. LITERATURE REVIEW

### A. Physiological Activity of indoles.

The normal degradation of tryptophan in animals occurs mainly by two means. One involves oxidation of tryptophan to kynurenine, which is converted to 3-hydroxyanthranilic acid and nicotinic acid. (I to VII)

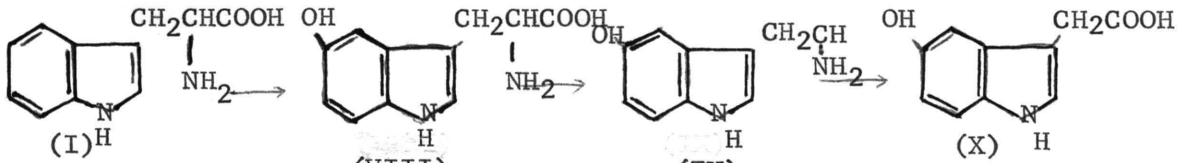


(I) (II) (III)



(IV) (V) (VI) (VII)

The other is the oxidation of tryptophan to 5-hydroxy-tryptophan and subsequent decarboxylation to 5-hydroxytryptamine (serotonin). The further metabolism of the latter compound to 5-hydroxyindole acetic acid generally follows (I, VIII, IX, X).



(I) (VIII) (IX) (X)

Many human disorders are considered in-born errors of metabolism and in certain cases are associated with abnormalities of amino acid metabolism. Discussed below are some of the metabolic disorders involving indole compounds. The indole compounds in many cases are derived from the amino acid tryptophan.

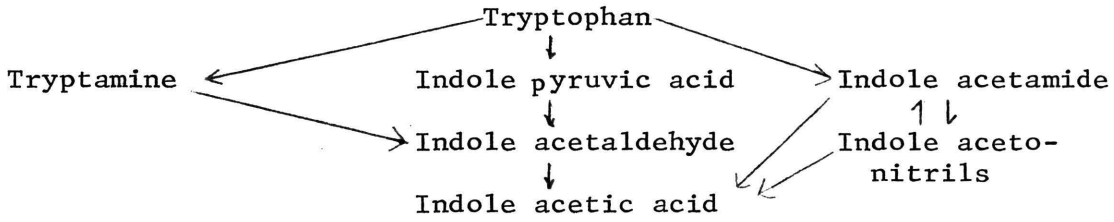
Hartnup disease has been found to be associated with mental retardation. The syndrome exhibits pellagra-like skin rashes and the excretion of indolyl-acetyl glutamine, indican and other indole compounds in the urine(Jepson 1956). These observations suggest an abnormality in tryptophan metabolism in the human body. The disorder is also related to a nicotinic acid deficiency.

In certain patients with advanced malignant carcinoma, tryptophan metabolism often takes place predominantly by the serotonin route (Sjoerdsma 1956). The patient excretes large amounts of 5-hydroxyindole acetic acid and the concentration of serotonin in the blood is higher than normal. There has been much speculation about the function of serotonin in the brain, and Woolley (1962) has recently summarized evidence concerning the possible relationship between the metabolism of serotonin and the development of psychoses.

Several investigators have found increased amounts of indole acetic, indole lactic and indole pyruvic acid in the urine of patients with phenylketonuria (Armstrong 1958). While in these same patients the amount of urinary 5-hydroxyindole acetic acid in reduced, indicating that perhaps there is some defect in the metabolic conversion of tryptophan to 5-hydroxytryptamine (serotonin). Retarded mental development frequently is exhibited in children with this metabolic defect.

A plant growth hormone from human urine has been identified as indole acetic acid. There is good evidence that tryptophan is the precursor of indole acetic acid (Gordon 1949) and several

plausible pathways for the conversion of tryptophan to indole acetic acid have been suggested. However, none is conclusive.




Unlike the other indolyl excretion products, microorganisms in the gut of animals are probably responsible for the formation of the indole acetic acid rather than the animals themselves.

B. Microbial Transformation of Indole Compounds.

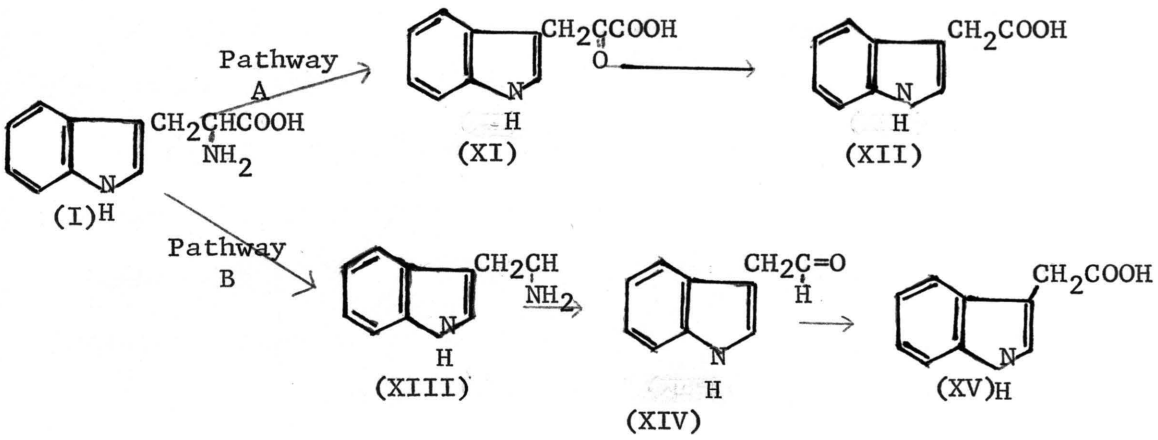
Investigations of the metabolic products of microorganisms have contributed substantially to the understanding of metabolic processes in general, and information thus derived may be applied to the related field of animal biochemistry. Studies of microbial biochemistry may offer clues to possible pathways for the production of indole excretion products associated with certain human diseases. Also it is possible that certain metabolites will accumulate in fungal culture which will never be observed in animals because either the product is extremely toxic or the product is further metabolized. Hence, certain metabolic pathways will go undetected in animals unless the pathways found in microorganisms are specifically sought in animals.

Other pathways than those known to occur in animals for the degradation of tryptophan are known in bacteria. Products formed by bacteria from tryptophan are indole, catechol, kynurenic acid and 5-hydroxytryptophan (Adelberg 1956 and Greenberg 1954)

Tryptophan catabolism in the fungus, Neurospora crassa, has been intensively investigated principally in relation to nicotinic acid biogenesis. These studies were reviewed by Bonner and Yanofsky (1950) and the suggested scheme for the formation of nicotinic acid from tryptophan is similar to that mentioned earlier (I to VII).

Evidence for this pathway comes primarily from the ability of certain compounds to replace nicotinic acid in the nutrition of deficient mutants of N. crassa and from the accumulation of intermediates by deficient strains. The steps from 3-hydroxyanthranilic acid to nictonic acid are still uncertain (V to VII).

Indole acetic acid (IAA) is a plant-growth hormone which is widespread in nature. Indole acetic acid was first isolated by Kögl and Haagen-Smit (1934) from a culture of the fungus Rhizopus suinus. The yield of indole acetic acid depended on a specific substance in the medium. This substance was found to be tryptophan. Although the mechanism of transformation has not been determined, it is postulated that tryptophan is first converted by decarboxylation to tryptamine which in turn is oxidized via the corresponding aldehyde to indole acetic acid (Pathway A, I, XI, XII). On the other hand, deamination of tryptophan by transamination would yield indole pyruvic acid which could give indole acetic acid by decarboxylation, as was suggested by Mehler (1955) and Went and Thimann (1937) (Pathway B, I, XIII, XIV, XV).




Horak (1964) found that 8 species of the obligate mycorrhiza fungus (Phlegmacium wunsch spp.) of spruce convert tryptophan through oxidative deamination into indole acetic acid. The transformation was thought to be by pathway B (I, XII, XIV, XV).

In the studies on Pseudomonas savatonoi (a bacterium), Beltra (1964) found that tryptophan is transformed into indole pyruvic acid and indole acetic acid. Indole pyruvic acid and other degradation products were identified by paper chromatography.

Endomycopsis vernalis (a yeast) transformed tryptophan into indole lactic acid, indole-3-aldehyde, indole-3-carboxylic acid and L-(-)-indole Iactic acid (Glombitza 1964).

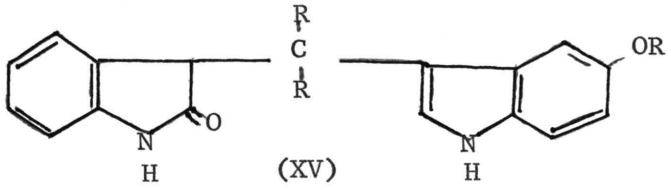
Recently Schuytema et al. (1966) reported that two species of basidiomycetes converted tryptophan into indole acetic acid and 4-species oxidized tryptophol into indole acetic acid.

Indole acetic acid is also produced by Asperillus niger and Phycomyces nitens (Boysen - Jensen 1931 & 1932) on peptone media. Experiments with other microorganisms have indicated the presence of indole pyruvic acid (Gordon 1949, Kaper 1958) and

indole-acetaldehyde (Larsen 1951), when indole acetic acid was formed from tryptophan.

Whereas tryptophan is converted to indole acetic acid, tryptamine was not reported to be utilized in seed plant tissue cultures. However, Schuytema et al. (1966) reported that 10 species of basidiomycetes transformed tryptamine to indole acetic acid and a species of basidiomycete hydroxylated the indole nucleus giving 5-hydroxyindole acetic acid. However, only two of the basidiomycete cultures surveyed in the report formed indole acetic acid from tryptophan.

