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Studies on the conversion of indole acetic acid and oxindole acetic acid by Hygrophorous conicus

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Department:

STUDIES ON THE CONVERSION OF INDOLE ACETIC ACID TO OXINDOLE ACETIC ACID BY HYGROPHOROUS CONICUS

BY

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A

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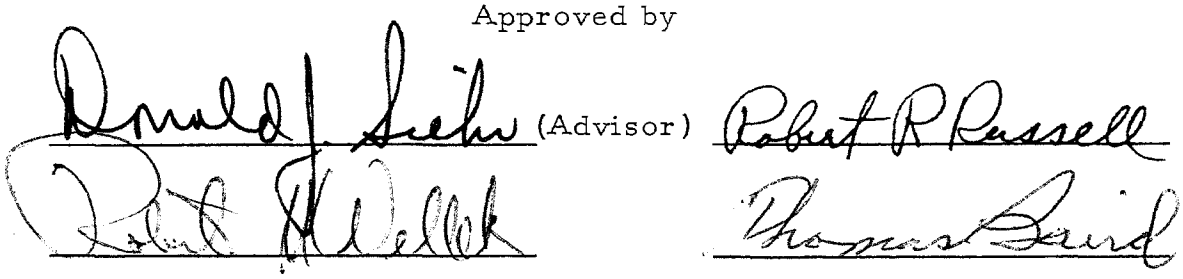

Abstract

The fungus, Hygrophorous conicus, produces the enzyme, indole acetic acid oxidase, when grown in the presence of indole acetic acid (IAA) or tryptamine. The IAA acts both as an inducer and a substrate for this intracellular enzyme. The product of the oxidation catalyzed by the IAA oxidase is oxindole acetic acid (OIAA).

Since the IAA and OIAA absorb maximally at two different wavelengths the use of spectrophotometric analysis was possible for assaying for the IAA oxidase activity. Paper chromatography was used for qualitative identification of the IAA and OIAA.

The rate of growth and the rate of formation of the enzyme by H. conicus were studied. The rate of growth was found to be similar to that of other fungi. The formation of the enzyme, however, proved to be somewhat different from that of other inducible enzyme systems.

The OIAA formed by the oxidation of IAA was also found to induce the formation of an enzyme in H. conicus. This enzyme catalyzed the destruction of OIAA.

Several methods were tried in an attempt to isolate the IAA oxidase from the cells of H. conicus, but these methods proved, for the most part, unsuccessful

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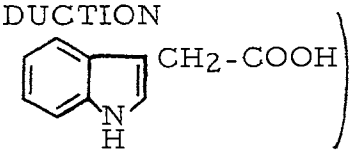
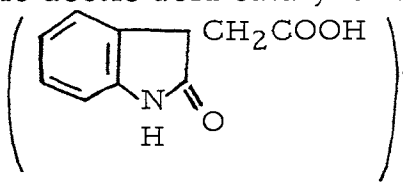
I. INTRODUCTION

Indole acetic acid  is a growth hormone which is widespread in nature. Indole acetic acid oxidase, an enzyme which catalyzes the oxidation of indole acetic acid, is almost as widespread in nature as indole acetic acid. Indole acetic acid oxidase has been found in many plants and in several fungi. One of the fungi in which this enzyme has been found is Hygrophorous conicus. The indole acetic acid oxidase of H. conicus is an induced, intracellular enzyme. The product of oxidation of indole acetic acid catalyzed by the H. conicus enzyme is oxindole acetic acid  . The H. conicus enzyme is of interest because of the unique oxidation product and also because it is one of the few indole acetic acid oxidase systems for which the oxidation product has been isolated and positively identified.

The purpose of this research project was to develop a more satisfactory method for assaying the indole acetic acid oxidase activity in H. conicus, and to develop a method for preparing a cell-free extract of the enzyme. Although the latter objective was not fully attained, several interesting facts were discovered concerning the fungus, H. conicus, and the enzyme activity.

II. LITERATURE REVIEW

Biological Oxidation

Biological oxidation proceeds by many different mechanisms and is catalyzed by several different groups of enzymes. Some of the groups which catalyze oxidations are oxidases, peroxidases, oxygenases, and dehydrogenases.

Oxidases catalyze reactions in which oxygen molecules serve as the immediate electron acceptor (Hayaishi, 1962). Classical oxidases may be divided into two categories. In the first category the enzyme catalyzes the transfer of two electrons from the substrate, S, to one molecule of oxygen-forming hydrogen peroxide as shown in equation (1).

$$SH_2 + O_2 \xrightarrow{\text{oxidase}} S + H_2O_2 \qquad (1)$$

In the second category two electrons are transferred to one atom of oxygen to produce water as seen in equation (2).

$$SH_2 + 1/2\ O_2 \xrightarrow{\text{oxidase}} S + H_2O \qquad (2)$$

Peroxidases are enzymes which use H_2O_2 as an acceptor of electrons as shown in equation (3).

$$SH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} S + 2H_2O \qquad (3)$$

In oxidation reactions in which oxygen is directly incorporated into the substrate molecule as in equations (4) and (5), the enzyme catalyzing the reaction is called an oxygenase.

$$S + 1/2\ O_2 \xrightarrow{\text{oxygenase}} SO \quad (4)$$

$$S + O_2 \xrightarrow{\text{oxygenase}} SO_2 \quad (5)$$

In several biological reactions in which the overall reaction appears as an addition of oxygen, it has been found that the oxygen atoms are derived from a water molecule rather than from molecular oxygen. (Hayaishi, 1962). An example of this mechanism is seen in equations (6) and (7). The substrate is first hydrated and then dehydrogenation occurs in the second reaction with A acting as an electron acceptor.

$$S + H_2O \rightleftharpoons SH_2O \quad (6)$$

$$SH_2O + A \underset{\text{dehydrogenase}}{\rightleftharpoons} SO + AH_2 \quad (7)$$

IAA Oxidases and Peroxidases

IAA oxidase is an enzyme which catalyzes the destruction of IAA (Hare, 1964). IAA oxidase has been found to be widespread in nature. It has been found in several fungi and in many plants such as pea shoots, pineapple, the Japanese radish, horseradish, turnips, etc.

Hygrophorous conicus, a basidiomycete, will produce IAA oxidase if the mycelia is grown in the presence of IAA or tryptamine (Siehr, 1961). Patel (1964) found that the IAA and tryptamine acted both as an inducer and a substrate for the IAA oxidase, and that the enzyme is intracellular. The product of the oxidation of the IAA by

the H. conicus enzyme has been isolated and shown to be oxindole acetic acid (Siehr, 1961).

The basidiomycete, Omphalia flavida, produces an extracellular, constitutive IAA oxidase (Ray and Thimann, 1956). Although they were unable to isolate the pure oxidation product, they postulated from infrared and ultraviolet spectrum evidence that 3-methyldioxindole was the product (Stowe, Ray and Thimann, 1954).

The formation of an IAA and phenol oxidase can be induced in another basidiomycete, Polyporous versicolor (Tonhazy and Pelczar, 1954, and Fåhraeus and Tullander, 1956). IAA and various phenolic compounds were found to act as both inducers and substrates for this extracellular enzyme. The oxidation product of IAA was not identified but was found to be of carbonyl nature. On the basis of theoretical considerations and various tests for higher aldehydes the product was postulated to be indole-3-aldehyde (Tonhazy, Pelczar, 1954).

Another basidiomycete, Marasmius scorodonius, has also been found to produce an enzyme which oxidizes IAA (Fåhraeus, Tullander, 1956). This extracellular enzyme was constitutive, but its production was increased when the organism was grown in the presence of IAA.

The enzyme was very similar to that found in Polyporous versicolor.

One of the many plant sources of the IAA oxidizing enzyme is the Japanese radish (Morita, Kameda, and Mizuno, 1962). The enzymes, peroxidases a and c, were found to be induced in Japanese radish by H_2O_2. When low concentrations of the peroxidases were used, the product of IAA oxidation was found to be similar to that proposed by Stowe, Ray and Thimann (1954) for Omphalia flavida. When high concentrations of the enzymes were used, the proposed oxidation product was indole-3-aldehyde.

Nature of the Enzyme

Ray and Thimann (1956) found that the IAA destroying reaction catalyzed by the enzyme from Omphalia flavida was stoichiometric, consuming 1 mole of O_2 and releasing 1 mole of CO_2 per mole of IAA destroyed. Spectrophotometric studies of the action of the enzyme on IAA indicated that the initial product of the reaction (equation 1) undergoes further transformation (Ray, 1956).

$$\text{IAA} \xrightarrow{\text{I}} \text{A} \xrightarrow{\text{II}} \text{B} \qquad (1)$$

Ray (1956) found that the first step is catalyzed by the enzyme, while the second step was spontaneous and acid catalyzed. The first step also consumes the 1 mole of oxygen per mole of IAA, and it was also deduced that the CO_2 was formed in the first step. The final

product appeared to consist of at least two, and possibly more, substances. As mentioned earlier 3-methyl-dioxindole was proposed as one of these products. It has also been found that H_2O_2 increases the rate of step I (Ray, 1962), but has no effect on step II (Ray, 1956).

The oxidation of IAA by purified IAA oxidase from the fungus, Polyporous versicolor, was found to consume 1 mole of O_2 and liberate 1 mole of CO_2 per mole of IAA (Tonhazy, Pelczar, 1954; Fåhraeus, 1961). Tonhazy and Pelczar (1954) found the oxidation to be stimulated by Mn^{++} but Fåhraeus (1961) found Mn^{++} to have no effect on the rate of oxidation. Both Tonhazy and Fåhraeus found that H_2O_2 had no effect on the oxidation and, therefore, concluded that the enzyme is not a peroxidase.