In the studies on Cromobacterium violacem, Mitoma et al. (1955) and Beer (1954) found that this fungus could convert tryptophan to 5-hydroxytryptophan. This compound is the precursor of the pigment violacein (XV) produced by this organism.




(XV)

Teuscher (1965) found 5-hydroxyindole acetic acid was the major product obtained after feeding indole acetic acid to an alkaloid-producing strain of Claviceps purpurea (an ergot fungus).

Horak (1964) found 2 species of the genus Phlegmacium (Phlegmacium elotum, Phlegmacium caesiostraminuem var. amarissium) which converted tryptophan to 5-hydroxyindole acetic acid. This genus belongs to the basidiomycetes and is found predominantly as mycorrhiza on firs.

Tryptophan also can be transformed to indole (Ichihara 1955) to tryptamine (Laidlaw 1912), to tryptophol (Ehrlich 1912), to indole propionic acid (Hopkins 1903) and to indole lactic acid (Ehrlich 1911, Kaper 1963, Glombitza 1964) by microorganisms.

D-Amino acids have not been found in the tissues of higher animals, and if D-amino acids are present, it is likely that their concentration is not very great. It is of interest that neither rats nor human beings can utilize N-acetyl-D-tryptophan (Rose 1954). About 75% of ingested N-acetyl-D-tryptophan in humans has been found in the feces.

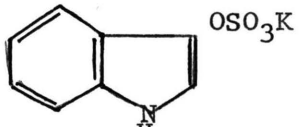
Luckner (1963) found that Penicillium viridicaturm accumulates in the medium D-kynurenine, γ-N-acetyl-D-kynurenine and N-acetyl-D-tryptophan when grown in the presence of D-tryptophan. When L-tryptophan was added to the medium L-kynurenine, anthranilic acid and 3-hydroxy-anthranilic acid were formed but did not accumulate.

Saccharomyces species, (yeasts) also can form N-acetyl-D-tryptophan from D-tryptophan (Hagemann 1964). Using S. cerevisiae S. carlsberg I and S. sake cells it was possible to acetylate D-tryptophan to produce N-acetyl-D-tryptophan.

Zenk and Schmitt (1964) found that a cell-free preparation of Saccharomyces cerevisiae can acetylate D-tryptophan to N-acetyl-D-tryptophan at pH 8.1 in the presence of coenzyme A (Co A) or acetylphosphate. They suggested that the enzyme catalyzing this reaction be called acetyl-Co A-D-tryptophan-δ-N-acetyl-transferase.

The indole nucleus is also found in indican (XVI), ergot alkaloids and toxins produced by fungi. There is good evidence that indoles are the starting point for these compounds and it is possible that the indole nuclei arise from tryptophan.

Miles (1956) and Falanghe (1962) found that indigo (XVII) is produced by the basidiomycetes Schizophyllum commune and Agaricus campestris.

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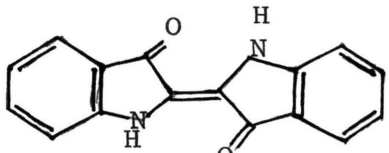



(XVII)

In addition to the human diseases mentioned previously and which are associated with faulty indole metabolism, certain human disorders can be caused by the ingestion of fungal secondary metabolites which contain an indole nucleus. For the most part these disturbances are associated with the central nervous system of humans.

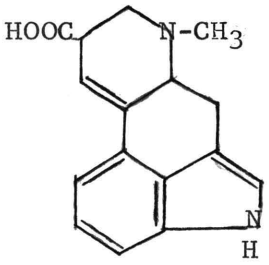
It has been shown that bread made from rye flour contaminated with the ergot fungus can cause illness and even death. It is now possible to grow strains of the organism Claviceps purpurea (Taber 1957) or closely related species in submerged culture and obtain the ergot alkaloids responsible for this disorder.

Lysergic acid (XVIII), derived from ergot alkaloids, is the basis of a compound, lysergic diethyl amide (LSD), that produces hallucinations and mental disturbance when administered to normal individuals in exceedingly low doses. Lysergic acid has been found in Aspergillus glaucus (Quilico 1955) in addition to

the Claviceps species.

Psilocybin (XIX), a hallucinogenic compound, has been isolated from fungi of the psilocybe group (basidiomycetes). It has been found in P. zapotecorum, P. mexicana and P. caerulescens etc. by Hofmann (1958).

Bufotenin (XX) has been found in Amanita citrina by Wieland (1963). It can produce hallucinations when ingested.



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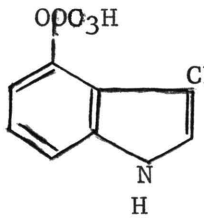

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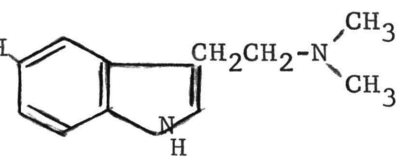
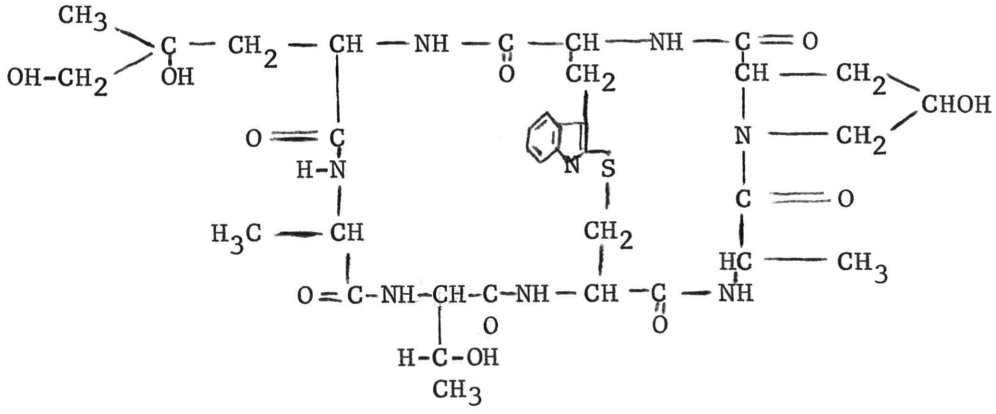


(XX)

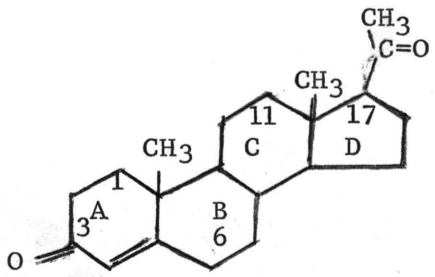
The substance phalloidine (XXI) and α- and β-amanitine are the toxic components of the deadly mushroom, Amanita phalloides. These are cyclic peptides containing an α-substituted tryptophan residue (Weiland 1963).



(XXI)

Specific microbiological transformations of steroids have been studied extensively. Schuytema et al. (1966) used progesterone (XXII) as a model for examining the transforming potential of some basidiomycetes. They have found that the modifications of progesterone by basidiomycetes includes 6-, 11- and 17-hydroxylation and oxidative cleavage of D-ring to form testololactone (XXIII).

A complete list of the hydroxylations of steroids by fungi and actinomycetes has been reviewed by Eppstein (1956). He noted that among the most frequent positions of attack are the 6 β-11α, 11β- and 17α-. Hydrogenation or oxidation of the side-chain has also been reported.

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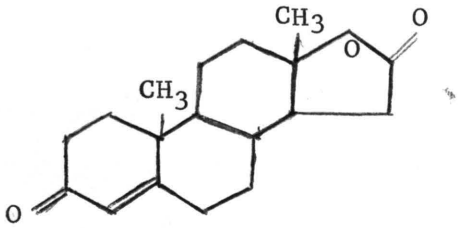
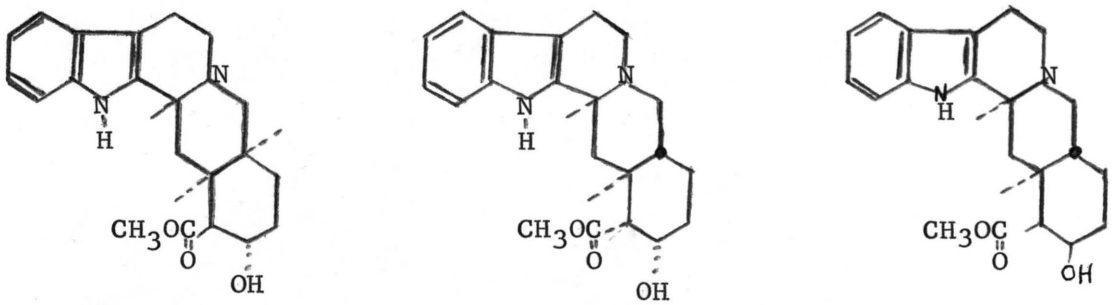



(XXIII)

Several transformations of indole alkaloids have also been reported. Pan and Meyers (196 ) reported that twelve basidiomycetes converted yohimbine (XXIV) into 10-hydroxyyohimbine.

Hartman and Drause (1964) reported that the most common transformation of α-, β-yohimbine and yohimbine was hydroxylation at either the 10- or 11-position. Without exception the transformation were monohydroxylations.

Oxidations of indole acetic acid by microorganisms have been extensively studied, primarily because of the importance of indole acetic acid as a plant-growth hormone.

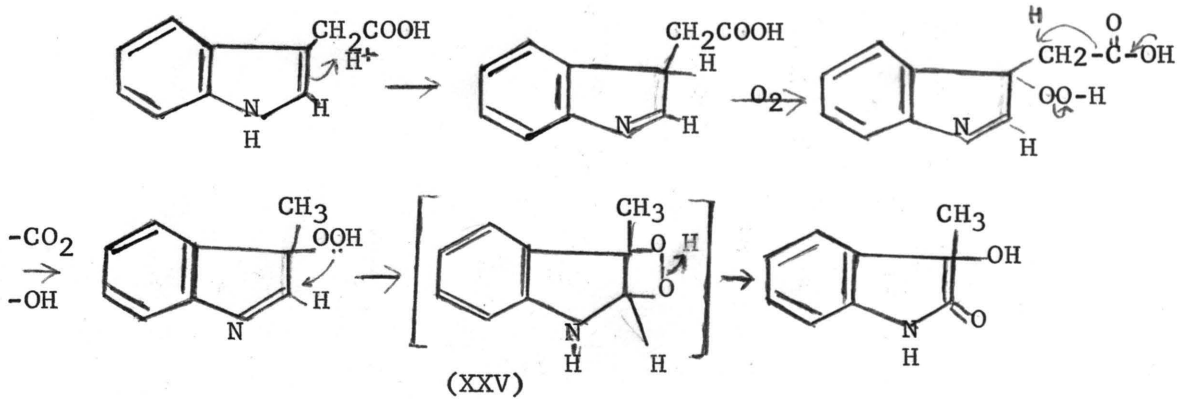


(α-XXIV) (XXIV) (β-XXIV)

The mechanism for the enzymic inactivation of indole acetic acid (loss of activity as a plant-growth hormone) is poorly understood at present, but is thought to involve oxidation in most cases. Little progress has been made and no general theory has yet been proposed for the mechanism by which indole acetic acid is inactivated.

Biological oxidations proceed by many different mechanisms and are catalyzed by several different groups of enzymes. Indole acetic oxidase, an enzyme which catalyzes the oxidation of indole acetic acid, has been found in many plants and in several fungi (Briggs & Ray 1956, Fahraeus & Tullander 1956, Sequeirs & Steeves 1954 and Tonhazy & Pelczar 1954).

Ray and Thimann (1956, 1958, 1960) studied the oxidation of indole acetic acid by an enzyme obtained from a culture of the fungus Omphalia flavida. The end product of the destruction was proposed to be 3-methyl dioxindole (XXV) on the basis of infrared and ultraviolet spectral evidence. They were not able, however, to isolate any 3-methyl dioxindole. They suggested the following mechanism for the transformation of 3-methyl dioxindole.



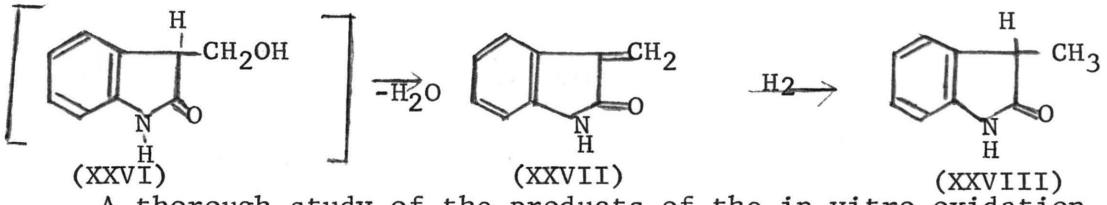

(XXV)

Tonhazy and Pelczar (1954) and Fahraeus and Tullander (1956) in their study of the destruction of indole acetic acid by an extracellular enzyme from Polyporus versicolor, could not identify the oxidation product, nor could they concentrate the enzyme. They postulated that the product was indole-3-aldehyde.

Another basidiomycete, Marasmins scorodimus, has also been found to produce an enzyme which oxidizes indole acetic acid (Fahraeus and Tullander 1956). This extracellular enzyme was constitutive but its production was increased when the organism was grown in the presence of indole acetic acid.

During a study of the metabolism of indoles by basidiomycetes in submerged culture, Siehr (1961) found oxindole-3-acetic acid to be the product of the metabolism of either tryptamine or indole acetic acid by Hygrophorus conicus. This is an intracellular, inducible enzyme.

Two oxidation products of indole acetic acid formed by the yeast, Schizosaccharomyces pombe have been reported by Fukuyama and Moyed (1964) to be 3-hydroxymethyloxindole (XXVI) and 3-methylene oxindole (XXVII). Both compounds could be converted to 3-methyl-oxindole (XXVIII). They suggested the following mechanism.




(XXVI) (XXVII) (XXVIII)

A thorough study of the products of the in vitro oxidation of indole acetic acid, catalyzed by horseradish peroxidase ( a relatively non-specific enzyme) in the absence of added hydrogen peroxide, has shown that at substrate concentrations of $2 \times 10^{-4}$ M and below, 3-methylene oxindole (XXVII) is the end product and oxindole-3-carbinol (XXVI) is its immediate precursor. This study was done by Hinman and Lang (1965).

Another oxidation reaction which has received considerable attention is the oxidative deamination of amino acids and amines. Although these are not strictly related to indole metabolism, the fact that both tryptophan and tryptamine are acted upon by de-aminases makes these enzymes of interest in a study of indole metabolism.

Numerous investigators have studied mono-amine oxidases, since they were first described by Hare (1928). They have been found in most organs and tissues where they are primarily localized in the mitochondria (Zeller 1955).

Horak (1964) suggested that to convert tryptamine into indole acetic acid some type of oxidative deamination had to occur. An enzyme catalyzing such a reaction is mono-amine oxidase.

$$R\text{-}CH_2NH_2 + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{mono-amine}} RCHO + NH_3 + H_2O_2$$

Mono-amine oxidase has usually been considered as a single enzyme (Sumner & Somer 1953) but some authors (Werle & Roewer 1952 and Alles & Heegaad 1943) have presented evidence indicating that their preparations contained more than one type of amino oxidase activity. As yet, few of these enzymes have been obtained in a state of high purity. Most studies with these enzymes have therefore been carried on with crude preparations.

While a number of mono-amine oxidases have been shown to be present in animal tissues, little is known about the distribution of such enzymes in higher plants or fungi.

Kenten and Mann (1952) reported an enzyme system in extracts of pea seedlings. It has been found that not only diamines but also certain mono-amines are oxidized by these extracts. It is not yet known whether this is due to the presence of a specific mono- and di-amine oxidases or a single enzyme. It is suggested that indole acetic acid may be formed by successive action on tryptamine of a plant amine oxidase followed by an aldehyde oxidase (Pathway B, p.6).

C. Qualitative and Quantitative Determination of Indoles.

"Indole chemistry has undergone a remarkable resurgence in the last decade, largely as a result of the elucidation of the structures of the hormone serotonin, of the plant growth auxins related to indole acetic acid, and the alkaloids of the ergot groups. Many of the advances in these fields have only been possible through the use of paper chromatography." (Smith 1958).

"Many of the methods proposed for the estimation of tryptophan exploited the striking tendency of the indole nucleus of the tryptophan residue to undergo color-forming reactions with a variety of chemical reagents, particularly upon treatment (a) with oxidizing agents such as iron III chloride, bromine, sodium nitrite, etc., in acidic solution; (b) with potassium nitrate and an aliphatic or aromatic aldehyde in concentrated hydrochloric acid; (c) with certain aromatic aldehydes, e.g., p-dimethylaminobenzaldehyde or p-nitrobenzaldehyde in 10% sulfuric acid. However, the color intensities often did not provide a very quantitative or precise measure of the amount of indole compounds and in many cases were further exacerbated by the relative instability of the amino acid under the reaction conditions imposed, a property which led to interfering side reactions both during the analysis and during the preparation of the sample for assay" (Greenstein 1961).

There is a very useful qualitative method for the determination of indole compounds and in spite of the above quotation, there is a good quantitative method for the determination of indole acetic acid.

Ehrlich reagent (p-dimethylaminobenzaldehyde in concentrated hydrochloric acid) is most useful as a general test for indolyl compounds. It reacts with a wide range of such compounds, yet with significant variations in color so as to aid in the identification of specific structures.

Salper reagent (perchloric acid and iron III chloride) which is modification of the iron III-sulphric acid method (Tang 1947)

is the most specific colorimetric method available for the quantitative determination of indole acetic acid. The Salper reagent method was described by Gordon and Weber (1951).

The availability of a sensitive technique for the detection of the indole nucleus on chromatograms, has made it an easy matter to study transformations on the intact indole nucleus. This property, coupled with the fact that the mechanisms of indole degradation in biological systems are poorly understood, has prompted the survey of the metabolism of tryptophan, tryptamine and indole acetic acid by basidiomycetes described in this thesis.

# III. EXPERIMENTAL

## A. Materials.

Malt extract and agar were obtained from the Difco Chemical Company, Detroit Michigan. p-Dimethylaminobenzaldehyde was obtained from the Matheson Chemical Company. Silica Gel G is a product of Brinkmann Instruments Co., Cantiague Road, Westbury, N. Y. Whatmann No. 1 chromatography paper was obtained from Aloe Scientific Co. Tryptamine, tryptophan and indole acetic acid were obtained from Sigma Chemical Company, St. Louis, Missouri. N,N-Dimethyl-tryptamine, $\alpha$-methyl-tryptamine and $\alpha$-ethyl-tryptamine were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Solvents employed were reagent grade. All chemicals were used without further purification.