IAA oxidases from several other plants and fungi have been reported, but in no case has any product been positively identified. Without identification of the products it is difficult to propose the mechanism of the reaction. Without more precise knowledge about the properties of the reactions obtained with the crude enzymes prepared from the various sources, it is impossible to conclude that the same type of reaction or enzyme is being studied (Mehler, 1962).

Preparation of a Cell-Free Enzyme

In order to study an enzyme it is desirable to liberate the

enzyme from the organism. If the enzyme is intracellular, this involves the rupturing of the cells. Several methods (each basically chemical, mechanical, or enzymatic) of liberating enzymes from the organism have been used, and all facilitate the removal of the enzyme by alteration of the cell wall or the cell membrane (Seaman, 1963).

Although several mechanical methods of cell rupturing have been devised, two procedures have been widely used in recent years: grinding the cells with alumina and breakage of cell structure by ultrasonic oscillations.

Sonic treatment of the cells (insonation) has proven successful in breaking ordinarily difficult-to-rupture cells. For instance, such refractory cells as staphylococci can be ruptured in a matter of minutes (Seaman, 1963). Antigens from various pathogenic fungi have been prepared by disrupting the cells by insonation (Sonifier News, 1964). Lion and Avi-Dor (1963) were able to prepare an active enzyme extract of NADH-oxidase from E. coli by insonating the cells for 30 minutes.

Quick drying of the cells from the frozen state can also result in sufficient alteration of the cell membrane to allow for easy extraction of the enzymes (Seaman, 1963). However, an oxidation-sensitive enzyme can be inactivated during freeze drying. (This is also true

for any method of preparation of cell-free extracts). For example, the viability of the organism E. coli was lost when freeze dried from distilled water (Lion and Bergmann, 1961). This lethal effect of air, however, could be reduced if certain protective substances were added before the freeze-drying. Three groups of these protective substances were found by Lion and Bergmann (1961): (1) thiourea and some of its derivatives, (2) sugars and (3) some simple inorganic salts.

A cardinal rule for handling all enzyme extracts, no matter which method of preparation is used, is to keep the material at low temperatures (0° - 4°C) during all manipulations. (Seaman, 1963).

Enzyme Assay

Since enzymes are biological catalysts which accelerate thermodynamically possible biological reactions, the activity of an enzyme is measured in terms of the influence it has on the rate of the reaction. In order to study the activity of an enzyme, assay procedures have been devised. Assay procedures are based on the quantitative determination of either the rate of disappearance of a reactant or the rate of formation of a reaction product. In case of the H. conicus enzyme, which was studied herein, the activity of the enzyme was studied in terms of the oxindole acetic acid formed per hour by the oxidation of the starting material, IAA.

There are several analytical methods which have been used extensively to study the enzymatic destruction of IAA. The methods used include (a) spectrophotometric analysis, (b) colorimetric analysis, (c) manometric analysis and (d) paper chromatography.

Spectrophotometric Analysis

Ray (1956) and Morita et al., (1962) followed the disappearance of IAA and the appearance of the products of the enzymatic reaction spectrophotometrically. They scanned the IAA solution at various times after addition of the IAA oxidase. They determined the enzyme activity on the basis of the disappearance of the IAA absorption peak at 280 mμ since the identity of all the products was uncertain.

As mentioned earlier the oxidation product of IAA by H. conicus was proven to be oxindole acetic acid, and this compound was found to have a maximum absorption peak at 250 mμ (Siehr, 1961). Since the substrate (IAA) and the product (OIAA) absorb maximally at two different wavelengths, it was possible to determine the enzyme activity either by the disappearance of IAA or the appearance of OIAA. The latter was chosen since IAA can be destroyed by non-enzymatic reactions.

There are several methods which may be used to calculate the concentrations of OIAA and IAA in an IAA - OIAA mixture using spectrophotometric absorption data.

One method is based on the calculation of a factor for the IAA and OIAA in various known mixtures of OIAA and IAA (Layne, 1957). The factors are calculated by the following equations:

$$F_{IAA} = \frac{\%\ IAA}{a_{IAA}}$$

$$F_{OIAA} = \frac{\%\ OIAA}{a_{OIAA}}$$

where % is weight per cent, i.e., $\frac{mg_{IAA}}{mg_{IAA} + mg_{OIAA}}$, and a is the absorptivity at the wavelength used. The factors were calculated at 280 and 250 mμ for both the IAA and OIAA. A plot of the ratio $(R \frac{280}{250})$ of the absorption values of the known mixtures at 280 mμ to the absorption values at 250 mμ versus the F_{IAA} and F_{OIAA} can then be constructed. The concentration of the OIAA or the IAA can be calculated as follows:

$$C_{IAA} = (F_{IAA})\ (A_{280}) \qquad (1)$$

$$C_{OIAA} = (F_{OIAA})\ (A_{250}) \qquad (2)$$

where C = concentration in mg/ml

F = factor

A = absorbance at the designated wavelength

The concentration of IAA and OIAA in unknown mixtures can be

determined by the calculation of the ratio of the absorbance values at 280 and 250 mμ, then finding the corresponding factors from the graph, and then applying equation (1) and (2).

Another method for analyzing a two component system is given by Vierordt (Morton, 1962). In this method the absorptivities of various known mixtures of IAA and OIAA are plotted against wavelength. The resulting curves will intersect at isobestic points (wavelengths at which the absorptivities of the mixtures are equal). In a two component system the intensity of the absorption at the isobestic points will depend on the total molar concentration (i. e. the sum of the molar concentrations of the two components). The total molar concentration, $C_{IAA + OIAA}$, at the isobestic point, λ_1, where the absorptivity is a and the absorbance is A_1 is given by the following equation:

$$C_{OIAA + IAA} = \frac{A_1}{a}$$

Now at a different wavelength, λ_2, where absorptivities of IAA and OIAA are a_{IAA} and a_{OIAA}, the absorbance A_2 will be equal to

$$A_2 = C_{OIAA}\ a_{OIAA} + [C_{IAA + OIAA} - C_{OIAA}]\ a_{IAA}$$

then

$$C_{OIAA} = \frac{A_2 - (C_{OIAA + IAA})\ a_{IAA}}{a_{IAA} - a_{OIAA}}$$

A third method for calculating OIAA and IAA concentrations in a mixture is based on the following two equations (Lingane and Collat, 1950):

$$A_{250} = {}_{250}a_{OIAA}\, C_{OIAA} + {}_{250}a_{IAA}\, C_{IAA} \qquad (a)$$

$$A_{280} = {}_{280}a_{OIAA}\, C_{OIAA} + {}_{280}a_{IAA}\, C_{IAA} \qquad (b)$$

A more detailed development of the equations (a) and (b) and their application are given in the Experimental section and in Appendix 1.

Colorimetric Analysis

Two reagents have been extensively used to assay for IAA oxidase activity by measuring residual IAA. The Salper reagent (Patel, 1964) which is a modified Salkowski reagent (Hare, 1964) is often used for colorimetric determination of IAA. The Salper reagent consists of perchloric acid and $FeCl_3$ instead of sulfuric acid and $FeCl_3$ as in the Salkowski reagent. Patel (1964) investigated the Ehrlich reagent (p-dimethylamino benzaldehyde) as an assay reagent for IAA oxidase activity, but found it to be insensitive to the product (OIAA) at low concentrations. It is also not specific for IAA since it reacts with many other indoles.

Manometric Methods

Monometric techniques are employed to assay IAA oxidase activity by measuring the oxygen uptake during the reaction.

Ray and Thimann (1956), Fåhraeus (1956) and Tonhazy and Pelczar (1954), among others, followed the enzyme activity by the O_2 uptake. They measured the uptake in a Warburg Respirometer using standard techniques.

Paper Chromatography

Paper chromatography has been found to be a useful qualitative tool for following IAA oxidase activity. Its use as a quantitative tool, however, is severely limited by the many difficulties encountered when used as such.

Paper chromatography can be used for the qualitative separation and identification of indoles (Block, Durrum and Zweig, 1958). Since IAA produces a blue-violet spot with Ehrlich reagent (Block, Durrum and Zweig, 1958) and OIAA, a blue-green spot (Siehr, 1961), paper chromatography proved to be a useful tool for qualitative determinations of enzyme activity in H. conicus.

Enzyme Induction

Although the theory of enzyme induction is beyond the scope of this thesis, the IAA oxidase of H. conicus is an induced enzyme and, therefore, it may be worthwhile to mention some general facts about enzyme induction.

It is known that enzyme synthesis is under gene control (Dixon,

Webb, 1964). For the formation of a given enzyme by a cell to occur the corresponding structural gene must be present. However, the mere presence of the gene in the cell does not suffice to guarantee the production of the enzyme. In some cases a small molecular substance which is related to the reaction catalyzed by the enzyme must be present for production of the enzyme to occur. This small molecular substance is called an inducer, and the enzyme whose formation it elicits is called an induced enzyme. In the case where the cell produces the enzyme without the presence of an inducer the enzyme is known as a constitutive enzyme.

Most of the known inducers are also substrates for the enzyme whose formation they elicit (Neilands, Stumpf, 1955). An example of this case is H. conicus in which IAA acts both as an inducer and as a substrate for the IAA oxidase (Patel, 1964). However, this does not mean that inducers and substrates are synonymous or that inducers owe their inductive effect to their properties as substrates. There are inducers known which cannot act as substrates, and there are substrates which have no ability to elicit enzyme formation.

In most cases if the inducer is removed or depleted the induced enzyme synthesis ceases (Dixon, Webb, 1964). One notable exception to this rule is the enzyme penicillinase whose formation is induced by penicillin in the micro-organism, Bacillus cereus. In this case the

production of penicillinase is continued for many generations after removal of the inducer, penicillin.