The cultures of Hygrophorus conicus, Naucoria confragosa, Lencopaxillus paradoxus and Tricholoma nudum were obtained from Abbott Laboratories, North Chicago, Illinois. The Cantharellus cibarius (NRRL 2370) culture was obtained from the Northern Regional Utilization Research and Development Laboratories, U.S. Department of Agriculture, Peoria, Illinois.

B. Apparatus.

Rinco Rotary Evaporator: Rinco Instrument Co. Greenville, Illinois.

Rotary Shaker: New Brunswick Scientific Company, New Brunswick, New Jersey, Model CS-62630.

Spectrophotometers:

Beckmann DK-2 Spectrophotometer, Beckmann Instruments Inc., Fullerton, California.

Hitachi Perkin Elmer Model 139 Spectrophotometer, Perkin Elmer Corporation, New York, N.Y.

IR5A Infrared Spectrophotometer, Beckmann Instruments Inc., Fullerton, California.

Sterilizer: Rectangular type, 24" x 36" x 48", (Steam heat) American Sterilizer Company, Erie, Pennsylvania.

Ultraviolet Lamp: Mineralite type, Ultraviolet Products Inc., Middleton, Wisconsin.

Thin Film Chromatography Spreader: Desage-Brinkmann Inc., Great Neck, New York.

Sonifier: Branson Sonic Power, Division of Branson Instruments Inc. Danbury, Conn. Model S-75.

Centrifuge, High Speed: Lourdes Instrument Corporation, Brooklyn, N. Y. Model LCA-1.

Waring Blendor: Winsted Hardware Manufacture Company, Winsted, Connecticut.

Melting Point Apparatus: Nalge Co. Rochester, N. Y.

Thomas, Kofler Micro Hot Stage : Authur H. Thomas Co. Philadelphia, Pennsylvania.

Gas Chromatography: F and M Model 720 Dual Column Thermoconductivity, Avonsdale, Pennsylvania.

C. Procedures.

1. Cultivation of Microorganisms:

The stock cultures were grown at approximately 25° in 250 ml Erlenmeyer flasks containing 70 ml of 4% malt extract media on a rotary shaker (240 rpm). The 4% malt media was autoclaved and stored at 5° before using. For the microbial transformation of tryptamine, tryptophan or other indole compounds, 30 mg of the indole was added at the time that the flasks were inoculated with the organism. All of the inoculations of microorganisms and chemicals were carried out in a sterile room to avoid possible contamination.

2. Chromatography.

Paper Chromatography: For two dimensional ascending chromatography, glass cylinders, ten inches in diameter, were used. The cylinders were closed at the open end with a twelve inch square of double strength window glass. Whatmann No. 1 chromatography paper cut into eleven inch squares and formed into cylinders held together with thread, was used. For the location of indole compounds, the dried papergrams were dipped into, or sprayed with, Ehrlich reagent (one gram of p-dimethylaminobenzaldehyde in 10 ml concentrated hydrochloric acid diluted with 40 ml reagent grade acetone). The developing solvents used for single and two dimensional paper chromatograms were: Solvent A: 2-propanol

water, ammonium hydroxide (density 0.88) (200: 10:30). Solvent B: butanol, glacial acetic acid, water (120:30:50). Solvent C: benzene, propionic acid, water (100:70:5).

Indole acetic acid on a papergram when sprayed with Ehrlich reagent gave a purple color which turned blue on standing. The $R_f$ values of IAA were 0.44 in Solvent A, 0.89 in Solvent B, and 0.86 in Solvent C. N-Acetyl-D-tryptophan gave a purple color on a papergram when sprayed with Ehrlich reagent. The spot turned blue on standing. The $R_f$ values for N-acetyl-D-tryptophan were 0.81 in Solvent A, 0.93 in Solvent B, and 0.71 in Solvent C. Oxindole acetic acid on a papergram when sprayed with Ehrlich reagent, gave a yellow green color which became an intense blue green on standing. The $R_f$ values of oxindole acetic acid were 0.34 in Solvent A and 0.87 in Solvent B. When 5-hydroxyindole acetic acid on a papergram was sprayed with Ehrlich reagent it gave a purple color which turned blue on standing. It gave a brown color when sprayed with Sulphanilic reagent (sulphanilic acid 9 gm. concentrated hydrochloric acid 90 ml, water 900 ml; sodium nitrate 5% in water; sodium carbonate anhydrous 10% in water (1:1:2). The $R_f$ values of 5-hydroxyindole acetic acid were 0.62 in Solvent A, 0.60 in Solvent B, and 0.30 in Solvent C.

Thin layer chromatography: Glass plates were thoroughly scrubbed and cleaned until they were completely free from grease. The plates were rinsed with water repeatedly and then dried at 100°C in an oven. The dried plates were put on

an evenly balanced guide bar (Brinkmann) and wiped carefully with an acidified solution of methanol. A suspension of 30 g of Silica Gel G powder in 75 ml of deionized water was prepared in a Waring blendor. The suspension was immediately transferred to a spreader set at a coating thickness of 0.25 mm for qualitative runs or 0.5 mm for preparative thin layer plates. Coating of the plates was accomplished holding the spreader with both hands and drawing it across the plates without applying much pressure. The coated plates were left until the surface became dry and then were heated for 30 min. in the drying oven at 110°. After cooling, the plates were put into a desiccator for storage.

3. Survey of the Transformation of Indole Compounds by Basidiomycetes.

For the study of the transformations of tryptamine, tryptophan, IAA and other indole compounds by basidiomycetes, 30 mg of the indole was added at the time that the flasks were inoculated with the organism. After shaking for four days on a rotary shaker at approximately 25°, the contents of the flasks were filtered. The filtrate was brought to pH 3 with 10% hydrochloric acid. The pH was checked using Hydrion paper. The acidified solution was extracted with three 50 ml portions of ethyl acetate and the ethyl acetate extract was concentrated to a gummy residue under vacuum. The residue was dissolved in sufficient ethyl acetate to give a final concentration of about 10 mg of solid per ml

of solution. Ten lambda of this solution were spotted on an 11 inch square of Whatmann No. 1 paper. The chromatogram in the form of a cylinder was developed in Solvent A in the first dimension and then in Solvent B in the second dimension, dried, and sprayed with Ehrlich reagent (cf. section 2 on chromatography). The results from these experiments are shown in Table I.

4. Isolation and Characterization of Indole Acetic acid.

A batch of four 250 ml Erlenmeyer flasks were each inoculated with Tricholoma nudum and 30 gm of tryptamine and were incubated for a period of four days. The contents of the flasks were filtered and the filtrate was brought to pH 3 with hydrochloric acid. The acidic aqueous phase was extracted with ethyl acetate and the extract concentrated nearly to dryness. Ten milliliters of 95% ethyl alcohol were then added and the solution evaporated to dryness in a Rinco evaporator under vacuum from a water aspirator. The residue was dried and then crystallized from hot benzene. The recovered material melted at 165°. Indole acetic acid is reported to melt at 166° (Merck Index 1960).

Ten milligrams of the crystalline material were dissolved in 1 ml of 95% ethanol. Ten lambda of this solution were spotted on Whatmann No. 1 paper. The chromatogram was developed in Solvent A and then in Solvent B in the second dimension and sprayed with Ehrlich reagent. A blue color typical of indole acetic acid appeared on the papergram with

$R_f$ values of 0.44 and 0.89 for Solvent A and Solvent B respectively. The recovered crystalline material could not be separated from a sample of known indole acetic acid by two dimensional paper chromatography.

The ether extract from the acidified culture fluid of T. nudum supplemented with tryptamine contained, as shown by paper chromatography, a second substance which gave a blue-gray spot when sprayed with Ehrlich reagent. The compound also reacted on a papergram with Sulphanilic Reagent. This information plus the fact that it had the same $R_f$ as 5-hydroxyindole acetic acid in two solvent systems was taken as proof that the compound was 5-hydroxyindole acetic acid. No attempt was made to isolate this compound.

5. Isolation and Characterization of N-acetyl-D-tryptophan.

A batch of five flasks each inoculated with Cantharellus cibarius and 30 mg of D-tryptophan were incubated for a period of four days on a rotary shaker at approximately 25°. The contents of the flasks were then filtered. The filtrate was brought to pH 3 with hydrochloric acid. The pH was checked using Hydrion paper. The acidified solution was extracted with three 50 ml portions of ethyl acetate and the ethyl acetate extract was concentrated almost to dryness. The residue was a pasty black-brown substance.

The residue was redissolved in sufficient ethyl acetate to give a final concentration of about 10 mg per ml. This solution was spotted onto three 20 x 20 cm 0.5 thick silica

gel G plates. Each plate carried approximately 40 mg of crude material. The plates were developed with Solvent A. A 5 x 20 cm silica gel G plate was spotted with 10 lambda of the extract and developed. The indole bands on the silica gel G plates were located by the use of a short wave length ultraviolet light and further ascertained by spraying the small plate with Ehrlich reagent. The ultraviolet fluorescent bands corresponding to the desired indole were scraped off the glass plates and eluted from the silica gel G with ethanol. The alcohol solution was evaporated nearly to dryness and then a small amount of ether was added. The ether solution was cooled and petroleum ether (b.p. 78-103) was added until the solution became turbid. Cooling with simultaneous stirring brought about crystallization. The crystals were collected and then recrystallized twice from hot water. The melting point of the recrystallized substance was 182°. The melting point of N-acetyl-D-tryptophan was reported (Beil. 22, II, 469) to be 189-190°. A known sample of N-acetyl D-tryptophan gave a melting point of 182°. When the unknown was mixed with an equal weight of known N-acetyl-L-tryptophan and recrystallized from water, the melting point was 203°. The melting point of N-acetyl-DL-tryptophan reported in the literature was 205-206° (Beil. 22, II, 469). The crystalline product could not be separated from synthetic N-acetyl-D-tryptophan by two dimensional paper chromatography. (cf. Table II).

A small amount of the crystalline material was dissolved in 1 ml of 6N hydrochloric acid and heated under reflux for 6 hours. The presence of tryptophan in the hydrolysis mixture was indicated by paper chromatography. The material obtained by extracting the hydrolysis mixture with ether and drying the solution with anhydrous sodium sulfate had the same retention time as an ethereal solution of acetic acid on a 6-ft. silicone oil columan on an F & M gas chromatography instrument.

A small amount of the crystalline material was suspended in liquid petroleum (Nujol) and throughly mixed. The infrared spectrum of the mixture was taken on a Beckmann IR5A spectrophotometer and compared to the spectrum obtained using a sample of known N-acetyl-D-tryptophan under the same conditions. These two spectra when compared were found to be closely matched except for the intensities of the major peaks. The characteristic intensisites were as follows:

TABLE I

The Characteristic Infrared Absorption of N-acetyl-D-tryptophan

| Frequency, $cm^{-1}$ | Nature of Vibration | Type of compound |
|---|---|---|
| 668 | =C--H | Alkene |
| 770 | $C_6H_5$-- | Aromatic |
| 1200 | -CH-N-CH- (H) | NH-group |
| 1550 | R-N-C=O (H) | Mono-substituted amide |
| 1650 | -C==C- | Alkene |
| 1700 | -C(=O)-OH | Carboxylic acid |
| 2800 | $-CH_2-$ | Alkane |
| 3350 | -NH- | Amine |

Cell-free Preparation of Mono-amine Oxidase.

A stock cluture of T. nudum was grown at 25° in 300 ml. Erlemeyer flasks containing 60 ml. of 4% malt medium on a rotary shaker (240 rpm). For the induction of the enzyme, 25 mg of tryptamine were added at the time the flasks were inoculated with a growing culture.

The induced cells were harvested by centrifugation and were washed twice with distilled water. The cells were suspended in 30 ml of sodium phosphate buffer pH 7.0 (32.2 ml 0.5 M $Na_2HPO_4$ + 17.5 ml 0.5 M $NaH_2PO_4$). The suspended cells were insonated with a Bronson Sonifier for 10 minutes at 7 amps. The cell suspension was cooled during insonation by placing the vessel containing the suspension in an ice-water bath. The suspension was centrifuged for 40 minutes at 10,000xg. The supernatant and the cell debris were transferred to separate flasks. The supernatant was used as the enzyme source.

Salper reagent was prepared by adding 250 ml of 35% perchloric acid to 50 ml of 0.5 M iron III chloride. The procedure for determining indole acetic acid was as follows:

To 2 ml of solution (in deionized water) containing 0.2 to 45 $\gamma$/ml (1 $\gamma = 10^{-6}$ gm) indole acetic acid, was added 4 ml of Salper reagent. The solution was mixed and the absorbance read after 30 minutes at 530 mu against a reagent blank (deionized water with Salper reagent), using a Bausch and Lomb Spectronic 20 Colorimeter.

The enzymic formation of indole acetic acid was followed in the manner described below.

To a test tube containing 6 ml 0.5 of sodium phosphate buffer (pH 7.0) 2 ml of the enzyme preparation and 2 ml of substrate (35γ of tryptamine/ml) were added. The solution was incubated at 30° in a water bath for 40 minutes. Four grams of $(NH4)_2SO_4$ and 1 ml of 4% $ZnSO_4$ was added to the enzyme solution to precipitate all of the protein. After separating the precipitate by centrifugation, 4 ml of Salper reagent was added to the supernatant and then after 40 min. the percent transmittance of the solution was read at 530 mμ against a buffer blank. An enzyme blank and substrate blank were also run. The results obtained by the above procedure were not satisfactory, due probably to the protein and buffer influence on the color formation. The procedure was found to be more satisfactory as follows.

To the test tube containing 6 ml of 0.5 M of sodium phosphate buffer (pH 7.0) and 2 ml of enzyme, 2 ml of substrate (35 γ of tryptamine) were added. The enzyme solution was incubated at 30° in a water bath for 40 minutes. Four grams of $(NH_4)_2SO_4$ and 1 ml of 4% $ZnSO_4$ were added to the enzyme solution to precipitate all the protein. After separating the precipitate by centrifugation the supernatant was extracted with absolute ether. The ether solution was evaporated to dryness and 2 ml of water and 4 ml of Salper reagent were added. After 30 minutes the percent transmittance of the solution was read at 530 mu against a reagent blank (6 ml of buffer and 4 ml of water). At the same time an enzyme and a

substrate blank were run.

The results obtained by the above procedure were not very satisfactory (cf. Table V-X). The readings were neither stable nor completely reproducible. However, the method did show some change in the concentration of IAA and possibly there was some formation of indole acetic acid in the cell-free preparation.

TABLE II

Transformation Products of Indole Compounds

| Organism | Substrate-Product | | |
|---|---|---|---|
| | Tryptamine | IAA | D-tryptophan |
| *Tricholoma nudum* | IAA<br>5-HOIAA | 5-HOIAA | N.T. |
| *Hygrophorus conicus* | OX-IAA<br>IAA | OX-IAA | N.T. |
| *Leucopaxillus paradoxus* | OX-IAA<br>UI | N.T. | OX-IAA* |
| *Naucoria confragosa* | 5-HOIAA<br>OX-IAA + UI | 5-HOIAA<br>OX-IAA | OX-IAA* |
| *Cantharellus cibaricus* | N.T. | N.T. | N-acetyl-D-tryptophan |

| | N,N-dimethyl tryptamine | α-methyl tryptamine | α-ethyl tryptamine |
|---|---|---|---|
| *Cantharellus cibaricus* | IAA<br>5-HOIAA | N.T. | N.T. |

Abbreviations

IAA----------Indole acetic acid.

5-HOIAA--------5-hydroxy-indole acetic acid

OX-IAA---------Oxindole acetic acid

UI----------Unidentified indole

N.T.----------No transformation

*----------Small amount

TABLE III

Standard Curve for Determination of IAA with Salper Reagent

| Concentration of IAA ($10^{-6}$g.)/2 ml. | Absorbance at 530 mμ |
|---|---|
| 1.0 | 0.003 |
| 2.0 | 0.005 |
| 3.0 | 0.015 |
| 4.0 | 0.032 |
| 5.0 | 0.035 |
| 10.0 | 0.118 |
| 15.0 | 0.215 |
| 20.0 | 0.315 |
| 25.0 | 0.405 |
| 30.0 | 0.515 |
| 40.0 | 0.618 |