III. EXPERIMENTAL

Materials

The malt extract and the agar were obtained from Difco Chemical Company. The indole acetic acid, the bovine serum albumin, and the reduced glutathione were obtained from the Sigma Chemical Company, and the p-dimethyl amino benzaldehyde was obtained from the Matheson Chemical Company. All of these materials were used without further purification. The oxindole acetic acid was prepared by Chung Y. Chen of this laboratory by the Lawson and Withop (1961) method and was used without further purification.

All inorganic salts and solvents were reagent grade and were used without further purification.

The culture, Hygrophorous conicus, was obtained from Abbott Laboratories, North Chicago, Ill.

Apparatus

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

Colorimeter, Spectronic 20. Bausch and Lomb Incorporated, Rochester, N. Y.

Freeze Drying Apparatus. (Lyophil). Ace Glass Incorporated, Vineland, N. J. Apparatus described by Campbell and Pressman (1944)

pH Meter. (No. 7664) Leeds and Northrup Company, 4901 Stanton Ave., Philadelphia 44, Pa.

Pressure Filtration Funnel (No. 4240) using a Gelman 2 Micron Multipore Filter. Gelman Instrument Company, Ann Arbor, Mich.

Rotary Evaporator. Rinco Instrument Company, Greenville, Ill.

Shaker, Rotary. New Brunswick Scientific Company, New Brunswick, N. J. Model CS-62630

Sonifier. Branson Sonic Power, Division of Branson Instruments Inc., Danbury, Conn. Model S75

Spectrophotometer. Beckman DK-2A. Scientific and Process Instruments Division, Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, Calif.

Spectrophotometer. Hitachi-Perkin Elmer Model 139. Perkin Elmer Corp., Church Street Station, New York 8, N. Y.

Sterilizer. Rectangular type, 24" x 36" x 48" (steam heat). American Sterilizer Co., Erie, Pa.

Vacuum Gauge. Hastings-Raydist Inc., Hampton, Virginia

Vacuum Pump. Duo Seal, Welch Scientific Co., 1515 Sedgwick, Chicago 10, Ill.

General Methods

Preparation of the Sodium Salt of Indole Acetic Acid

The sodium salt of indole acetic acid (IAA) was prepared by suspending IAA in ten times its weight of water containing an excess of sodium hydroxide. The mixture was stirred continuously at 60°C for three hours. The solution was filtered, and acetone was added to precipitate the salt. Twenty five volumes of acetone per volume of salt solution were necessary. The sodium indole acetate (NaIAA) was collected by filtration and washed with acetone. The salt was redissolved in a minimum amount of warm water (60°C) and reprecipitated with acetone. Portions of the salt were recrystallized a second and a third time. The purity of the 2x and 3x recrystallized salts was determined spectrophotometrically. A comparison was made between the absorption peak at 280 mμ of a solution of the Na IAA and that of a solution containing an equivalent amount of IAA. This showed the 2x and 3x recrystallized salts to be 94.7% and 96.5% pure, respectively. The 3x recrystallized salt was not further purified. Calculations involving Na IAA were corrected for the impurity.

Cultivation of H. Conicus

Stock cultures of H. conicus were grown at 25°C in a medium of 4% malt extract. Three hundred ml. Erlenmeyer flasks containing sixty ml. of the malt medium were autoclaved at 125°C and 15 psi for twenty minutes before inoculation with the culture.

The stock cultures of H. Conicus were prepared once a month by inoculation of the autoclaved medium with a culture from a malt-agar slant. The stock cultures were maintained by transferring three ml. of mycelial suspension from the stock culture to fresh malt medium weekly. Immediately after inoculation the stock culture flasks were placed on a rotary shaker (240 r.p.m.) where they remained during the entire period of growth.

Induction of IAA oxidase in H. conicus was accomplished by adding the inducer, IAA or NaIAA, to the malt medium at the time of inoculation. NaIAA (28.2 mg per flask) was found to be much more effective inducer than the equivalent amount of IAA (25.0 mg per flask). The comparison of the effectiveness of IAA and NaIAA as inducers is given in Table 1. The enzyme activity is expressed in μg of oxindole acetic acid (OIAA) formed per hour.

H. conicus was grown periodically on slants or plates to check for contamination of the culture. The solid medium used for the slants and plates was a 4% malt extract solution to which 1 1/2% agar was added. The medium was prepared by heating the agar in the malt solution until the agar was dissolved. The hot medium was poured into culture tubes and autoclaved. Following sterilization the hot medium was poured into sterile Petri dishes for plates or allowed to cool and solidify in the tubes for slants. A wire loop was used to inoculate the slants or the plates with H. conicus from the stock

TABLE 1

COMPARISON OF IAA AND NaIAA AS INDUCERS

INDUCER	SUBSTRATE	*ABSORBANCY AT 280 mμ	*ABSORBANCY AT 250 mμ	μg OIAA FORMED PER HOUR
25 mg IAA	2.82 mg NaIAA	0.552	0.407	267
28.2 mg NaIAA	2.82 mg NaIAA	0.486	0.624	578
28.2 mg NaIAA	2.82 mg NaIAA	0.489	0.641	578

*BLANK SUBSTRACTED

culture. The culture was allowed to grow for several days at room temperature and examined for contamination. The uncontaminated slants were stored in the refrigerator and were later used to prepare stock cultures. The plates were autoclaved and discarded.

Preparation of Two Dimensional Paper Chromatograms

Paper chromatograms were used for the separation and qualitative determination of IAA and OIAA. The spent malt medium from which the mycelia had been removed by centrifugation was used in the preparation of the chromatograms. The malt medium was first acidified with 5% HCl to a pH of 3 and was then extracted with three, 30 ml portions of ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the extract was evaporated to dryness at 45°C. The solid was weighed and then dissolved in a volume of ethyl acetate that would give a concentration of approximately 10 mg/ml of OIAA. In order to make this dilution it was assumed that half of the IAA was converted to OIAA. Five lambda (5×10^{-3} ml) of the solution was spotted in a corner, 1.5 cm from both edges, of an 11 inch square sheet of chromatographic paper (Whatman No. 1 filter paper). The spot was dried with a hand hair dryer, and then two edges of the chromatogram were stitched together with thread to form a cylinder. The chromatogram was placed in a glass cylinder which contained

Solvent A (300 ml isopropanol, 15 ml of concentrated ammonia, and 30 ml of water). The container was covered and sealed, and the solvent was allowed to rise to within a few centimeters of the top edge of chromatogram (usually overnight). The chromatogram was removed from the glass cylinder, the threads were cut, and the paper was hung to dry. The chromatogram was again formed into a cylinder, and the two edges which were formerly the top and bottom were stitched together. The chromatogram was placed in Solvent B (180 ml of n-butanol, 45 ml of glacial acetic acid, and 75 ml of distilled water), and this solvent was allowed to rise about 8 1/2 inches from the bottom edge (usually five hours). The chromatogram was removed from the cylinder and allowed to dry.

The dried chromatogram was examined under ultraviolet light. The OIAA spot fluoresced blue while the IAA spot absorbed in the U. V. If 5-hydroxy IAA were present, it fluoresced as a pink spot under U. V. The spots seen under U. V. were marked for comparison with the spots formed when the chromatogram was dipped in Ehrlich reagent.

After dipping the dried chromatogram in Ehrlich reagent (2.0 g p-dimethylamino-benzaldehyde, 160 ml of acetone, and 20 ml of concentrated HCl) a violet spot appeared almost immediately at the same location as the U. V. absorbing spot (IAA). If 5-hydroxy IAA were present, then a violet spot which eventually turned blue would also

immediately appear at the same place as the pink fluorescent spot. After five or ten minutes a blue-green spot appeared at the same location as the blue fluorescent spot (OIAA).