FIGURE III

STANDARD CURVE OF DETERMINATION OF IAA

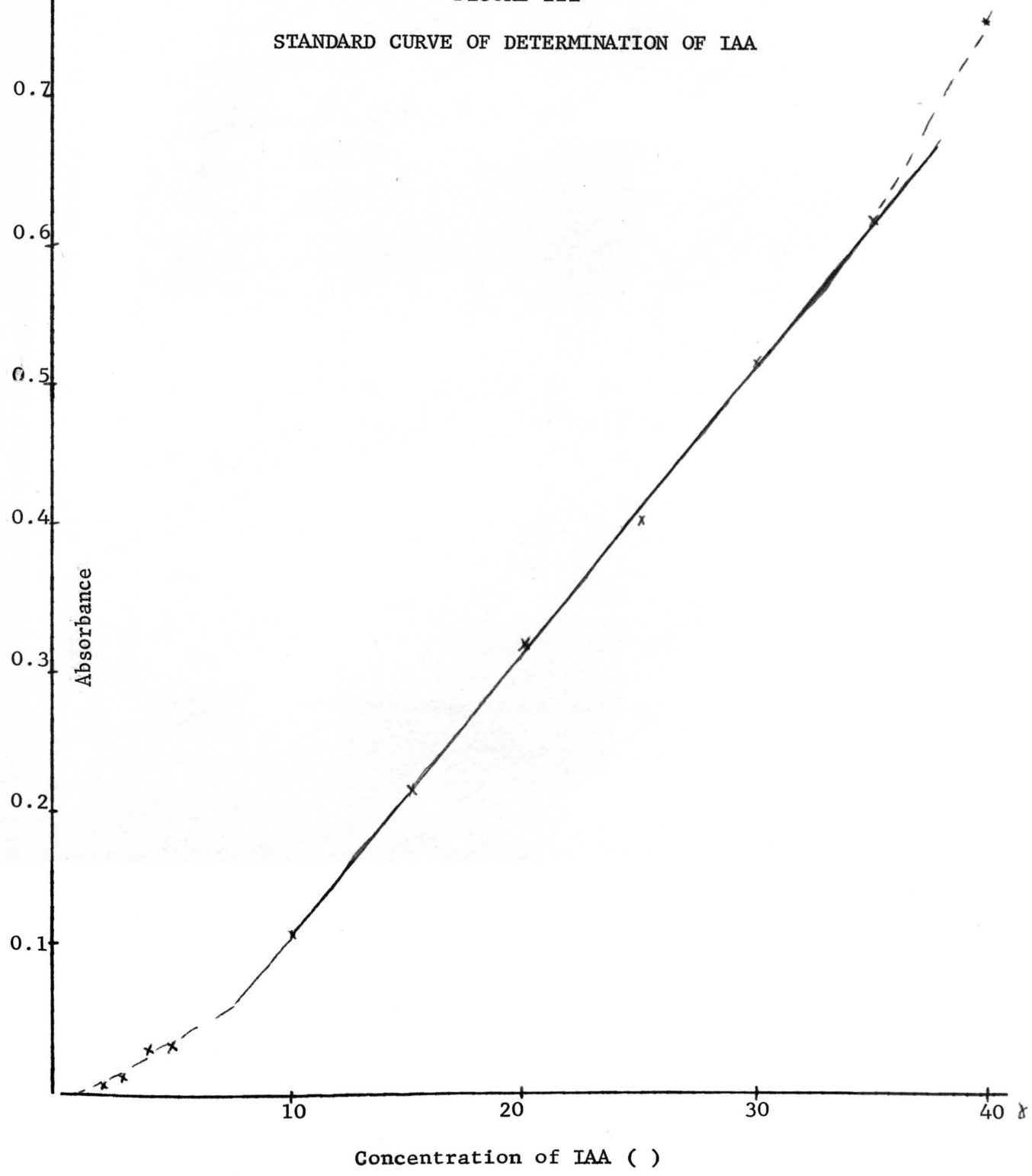


TABLE IV

Tryptamine Oxidase Activity of T.nudum*

| | Time | $Na\text{-}PO_4$ Buffer pH 7.0 ml. | Enzyme Preparation ml. | Tryptamine 35 γ/ml ml. | Salper Reagent ml. | Reading at 530 mμ | IAA formed m γ |
|---|---|---|---|---|---|---|---|
| Induced Enzyme | 0 | 4 | 2 | 2 | 6 | 0.61 | Too High Reading to obtain value from Standard Curve |
| | 10 | 4 | 2 | 2 | 6 | 0.61 | |
| | 20 | 4 | 2 | 2 | 6 | 0.82 | |
| | 30 | 4 | 2 | 2 | 6 | 0.89 | |
| | 40 | 4 | 2 | 2 | 6 | 0.93 | |
| Non Induced Enzyme | 0 | 4 | 2 | 2 | 6 | 0.58 | |
| | 10 | 4 | 2 | 2 | 6 | 0.58 | |
| | 20 | 4 | 2 | 2 | 6 | 0.58 | |
| | 30 | 4 | 2 | 2 | 6 | 0.57 | |
| | 40 | 4 | 2 | 2 | 6 | 0.54 | |

* Activity Determined by procedure 1.

TABLE V
Tryptamine Oxidase Activity of T. nudum*
Effect of pH

| pH | $Na\text{-}PO_4$ Buffer ml | Enzyme Preparation ml | Tryptamine 35 γ/ml ml | Salper Reagent ml | Reading** at 530 mμ | IAA (γ) formed at 40 min. |
|---|---|---|---|---|---|---|
| 6.0 | 6 | 2 | 2 | 4 | 0.092 | 15 |
| 6.5 | 6 | 2 | 2 | 4 | 0.102 | 16 |
| 7.0 | 6 | 2 | 2 | 4 | 0.130 | 19 |
| 7.0 | 6 | 2 | 2 | 4 | 0.120 | 18 |
| 7.0 | 6 | 2 | 2 | 4 | 0.135 | 19.5 |
| 7.5 | 6 | 2 | 2 | 4 | 0.119 | 18 |
| 8.0 | 6 | 2 | 2 | 4 | 0.145 | 21 |
| 7.0 | 6 | 2 | 2 | 4 | 0.110 | 16.5 |
| 7.0 | 6 | 2 | 0 | 4 | 0.005 | 1 |
| 7.0 | 6 | 0 | 0 | 4 | 0.000 | 0 |

* Activity determined using procedure 1

** Multiple by 14/6 = 2.3

TABLE VI

Tryptamine Oxidase Activity of T. nudum*

| Run | $Na\text{-}PO_4$ Buffer pH 7.0 ml | Enzyme Preparation ml | Tryptamine 35 γ/ml ml | Salper Reagent ml | Reading at 530 mμ | IAA (γ) formed at 40 min. |
|---|---|---|---|---|---|---|
| 1 | 6 | 2 | 2 | 4 | 0.64 | Too High Reading to obtain value from Standard Curve |
| | 6 | 2 | 2 | 4 | 0.62 | |
| | 6 | 2 | 2 | 4 | 0.74 | |
| | 6 | 2 | 2 | 4 | 0.45 | |
| | 6 | 2 | 2 | 4 | 0.45 | |
| | 6 | 0 | 2 | 4 | 0.02 | |
| | 6 | 0 | 0 | 4 | 0.00 | |
| | 6 | 0 | IAA 20 γ | 4 | 0.67 | |
| | 6 | 2 | IAA 20 γ | 4 | 1.30 | |
| 2 | 6 | 2 | 2 | 4 | 0.30 | |
| | 6 | 2 | 2 | 4 | 0.56 | |
| | 6 | 2 | 2 | 4 | 0.48 | |
| | 6 | 2 | 0 | 4 | 0.10 | |
| | 6 | 0 | 2 | 4 | 0.05 | |
| | 6 | 0 | 0 | 4 | 0.00 | |
| | 6 | 2 | IAA 20 γ | 4 | 0.60 | |
| | 6 | 0 | IAA 20 γ | 4 | 0.325 | |

*Activity determined using procedure 1

TABLE VII

Tryptamine Oxidase Activity of T. nudum*

| Run | $Na\text{-}PO_4$ Buffer pH 7.0 ml | Enzyme Preparation ml | Tryptamine 35 γ/ml ml | Salper Reagent ml | Reading at 530 mμ | IAA (γ) formed at 40 min. |
|---|---|---|---|---|---|---|
| 3 | 6 | 2 | 2 | 4 | 0.435 | Too High Reading to obtain value from Standard Curve. |
| | 6 | 2 | 2 | 4 | 0.49 | |
| | 6 | 2 | 0 | 4 | 0.029 | |
| | 6 | 0 | 2 | 4 | 0.001 | |
| | 6 | 0 | 0 | 4 | 0.000 | |
| | 0 | 2 | 2 | 4 | 0.31 | |
| | 0 | 2 | 2 | 4 | 0.25 | |
| | 0 | 0 | IAA 20 γ | 4 | 0.65 | |
| 4** | 6 | 2 | 2 | 4 | 0.51 | |
| | 6 | 2 | 2 | 4 | 0.34 | |
| | 6 | 2 | 0 | 4 | 0.48 | |
| | 6 | 0 | 2 | 4 | 0.005 | |
| | 6 | 0 | 0 | 4 | 0.00 | |
| | 0 | 2 | 2 | 4 | 0.14 | |
| | 0 | 2 | 2 | 4 | 0.17 | |
| | 0 | 0 | IAA (40r) | 4 | 0.03 | |
| | 0 | 2 | IAA (40r) | 4 | 0.75 | |

**Possibly the enzyme activity was lost.

*Activity determined using procedure 1.

TABLE VIII

Tryptamine Oxidase Activity of T. nudum

Effect of pH*

| pH | $Na\text{-}PO_4$ Buffer ml | Enzyme Preparation ml | Tryptamine 35 γ/ml ml | Salper reagent ml | Reading at 530 mμ | IAA (γ) formed 40 min. |
|---|---|---|---|---|---|---|
| 6.0 | 6 | 2 | 2 | 4 | 0.145 | 11.5 |
| 6.5 | 6 | 2 | 2 | 4 | 0.135 | 11 |
| 7.0 | 6 | 2 | 2 | 4 | 0.230 | 16 |
| 7.5 | 6 | 2 | 2 | 4 | 0.132 | 11 |
| 8.0 | 6 | 2 | 2 | 4 | 0.178 | 13 |
| 7.0 | 6 | 2 | 2 | 4 | 0.135 | 11 |
| 7.0 | 6 | 0 | 0 | 4 | 0.005 | 7 |
| 7.0 | 6 | 0 | 0 | 4 | 0.000 | 0 |
| | 0 | 0 | IAA 10γ | 4 | 0.095 | 9 |

*Activity determined using Procedure 2

TABLE IX

Tryptamine Oxidase Activity of T. nudum*

| $Na\text{-}PO_4$ Buffer pH 7.0(ml) | Enzyme Preparation ml | Tryptamine 35 γ/ml ml | Salper Reagent ml | Reading at 530 mμ | IAA (γ) formed in 40 min. |
|---|---|---|---|---|---|
| 6 | 2 | 2 | 4 | 0.250 | 17 |
| 6 | 2 | 2 | 4 | 0.180 | 13.5 |
| 6 | 2 | 2 | 4 | 0.200 | 14 |
| 6 | 2 | 0 | 4 | 0.145 | 11.5 |
| 6 | 2 | 2 | 4 | 0.006 | 7 |
| 6 | 2 | 0 | 4 | 0.000 | 0 |
| 0 | 0 | IAA 10γ | 4 | 0.097 | 9 γ |

*Activity determined using procedure 2.