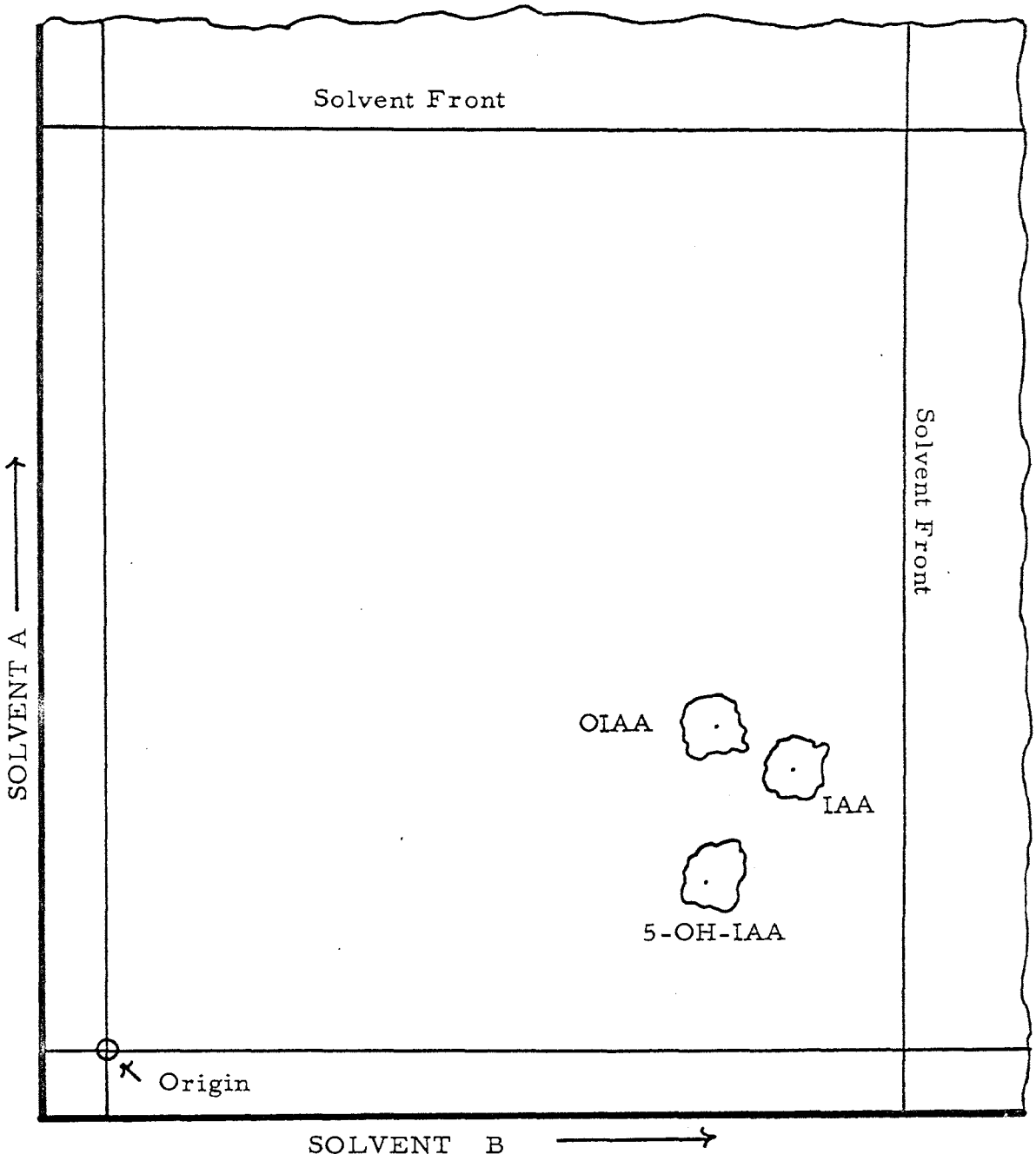
As a check of technique, a chromatogram of known indoles was always prepared and analyzed along with the chromatograms prepared from the spent malt medium. The known indole solutions were prepared by dissolving 10 mg each of IAA, OIAA and 5-hydroxy IAA in one ml of ethanol. Five lambda of each of the solutions were spotted on chromatographic paper, and the chromatogram was treated as described above.

Figure 1 is an illustration of a typical chromatogram and shows the R_f values for IAA, OIAA, and 5-hydroxy IAA in Solvents A and B.

Enzyme Assay

Since OIAA and IAA absorb strongly in U. V. at different wavelengths, 250 mμ and 280 mμ respectively, a simultaneous spectrophotometric analysis of the two substances was possible. Absorbance values for OIAA and IAA at various wavelengths are given in Table 2 and are shown plotted in Figure 2.

In order to ascertain whether or not IAA and OIAA adhered to Beer's law, solutions of various concentrations of OIAA and IAA were prepared, and their absorbances at 280 and 250 mμ were



Rf VALUES

	Rf IAA	Rf OIAA	Rf 5-OH-IAA
Solvent A	30.4	34.4	18.2
Solvent B	86.0	76.2	74.8

Figure 1. TYPICAL CHROMATOGRAM OF IAA, OIAA, and 5-HYDROXY IAA

TABLE 2

ABSORBANCE VALUES OF IAA AND OIAA IN WATER
AT VARIOUS WAVELENGTHS
DETERMINED USING THE BECKMAN DK-2A
SPECTROPHOTOMETER

Wave Length (mμ)	Absorbance of IAA (25 $\frac{mg}{L}$)	Absorbance of OIAA ($\frac{18\ mg}{L}$)
230	-	0.428
235	0.470	0.510
240	0.260	0.675
245	0.240	0.725
250	0.270	0.760
255	0.368	0.675
260	0.480	0.536
265	0.601	0.310
270	0.702	0.200
275	0.740	0.170
280	0.780	0.152
283	0.730	-
285	0.669	0.121
288	0.665	-
290	0.610	0.090
292	0.470	-
295	0.300	0.050
300	0.130	0.029
310	0.015	0.015
320	0.009	0.009
330	0.005	0.005
340	0.000	0.000

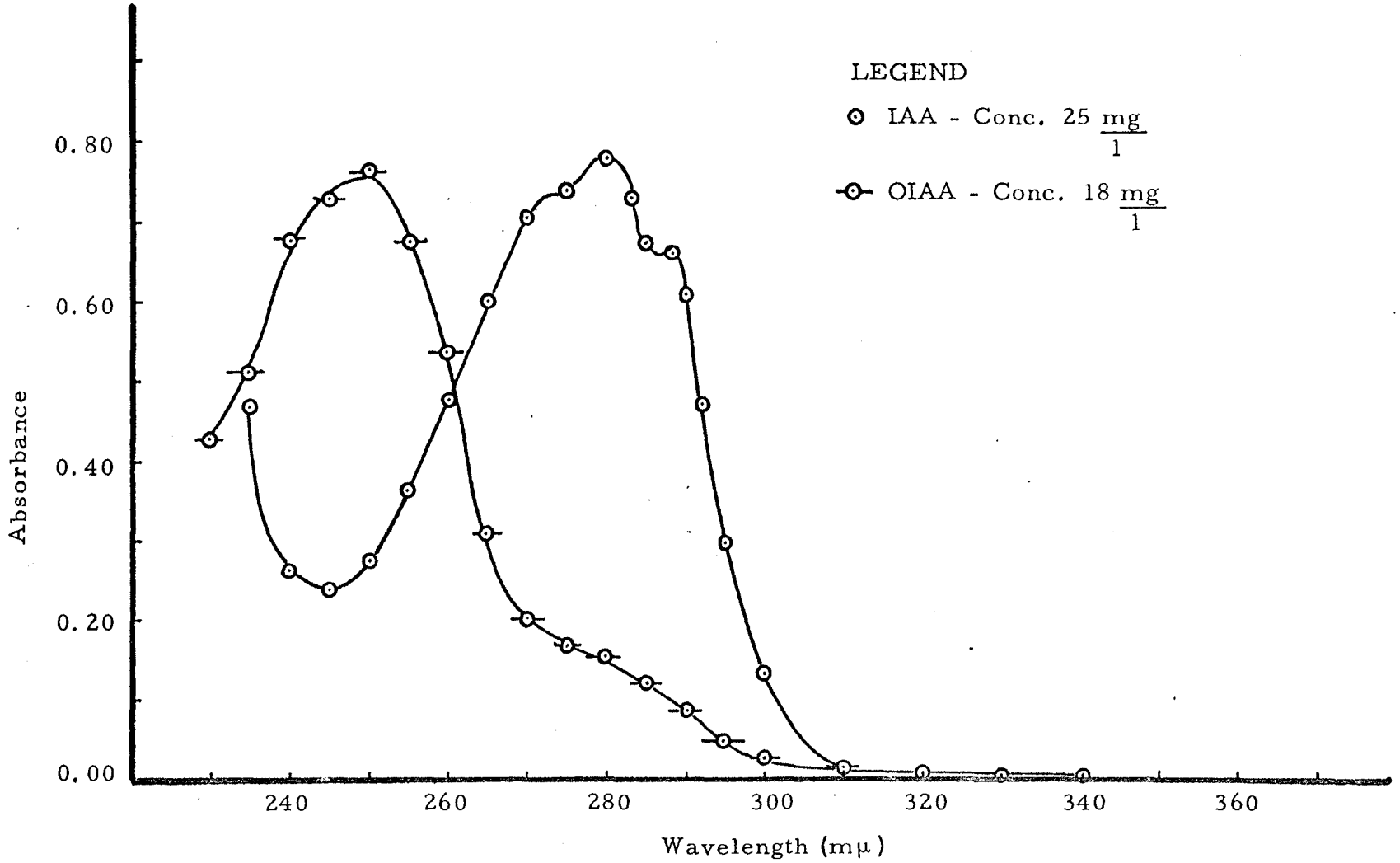


Figure 2. ABSORBANCE OF IAA AND OIAA IN WATER DETERMINED ON THE BECKMAN DK-2A

determined (Table 3). The plots of these absorbance values of IAA and OIAA at both 250 and 280 mμ versus concentration (Figures 3 and 4) are straight lines; therefore, solutions of IAA and OIAA do adhere to Beer's law. Since IAA is relatively insoluble in water, a pH 8.0 phosphate buffer was used in the preparation of both the IAA and the OIAA solutions. Various mixtures of IAA and OIAA were also prepared, and the absorbances of these solutions at 250 and 280 mμ were determined (Table 4). Figure 5 shows that the mixtures also conform to Beer's law.

The spectrophotometric analysis was conducted on a Beckman DK-2A recording spectrophotometer. The solutions were usually scanned from 340-230 mμ. Before scanning the sample the matched, quartz cuvettes were cleaned with 50% ethanol solution containing 10% ammonia. The sample and reference cuvettes were checked for cleanliness by comparing them filled with distilled water in the wavelength span used for the sample determination. During scanning the reference cuvette was filled with the same solvent as used in the preparation of the sample.

In order to assay for enzyme activity using the described spectrophotometric analysis, the basidiomycete cells were first harvested from the malt extract medium containing the inducer. The cells were usually grown in the presence of the inducer for

TABLE 3

ABSORBANCE VALUES FOR VARIOUS CONCENTRATIONS
OF IAA AND OIAA IN WATER

IAA $\frac{mg}{l}$	Absorbance at 280 mμ	Absorbance at 250 mμ	OIAA $\frac{mg}{l}$	Absorbance at 280 mμ	Absorbance at 250 mμ
25.0	0.778	0.278	18.0	0.154	0.764
22.5	0.708	0.257	16.2	0.140	0.798
20.0	0.629	0.228	14.4	0.122	0.610
17.5	0.550	0.201	12.6	0.110	0.540
15.0	0.470	0.173	10.8	0.095	0.462
12.5	0.391	0.141	9.0	0.080	0.388
10.0	0.310	0.117	7.2	0.065	0.308
7.5	0.234	0.086	5.4	0.049	0.230
5.0	0.155	0.055	3.6	0.037	0.156
2.5	0.082	0.030	1.8	0.019	0.078

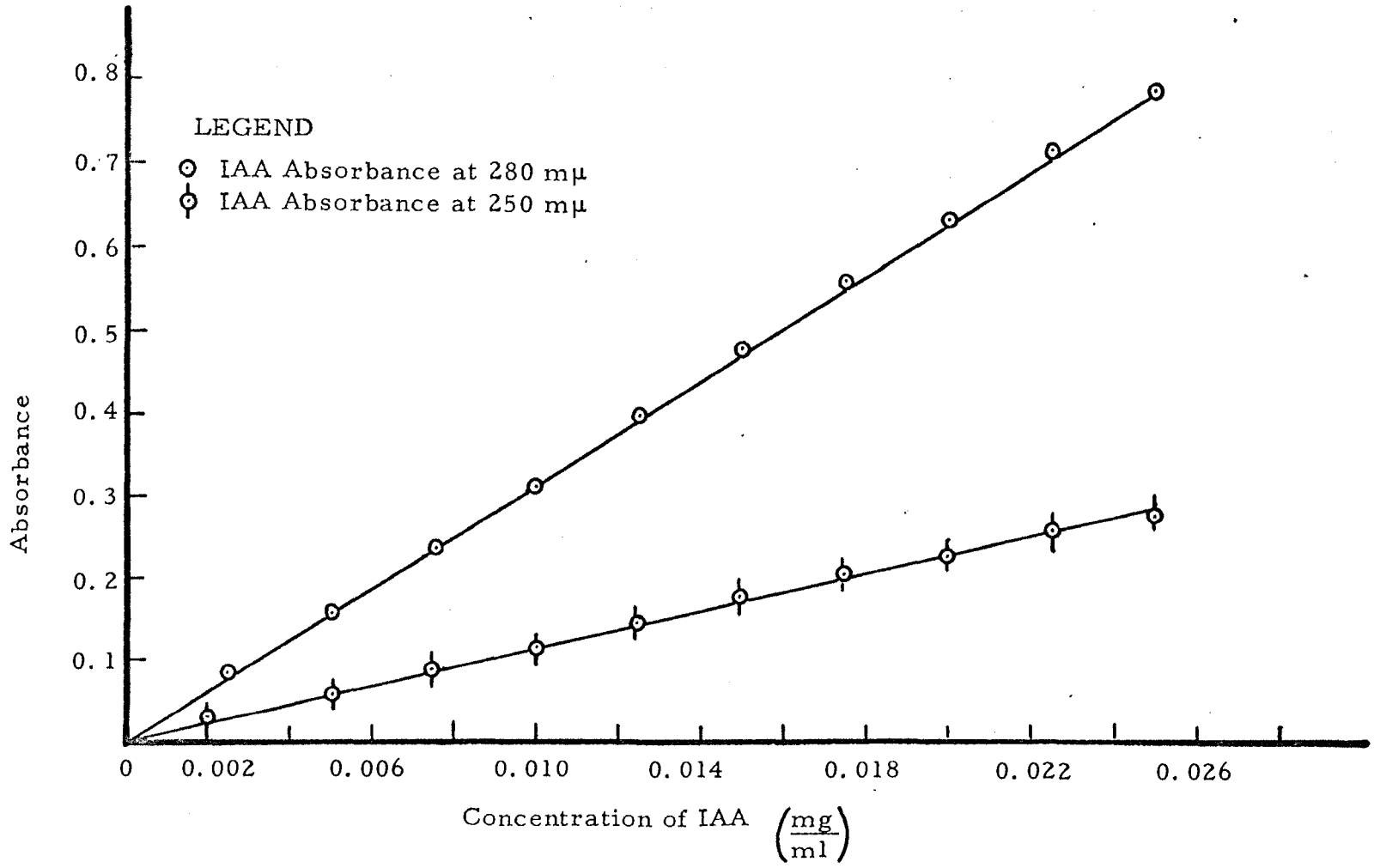


Figure 3. ABSORBANCE OF VARIOUS CONCENTRATIONS OF IAA AT 280 and 250 mμ

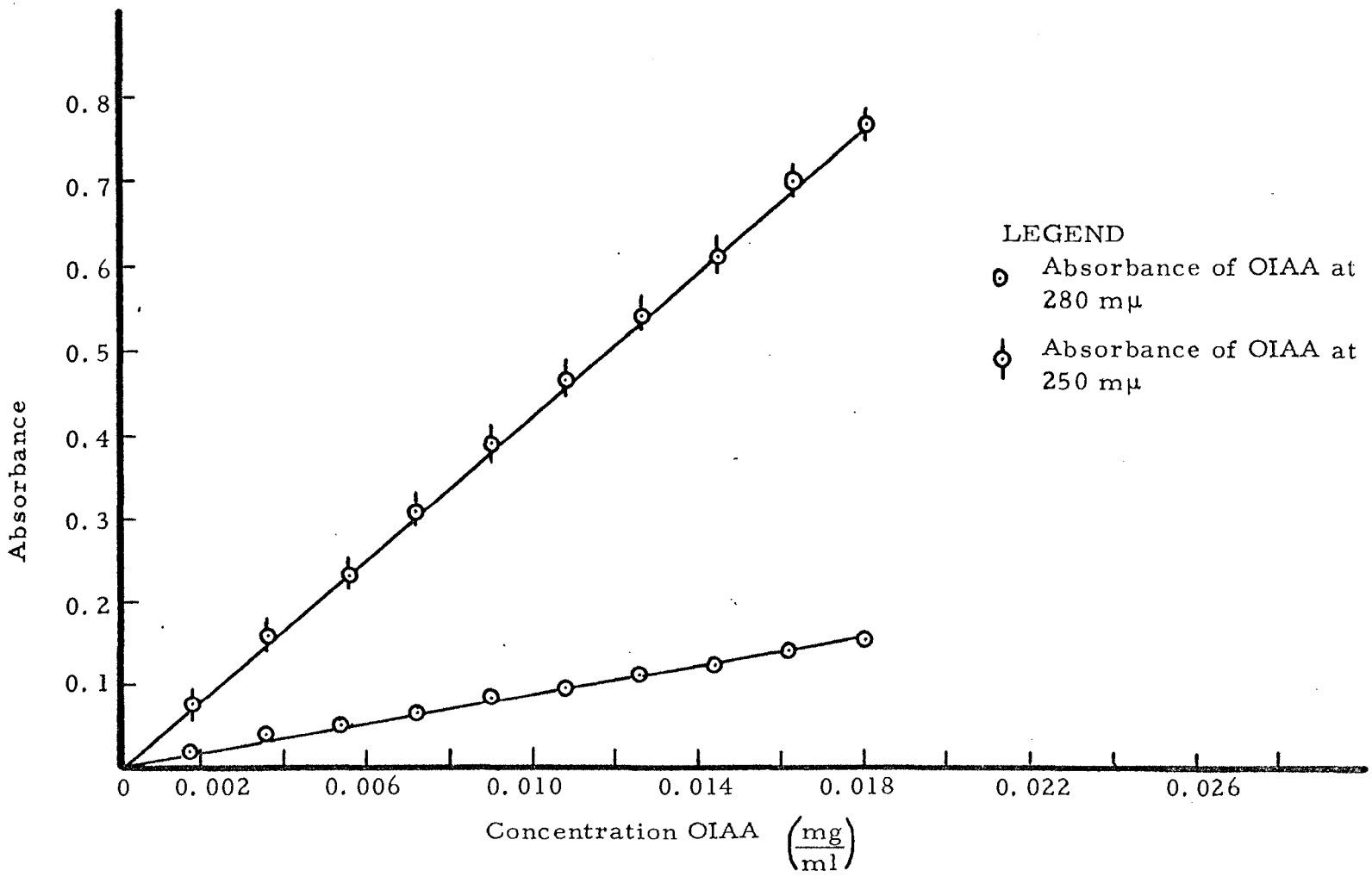


Figure 4. ABSORBANCE OF VARIOUS CONCENTRATIONS OF OIAA AT 250 and 280 mμ

TABLE 4

ABSORBANCE VALUES FOR MIXTURES OF
INDOLE ACETIC ACID AND OXINDOLE ACETIC ACID

% OIAA By Weight	OIAA $\frac{mg}{ml}$	% IAA By Weight	IAA $\frac{mg}{ml}$	Absorbance at 280 mμ	Absorbance at 250 mμ
0	0.000	100.0	0.0250	0.789	0.282
7.4	0.0018	92.6	0.0225	0.730	0.330
15.3	0.0036	84.7	0.0200	0.665	0.380
23.6	0.0054	76.4	0.0175	0.598	0.428
32.4	0.0072	67.6	0.0150	0.538	0.481
41.8	0.0090	58.2	0.0125	0.474	0.530
51.9	0.0108	48.1	0.0100	0.408	0.578
62.7	0.0126	37.3	0.0075	0.341	0.626
74.4	0.0144	25.6	0.0050	0.278	0.672
86.6	0.0162	13.4	0.0025	0.215	0.723
100.0	0.0180	0	0	0.155	0.760