## IV. DISCUSSION

A survey (Table II) of the ability of basidiomycetes to convert tryptamine and tryptophan to other acidic indoles was made. It was found that oxindole acetic acid was the transformation product when tryptamine, or D-tryptophan were added to the culture of L. paradoxus. This was shown by paper chromatography. Oxindole was also found in the culture of N. confragosa when tryptamine of indole acetic acid was added. 5-Hydroxy-indole acetic acid was a product when tryptamine or indole acetic acid were added to a culture of N. confragosa. None of the above organisms were found to transform L-tryptophan into other acidic indole compounds.

The so-called tryptamine pathway has been suggested as one of several pathways for the biosynthesis of indole acetic acid in microorganisms and higher plants.

Tryptophan-->Tryptamine-->Indole Acetaldehyde-->Indole Acetic Acid

The first step in the sequence is the decarboxylation of tryptophan to tryptamine. Tryptamine in turn can be oxidized to indole acetaldehyde. Oxidation of indole acetaldehyde yields indole acetic acid.

Since the organisms studied in this work are incapable of transforming tryptophan in any appreciable amount to other indolyl acids, it can be assumed that basidiomycetes do not possess enzymes capable of decarboxylating tryptophan. However, the formation of indole acetic acid from tryptamine by a number of basidiomycetes suggest that these organisms either can carry out oxidative deamination in a single step or can convert tryptamine in a two-step

process as indicated above. An analogous system has been reported in animals. Udenfriend (1956) has suggested that 5-hydroxytryptamine (serotonin) in animals is oxidized by a mono-amine oxidase to 5-hydroxy-indole acetaldehyde, which in turn is oxidized to 5-hydroxy-indole acetic acid by an aldehyde dehydrogenase. No tryptamine as such is present in animals though.

No attempt was made in this study to determine the mechanism by which basidiomycetes convert tryptamine to indole acetic acid. Tricholoma nudum, one of the organisms studied, readily converted tryptamine to indole acetic acid. When N,N-dimethyl-tryptamine was added to the medium, indole acetic acid was found in the spent medium. However, no transformations were observed when either α-methyl-tryptamine or α-ethyl-tryptamine were added to the culture. Although there is no direct proof, it is possible that there is a single tryptamine oxidase produced by T. nudum which can catalyze the oxidative deamination of a tryptamine provided that the amine is attached to a terminal carbon atom in a linear chain. Primary amines on a secondary carbon atom are not attacked. The specificity of the enzyme is also such that methyl substitution on the amino group is without influence.

When tryptamine was added to a culture of either T. nudum or Naucoria confragosa, 5-hydroxyindole acetic acid was detected in the spent medium. It was further shown that indole acetic acid can be converted to 5-hydroxyindole acetic acid (5-HOIAA) by these organisms. This would indicate that the hydroxylation of the indole nucleus occurred at the level of IAA rather than that of the tryp-

tamine. The evidence clearly indicates that in animals 5-hydroxy-tryptamine (Udenfriend 1956) is derived from tryptophan rather than from tryptamine. Since tryptamine is not formed from tryptophan in animals, it would appear that the 5-HOIAA found in the urine of animals came from the oxidation of serotonin rather than from the hydroxylation of IAA as observed in this study.

In Claviceps purpurea, an ergot fungus (Teuscher 1965), there is also evidence that indicates that indole acetic acid can hydroxylate to 5-hydroxy-indole acetic acid directly.

Cantharellus cibarius, one of the organisms studied, converted D-tryptophan to N-acetyl-D-tryptophan. The questions concerning this reaction are: Why does this microorganism acetylate D-tryptophan and what is N-acetyl-D-tryptophan doing for this microorganism?

It is a possibility that the formation of N-acetyl-D-tryptophan is a step in the conversion of D-tryptophan to L-tryptophan. The racemase which would then convert the N-acetyl-D-tryptophan to the L-isomer might be lacking in C. cibarius and hence any N-acetyl-D-tryptophan would accumulate. Below are described three observation which form the basis for this speculation.

First, the common chemical means of separating the racemic mixture of D,L-tryptophan is via the acetylated derivatives.

Secondly, in studies on the enzymic oxidation of 5-hydroxy-tryptamine (serotonin) to 5-hydroxy-indole acetaldehyde by a monoamine oxidase, inhibition of the reaction by the administration

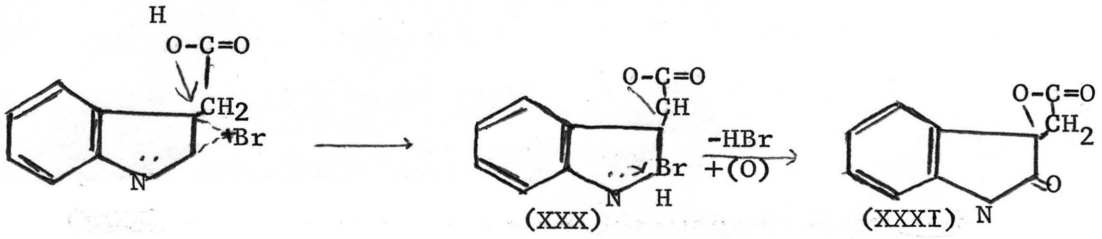
of a mono-amine oxidase inhibitor, such as 2-phenylcyclopropyl-amine, leads to the increased formation of the N-acetyl and N-methyl derivatives of serotonin (Udenfriend 1955). There is a possibility that the formation of N-acetyl-D-tryptophan by micro-organisms is evidence for an inhibitor of the D-tryptophan recemase system.

Third, Lactobacillus casei (Koser 1957) can utilize D-tryptophan only when it is first acetylated. Also racemases which do not require pyridoxal (Snoke 1962) have been reported to occur in microorganisms. These observation give credence to the speculation that C. cibarius contains only one of the two possible enzymes necessary for the conversion of D-tryptophan to L-tryptophan. It, therefore, is conceivable that the formation N-acetyl-D-tryptophan is the first step in the conversion of D-tryptophan to L-tryptophan.

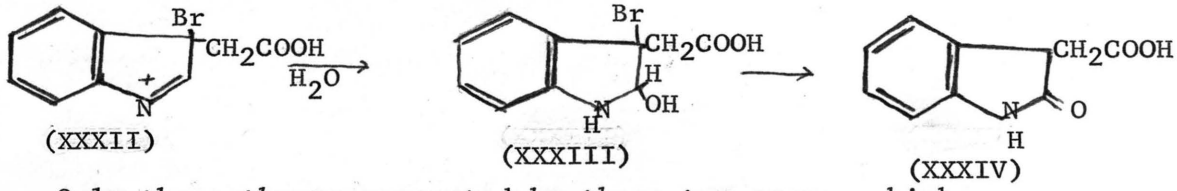
A product of the microbial destruction of IAA has been reported to be 3-methyl-oxindole. A different product, oxindole acetic acid reported here indicates that the IAA oxidase produced by H. conicus and N. confragosa differ from that of O. flavida which converts IAA to 3-methyl-oxindole (Fukuyama 1964).

The mechanism for the oxidation of IAA by N-bromosuccinimide and bromine is of interest in these studies. It is possible that the chemical reaction is a model for the enzymic reaction in H. conicus and N. confragosa.

The mechanism proposed by Witkop (1961) assumes the formation of a bromonium ion intermediate.




On the other hand, Hinman(1964) proposed 3-bromoindolenine (XXXIII) on grounds of economy as the first step in the reaction.




Only the pathways suggested by these two groups which are closely allied to that of the possible enzymic reaction are described above.

It would be of interest to determine the source of the oxygen incorporated into the oxindole acetic acid. This knowledge would make it possible to select one of the two proposed models as the one most likely to be operating in the enzymic catalyzed reaction.

The result from the studied of T.nudum indicates that a mono-amine oxidase is possibly the enzyme which converts tryptamine to IAA. Three analytical methods are of possible use in the study of mono-amine oxidases.

The manometric method should be useful for determining the oxygen uptake in the conversion of tryptamine to IAA provided that molecular oxygen is involved in the oxidation. Theoretically

1 micro-mole of oxygen should be utilized for every micro-mole tryptamine transformed to IAA. We did not find any oxygen uptake in the cell-free preparation. Although, it was possible that faulty technique was the reason for this failure, this method was not pursued.

Spectrophotometric methods (Hershenson 1961) for measuring enzyme activity are based on the fact that the substrate and the product absorb light at two different wave lengths. Since tryptamine and IAA absorb light at the same wave length, this assay method is of no use in this study.

A colorimetric method is possible and it was used to measure the conversion of substrate into product. The method is known as the Salper reagent method. Since Salper reagent is the most specific colorimetric method for IAA (Gordon 1954) (insensitive to tryptamine), it should have been useful for measuring the activity of the mono-amine oxidase in T. nudum. Unfortunately, the results were not encouraging in this case (cf. Table V-X). It is possible that either the sodium phosphate buffer or the protein precipitants (4% $ZnSO_4$ and $(NH_4)_2SO_4$) interfere with the color formation.

In spite of the inconsistant results it was possible to detect some activity in a cell free preparation from T. nudum growth in the presence of tryptamine (Table V-X). No enzymic activity was detected in the mycelium of stock cultures initially but only after the organism had been grown in the presence of tryptamine (Table V). Thus, it was concluded that the enzyme under study was an inducible enzyme.

Two procedures were tried for measuring the activity of tryptamine oxidase in a cell-free preparation of T. nudum. Inconsistent results were obtained when the Salper reagent was added directly to the incubation mixture after removal of precipitated proteins (Table V-VIII). Less variable, but still unsatisfactory, results were obtained when the supernatant after precipitation of proteins was extracted with ether and the ether solution assayed for IAA (Table IX-X). The failure to quickly find a suitable assay procedure for the tryptamine oxidase, discouraged further work. It was felt that too much time would be involved in finding a convenient procedure and the work on this point was suspended. The study of the tryptamine oxidase of T. nudum should form the basis of another thesis.