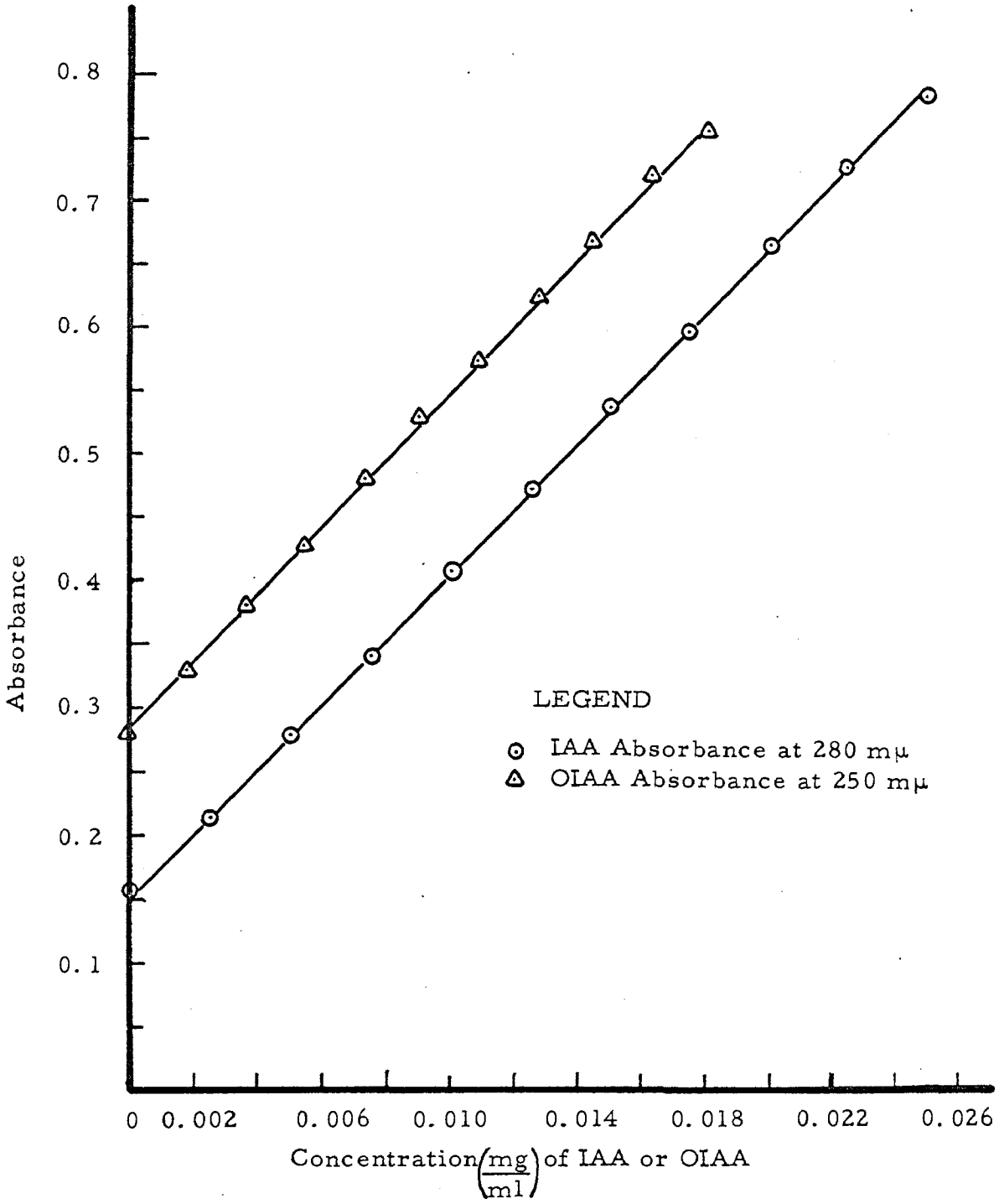


Figure 5. ABSORBANCE OF VARIOUS MIXTURES OF IAA AND OIAA

48 hours before harvesting. The cells were harvested by centrifugation at 6000 r.p.m. for 12 minutes. A Lourdes high speed centrifuge, which was located in the cold room (5°), was used. The cells were washed twice with distilled water to remove all traces of the malt medium and inducer. The cells were then suspended in 40 ml of distilled water, and the suspension added to 10 ml of distilled water containing 2.82 mg NaIAA. The cell suspension was incubated with the substrate (NaIAA) in an Erlenmeyer flask on the rotary shaker for two hours at 25°C. After incubation the assay sample was prepared for U. V. spectrophotometric analysis by one of two methods.

Method 1: Extraction

The cells were removed from suspension by centrifuging at 6000 r.p.m. for 12 minutes and were washed twice with distilled water. The supernatant and washings were combined and acidified to a pH of 3 (hydrion paper) with 5% HCl. The solution was extracted with three, fifteen ml portions of diethyl ether. The combined fractions were extracted with three, ten ml portions of 2% Na_2CO_3 solution. The Na_2CO_3 portions were combined, and the pH was adjusted to 7.0 (Leeds and Northrup pH meter) with 5% HCl. The solution was diluted to 100 ml in a volumetric flask. The absorbance of the solution was determined by scanning it on the Beckman DK-2A. The recovery of OIAA and IAA by this method was determined by preparing 50 ml solutions containing 2.5 mg of IAA or 1.8 mg of OIAA and extracting

them as described above. The recovery of IAA was found to be 90%, while the recovery of OIAA was only 26%. In order to improve the recovery of OIAA, the OIAA solutions were saturated with NaCl before extraction. The recovery of OIAA was 70% in this case.

Method 2: Filtration

The cells were removed from suspension by centrifugation and were washed twice with distilled water. Instead of extracting the supernatant as in Method 1, it was filtered through a Gelman 2 Micron multipore filter to remove any turbidity and then was diluted to 100 ml. This method was used whenever possible since the loss of IAA and OIAA was small, and the method was less time consuming.

The concentrations of IAA and OIAA in the assay sample were calculated from the net absorbance of the sample at 250 and 280 mμ by use of the equations:

$$C_{IAA} = 3.44 \times 10^{-2} A_{280} - 6.91 \times 10^{-3} A_{250} \quad (1)$$

$$C_{OIAA} = 2.57 \times 10^{-2} A_{250} - 9.24 \times 10^{-3} A_{280} \quad (2)$$

where C = concentration in mg/ml

A = net absorbance of the sample at the designated wavelength

The development of these two equations is given in Appendix 1.

The net absorbance of the sample was obtained by subtraction

of the absorbance of the blank at 250 and 280 mμ from the total absorbance of the sample. The blank was prepared in the same way as described for the sample except no substrate was added during the assay incubation of the induced cells.

A standard was also scanned with each group of samples. The standard was prepared by diluting 10 ml of the 0.282 mg/ml NaIAA to 100 ml in a volumetric flask. The NaIAA solution which was used in the preparation of the standard was the same solution that was used as the substrate in the assay incubation.

The Rate of Growth of H. Conicus and Formation of the Enzyme

The 4% malt extract medium was prepared as described under Cultivation of H. conicus except the medium and the culture flasks were autoclaved separately. The insoluble material formed in the medium during autoclaving was allowed to settle out. Only the clear malt solution was transferred to the sterile flasks. It was necessary to remove the insoluble material in order to obtain an accurate cell weight, especially in the early samples. A 28.2 mg/ml solution of NaIAA was prepared by dissolving the NaIAA in sterile water, and one ml. of this solution was pipetted into each of the flasks. The flasks were inoculated with 3 ml of one week old stock culture. Flasks were harvested at 6, 12, 18, 24, 36, 48, 60 and 72 hours. Three flasks were prepared for each of these sampling times--one for a blank and two duplicates. The zero hour sample was prepared by

transferring 3 ml of the inoculum into 60 ml of distilled water. The cells of the zero hour sample were collected by filtration through the Gelman 2 Micron multipore filter and were washed twice with distilled water. Because of the small cell volume, the cells of the 6 hour sample were also harvested and washed by filtration through the Gelman filter. The 12 to 72 hour samples were all harvested by the usual centrifugation procedure, and were washed twice with distilled water. The washed cells of all the samples were transferred to flasks containing either 50 ml of distilled water (blanks) or 40 ml of distilled water plus 10 ml of 0.282 mg/ml NaIAA solution. The samples were incubated for two hours at 25°C on the rotary shaker. Following incubation the cells of the zero and 6 hour samples were again collected by filtration through the Gelman filter, and the cells from the 12 to 72 hour samples were collected by centrifugation. The supernatants from the 12 to 72 hour samples were filtered through the Gelman filter. The filtrates of all the samples were diluted to 100 ml and analyzed spectrophotometrically. The cells from all the samples were transferred to Gooch crucibles which had been dried to a constant weight in an oven at 100-110°C. The cells and Gooch crucibles were dried at 100-110°C until they reached constant weight. The dry weight of the cells at various sampling times are tabulated in Table 5. Figure 6 is a plot of the increasing cell weight with time. The absorbance of the samples, the calculated

TABLE 5

DRY WEIGHT OF MYCELIA AT VARIOUS SAMPLING TIMES

Time of Sample	RUN 1		RUN 2	
	Dry Wt. Cells (mg)	Avg. Wt.	Dry Wt. Cells (mg)	Avg. Wt.
Zero Hr.	14.4		16.1	
" "	15.0	13.9	17.3	17.5
" "	12.4		19.0	
6 Hour	18.4		16.5	
" "	13.3	15.2	18.9	18.5
" "	14.0		20.2	
12 Hour	39.5		29.1	
" "	34.9	37.8	29.4	27.1
" "	39.0		22.8	
18 Hour	83.9		70.0	
" "	87.7	82.8	29.8 *	66.1
" "	76.7		62.2	
24 Hour	111.7		23.6 *	
" "	74.6	99.2	63.1	88.6
" "	111.6		114.1	
36 Hour	166.9		132.4	
" "	167.2	156.8	179.3	159.3
" "	136.2		166.2	
48 Hour	191.4		155.6	
" "	188.9	193.1	176.9	175.5
" "	198.9		193.9	
60 Hour	196.9		179.5	
" "	193.4	191.9	186.8	185.1
" "	185.5		188.9	
72 Hour	173.8		181.8	
" "	237.6	207.7	202.4	209.6
" "	211.7		244.6	

*Not Averaged

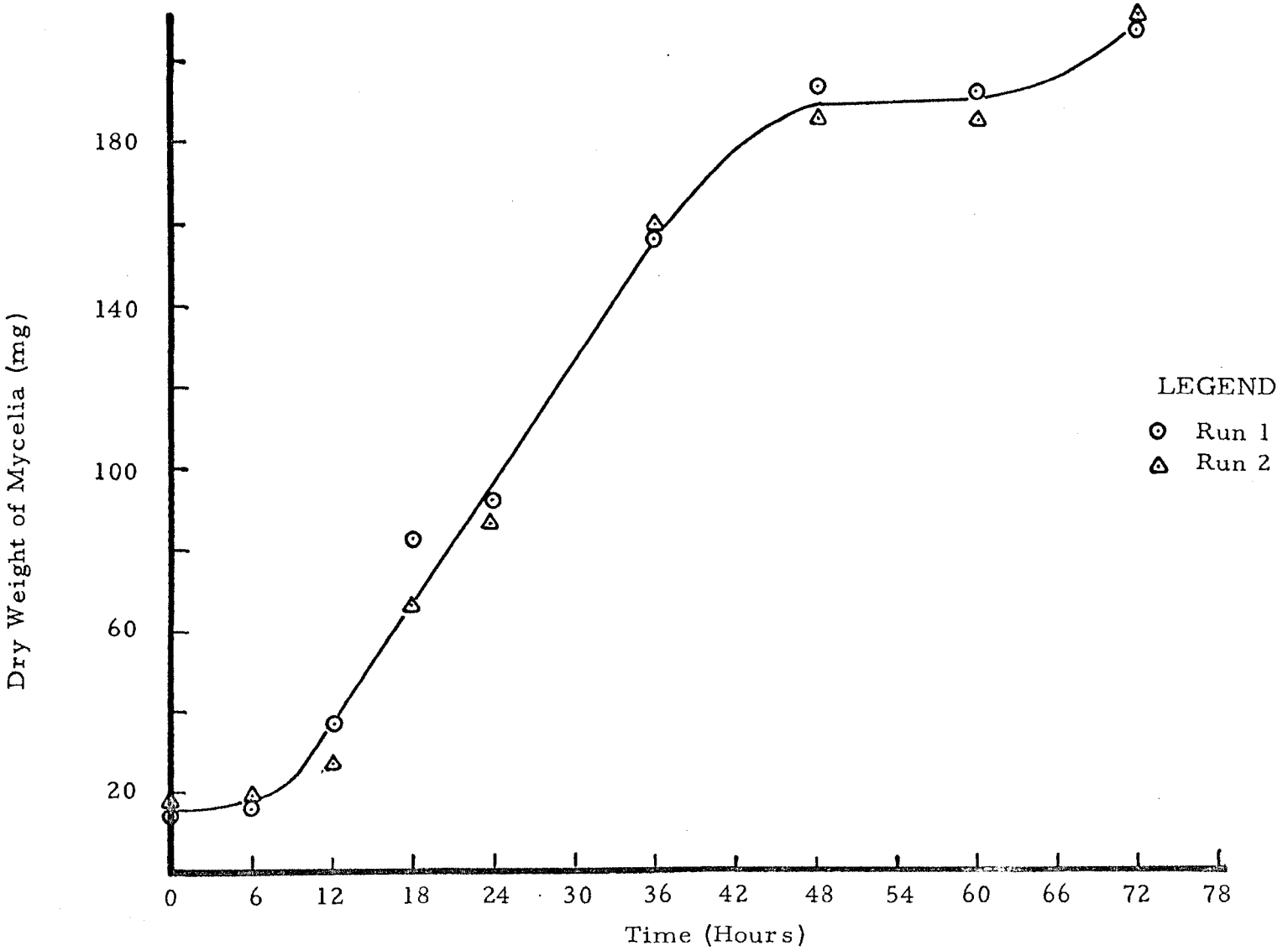


Figure 6. PLOT OF DRY WEIGHT OF MYCELIA VERSUS SAMPLING TIME

concentrations of OIAA and IAA, and the enzyme activities are given in Table 6 and 7. The enzyme activity is reported in enzyme units/ mg dry weight of cells. An enzyme unit is defined as the formation of one microgram of OIAA per hour. The specific enzyme activity (μg OIAA/hr/dry weight of cells) is shown plotted as a function of time in Figure 7.

Paper chromatograms were prepared from the spent malt medium and also from several of the samples after spectrophotometric analysis. These results are shown in Table 8.

OIAA Induced Enzyme

The results of the 60 and 72 hour samples described in the previous section suggested the possibility of an OIAA induced enzyme; therefore, an experiment was conducted to investigate this possibility.

Six culture flasks, three containing 28.2 mg of NaIAA as an inducer and three containing 18 mg of OIAA as an inducer, were inoculated with mycelia, and the cells were grown at 25°C on the rotary shaker for 48 hours. The induced cells were collected by centrifugation and were washed twice with distilled water. The washed cells were transferred to culture flasks for the enzyme assay. The contents of the flasks were as shown in Table 9. After two hours of incubation at 25° on the rotary shaker, the contents of the six flasks were centrifuged, and the cells were washed twice with distilled

TABLE 6

ENZYME ACTIVITY AT VARIOUS TIMES
FOR RUN 1

Time of Sample	*Absorbance at 280 mμ	*Absorbance at 250 mμ	μg of IAA in Sample	μg of OIAA in Sample	μg OIAA Formed Per Hour	Enzyme Units per mg Dry Wt of Mycelia	Avg Enzyme Units per mg Dry Wt
Zero Hour	0.747	0.279	2.37×10^3	27	13.5	0.94	1.13
" "	0.745	0.279	2.37 "	29	14.5	1.32	
6 Hour	0.734	0.271	2.34 "	18	9.0	0.68	1.12
" "	0.737	0.282	2.35 "	44	22.0	1.57	
12 Hour	0.713	0.270	2.26 "	35	17.5	0.50	0.51
" "	0.728	0.278	2.31 "	41	20.5	0.52	
18 Hour	0.706	0.274	2.23 "	52	26.0	0.30	0.28
" "	0.720	0.275	2.28 "	42	21.0	0.27	
24 Hour	0.719	0.276	2.28 "	45	22.5	0.30	0.131
" "	0.632	0.430	1.88 "	521	26.0	2.33	
36 Hour	0.463	0.590	1.185"	1088	544.0	3.25	0.198
" "	0.675	0.317	2.10 "	191	95.5	0.70	
48 Hour	0.403	0.681	0.917"	1378	689.0	3.65	3.60
" "	0.387	0.689	0.857"	1413	706.5	3.55	
60 Hour	0.463	0.217	1.444"	130	65.0	0.34	0.34
" "	0.448	0.211	1.393"	128	64.0	0.34	
72 Hour	0.547	0.231	1.725"	87	43.5	0.18	0.19
" "	0.571	0.239	1.795"	86	43.0	0.20	

*Blank Subtracted

TABLE 7

ENZYME ACTIVITY AT VARIOUS TIMES
FOR RUN 2

Time of Sample	*Absorbance at 280 mμ	*Absorbance at 250 mμ	μg of IAA in Sample	μg of OIAA in Sample	μg OIAA Formed per Hour	Enzyme Units per mg dry Wt. of Mycelia	Avg. Enzyme Units per mg dry Wt.
Zero Hr.	0.732	0.279	2.32×10^3	41	20.5	2.37	
" "	0.724	0.265	2.30 "	12	6.0	0.63	1.50
6 Hour	0.727	0.270	2.31 "	22	11.0	1.16	
" "	0.728	0.268	2.31 "	16	8.0	0.79	0.98
12 Hour	0.712	0.269	2.26 "	33	16.5	1.12	
" "	0.733	0.281	2.32 "	45	22.5	1.97	1.54
18 Hour	0.724	0.278	2.29 "	45	22.5	1.51	
" "	0.721	0.272	2.29 "	33	16.5	0.53	1.04
24 Hour	0.744	0.307	2.34 "	102	51.0	1.62	
" "	0.723	0.323	2.26 "	162	81.0	1.42	1.52
36 Hour	0.727	0.332	2.27 "	181	90.5	1.01	
" "	0.703	0.399	2.14 "	375	187.5	2.22	1.62
48 Hour	0.390	0.807	0.787 "	1734	867.0	9.80	
	0.300	0.935	0.390 "	2126	1063.0	10.96	10.38
60 Hour	0.448	0.280	1.346 "	306	153.0	1.64	
" "	0.389	0.297	1.063 "	661	330.0	3.50	2.57
72 Hour	0.541	0.252	1.684 "	148	74.0	0.731	
" "	0.584	0.240	1.839 "	77	39.5	0.315	0.52