The tryptamine oxidase of T. nudum was sensitive to pH as shown by the results given in Table VI-IX.

Paper chromatography was used for the separation and partial characterization of the products of the transformation of indole compounds. The blue and purple colors developed on the papergrams after spraying with Ehrlich reagent provided a good means of detecting indole compounds.

Dimethylaminocinnameldehyde (DMCA) (Harley 1959) has been reported to be a more sensitive reagent for detecting indole compounds but as Ehrlich reagent proved to be satisfactory in the present case, DMCA was not used.

Two dimensional papergrams were used as a tool for checking

technique. An inseparable spot of a sample and a known compound indicated that the two compounds under study were the same.

It was found that N-acetyl-D-tryptophan can be easily identified on a single dimensional papergram using the benzene-propionic acid-water solvent system (Solvent C). In this system it is readily separated from IAA and indole lactic acid (ILA), two compounds difficult to separate from N-acetyl-D-tryptophan with Solvents A and B.

Thin layer chromatography was found useful for separating quantities of N-acetyl-D-tryptophan during this investigation. The N-acetyl-D-tryptophan could be easily eluted from the Silica Gel G. by ethanol. A dark brown substance also eluted from the Silica Gel G with the N-acetyl-D-tryptophan makes the crystallization of N-acetyl-D-tryptophan more difficult.

The preparation of useful Silica Gel G thin layer plates depends upon two important factors. One is the ratio of water to Silica Gel G. Usually the ratio of 30/85 (gm/ml) was satisfactory in our work. It was also found that the time taken to spread the Silica Gel G suspension should not be longer than three minutes.

In the literature (Beil. 22,II, 469) the melting point of N-acetyl-D-tryptophan was reported to be 189-190$^{o}$ on recrystallization from water. In the present case, the melting point of N-acetyl-D-tryptophan isolated from the spent media was 182-183$^{o}$.

TABLE X

Mixed Melting Point Determination of D, L and N-acetyl-D,L-tryptophan

| Compounds and Synthetic Mixtures | Melting point |
|---|---|
| Isolated compound | 182-183° |
| Isolated compound + Known N-acetyl-D-tryptophan | 182-183° |
| Isolated compound + Known N-acetyl-L-tryptophan | 203-203° |
| Recrystallized mixture of isolated compound + Known N-acetyl-L-tryptophan | 203-204° |
| Known N-acetyl-D,L-tryptophan | 203-204° |

The ordinary physical properties of the D, and L-form are identical, but are different from the physical properties of the racemic mixture. The results from the mixed melting point determinations show that the melting point of the racemic compound is higher than pure D, or L-form. The observations listed in Table III indicate that the transformation product of D-tryptophan by C.cibarius is N-acetyl-D-tryptophan. It is not the DL or L-form.

Infrared spectroscopy was used for the qualitative analysis of isolated N-acetyl-D-tryptophan. A comparison was made of the spectra of the isolated N-acetyl-D-tryptophan with that of synthetic N-acetyl-D-tryptophan. The absorption spectra were identical.

# V. CONCLUSIONS

A survey of the ability of basidiomycetes (fungi) to convert indoles was made. Tryptamine, D- and L-tryptophan and indole acetic acid were used with the organisms Tricholoma nudum, Cantharellus cibarius, Hygrophorus conicus, Leucopaxillus paradoxus and Naucoria confragosa.

The products formed from tryptamine and N,N-dimethyl-tryptamine by T. nudum have been identified as indole acetic acid and a small amount of 5-hydroxy-indole acetic acid. C. cibarius converts D-tryptophan to N-acetyl-D-tryptophan. H. conicus formed oxindole acetic acid from either tryptamine or indole acetic acid. L. paradoxus transformed tryptamine into oxindole acetic acid and one unidentified indole compound. It also transformed D-tryptophan to oxindole acetic acid but in very low yield. N. confragosa transformed tryptamine and indole acetic acid into oxindole acetic acid and 5-hydroxy-indole acetic acid and one unidentified indole compound. It also transformed D-tryptophan to oxindole acetic acid but in very low yield.

Crystalline N-acetyl-D-tryptophan and indole acetic acid were isolated from the culture fluid of C. cibarius and T. nudum respectively.

An attempt to develop an assay method for the mono-amine oxidase of T. nudum was made, without notable success.

## VI. RECOMMENDATIONS

The following is a list of suggestions for the further study of the transformation of indole compounds by basidiomycetes and for the mono-amine oxidase of T. nudum.

(1) Determine the unidentified indole compound found in L. paradoxus and N. confragosa cultures when tryptamine was added.

(2) Determine whether C. cibarius will acetylate other D-amine acids.

(3) Develop a colorimetric enzyme assay method to measure the mono-amine oxidase in T. nudum.

(4) An attempt should be made to isolate the mono-amine oxidase from the mycelium of T. nudum.

(5) Determine the effect of buffers on the mono-amine oxidase activity from the mycelium of T. nudum.

(6) Use $O_2^{18}$ or $H_2O^{18}$ to determine the source of the oxygen in the oxindole acetic acid formed from indole acetic acid by H. conicus.

(7) Determine the rate of growth and the rate of enzyme production of T. nudum on malt medium supplemented with tryptamine.

(8) Determine the effect of activators such as $Mn^{++}$, $Cu^{++}$, $H_2O_2$ and catalase on the enzyme activity of mono-amine oxidase.

(9) Try to determine the specificity of the mono-amine oxidase T. nudum by using substituted tryptamines as substrates.

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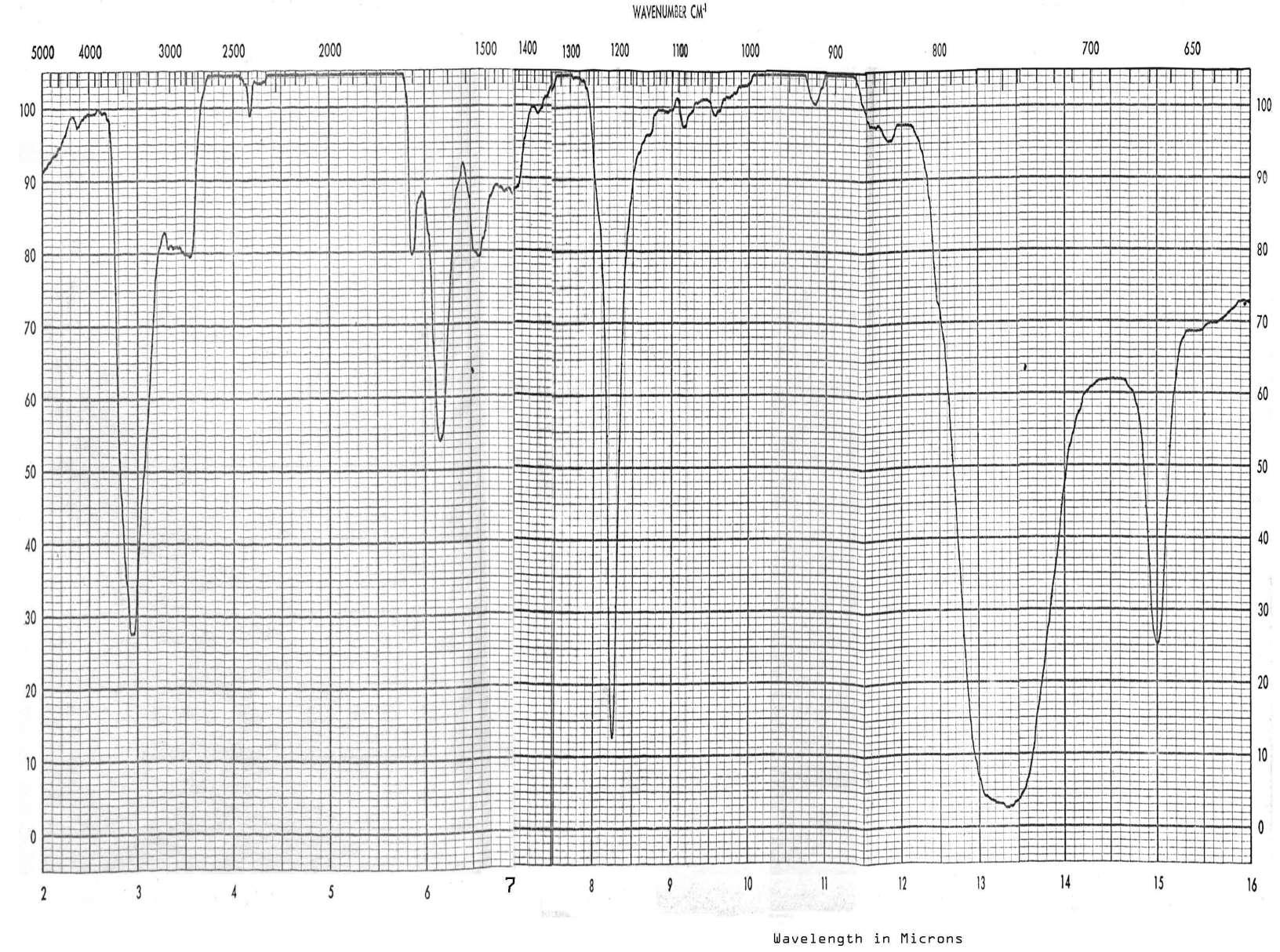

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