*Blank Substracted

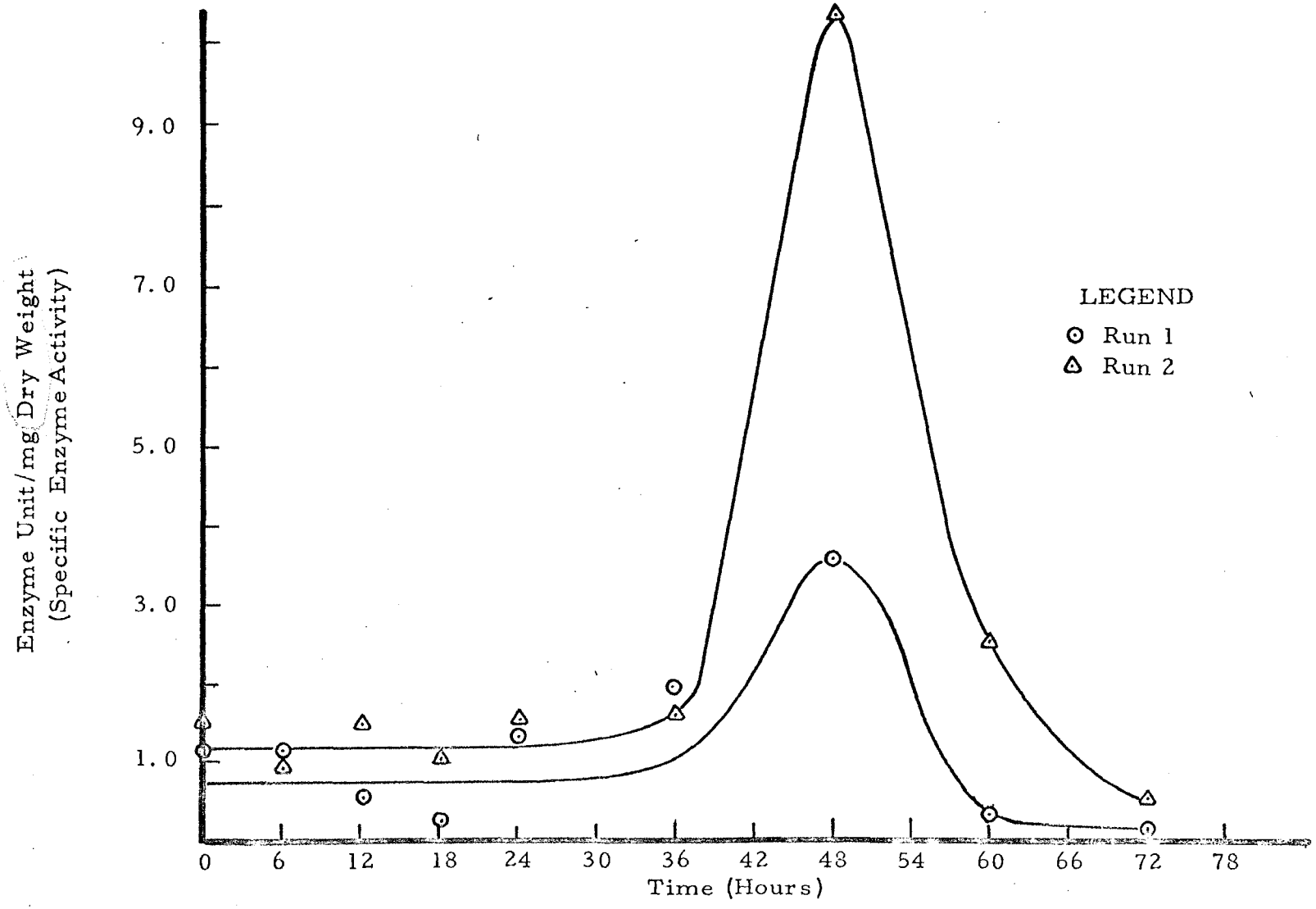


Figure 7. RATE OF FORMATION OF IAA OXIDASE

TABLE 8

SPOTS FOUND ON PAPER CHROMATOGRAMS
PREPARED FROM THE SPENT MALT MEDIUMS
AND ASSAY SAMPLES

Run 1		Run 2	
Time (Hours)	Spent Malt Medium	Spent Malt Medium	Sample
24	-	IAA (Large Quantity)	IAA
36	-	IAA OIAA (Small Quantity)	-
48	IAA 5-OH IAA OIAA	-	IAA (Small Amount) OIAA (Large Amount)
60	-	IAA (Small Amount) OIAA	OIAA (Small Amount)
72	IAA (Small Amount) OIAA (Small Amount)	IAA (Small Amount) OIAA 5-OH-IAA	-

TABLE 9

CONTENTS OF FLASKS USED FOR ASSAY IN OIAA INDUCED ENZYME EXPERIMENT

Flask	Cells	Substrate
1	NaIAA Induced	50 ml of Distilled H_2O (Blank)
2	" "	2.82 mg NaIAA + 50 ml H_2O
3	" "	1.80 mg OIAA + 50 ml H_2O
4	OIAA Induced	50 ml of Distilled H_2O (Blank)
5	" "	2.82 mg NaIAA + 50 ml H_2O
6	" "	1.80 mg OIAA + 50 ml H_2O

water. The supernatant of each of the six samples was filtered through the Gelman multipore filter, diluted to 100 ml, and analyzed spectrophotometrically.

Duplicate paper chromatograms were prepared from the spent malt medium. The chromatograms were either dipped in Ehrlich reagent or sprayed with Pauly reagent after removal from Solvent B. Pauly reagent was prepared by adding 25 ml of freshly prepared 5% $NaNO_2$ slowly at $0^{\circ}C$ to 5 ml of sulfanilic acid solution (0.9 of sulfanilic acid and 9 ml of concentrated HCl, diluted to 100 ml with water). While the chromatogram was still damp following the spraying with Pauly reagent, it was sprayed with 20% Na_2CO_3. Pauly reagent produces color with aromatic amines, hydroxy compounds, and the imidazole ring.

The results of the enzyme assay and the paper chromatograms are shown in Table 10.

Effect of pH on Enzyme Activity

The enzyme assay was conducted in buffers of different pH to determine the effect of pH on enzyme activity. The following buffers were used: potassium hydrogen phthalate/HCl buffer (pH 3.0 and 4.0), phosphate buffer (pH 5.0, 6.0 and 7.0), and sodium citrate/HCl buffer (pH 3.0 and 4.0). The preparation of each of these buffers is given in Table 11 and 11A.

TABLE 10

ENZYME ACTIVITY AND RESULTS OF PAPER CHROMATOGRAMS
FOR THE OIAA INDUCED ENZYME EXPERIMENT

Flask	*Absorbance at 280 mμ	*Absorbance at 250 mμ	μg OIAA Produced per Hour	Spots Found with Ehrlich Reagent	Spots Found with Pauly Reagent
1 (Blank)	0.056	0.071	-	-	-
2	0.414	0.839	890	IAA, OIAA	5-OH IAA
3	0.132	0.658	-116	5-OH IAA	
4 (Blank)	0.071	0.099	-	-	-
5	0.679	0.293	63	OIAA	No Spot
6	0.114	0.242	-609		

* Blank Subtracted

TABLE 11

PREPARATION OF BUFFERS *

Solutions Needed for Preparation of Buffers

A. Potassium Acid Phthalate/HCl Buffer

1. 40.836 g KH Phthalate in Liter = 0.2 M

2. 0.2 M HCl

B. Sodium Citrate/HCl Buffer

1. 21.008 g Citric Acid (Monohydrate) + 200 cm^3 1 N NaOH in Liter = 0.1 M Na Citrate

2. 0.1 N HCl

C. Potassium Dihydrogen Phosphate/Disodium Hydrogen Phosphate Buffer

1. 9.0727g KH_2PO_4 in Liter = M/15

2. 9.4646g $Na_2H\ PO_4$ in Liter = M/15

* Taken from Rauen (1956)

TABLE 11 A*

Preparation of Various pH Buffers

Buffer	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
Potassium Acid Phthalate/HCl	50 ml 0.2 M Phthalate + 21.5 ml 0.2 M HCl Diluted to 200 ml	50 ml 0.2 M Phthalate + 0.25 ml 0.2 M HCl Diluted to 200 ml			
Na Citrate/HCl	59.7% 0.1 N HCl 40.3% 0.1 N Na Citrate	44.0% 0.1 N HCl 56.0% 0.1 N Na Citrate			
KH_2PO_4/Na_2HPO_4			0.95% M/15 Na_2HPO_4 99.05% M/15 KH_2PO_4	12.1% M/15 Na_2HPO_4 87.9% M/15 KH_2PO_4	61.2% M/15 $NaHPO_4$ 58.8% M/15 KH_2PO_4

*Taken from Rauen (1956)

The induction of the enzyme and the enzyme assay were conducted as described in Cultivation of H. conicus and Enzyme Assay. The only changes in the procedure were the cells were washed after centrifugation with buffer solution instead of water, and the cells were suspended in buffer solution containing the substrate for incubation. The pH of the buffer solutions were measured before and after incubation. The results of this experiment are tabulated in Table 12. The phthalate buffer was found to absorb strongly in the U. V. range used; therefore, a second experiment was conducted in which a sodium citrate buffer (pH 3.0 and 4.0) was used instead of the phthalate buffer.

Methods for the Preparation of Cell-Free Enzyme

Previous attempts to prepare a cell-free enzyme by rupturing the cells by grinding them with levigated alumina in a mortar and pestle proved to be unsuccessful. Further attempts were made to rupture the cells using two other methods. The first method involved freeze drying of the cells followed by grinding them with levigated alumina, and the second method involved insonation of the cells.

Since protein is released when the cells are ruptured, measurement of the released protein can give an indication as to the extent of cell breakage. The protein released by freeze drying followed by grinding of the cells was determined colorimetrically using the biuret

TABLE 12

EFFECT OF pH ON ENZYME ACTIVITY

Buffer	Absorbance at 280 mμ	Absorbance at 250 mμ	Absorbance of Blank at 280 mμ	Absorbance of Blank at 250 mμ	μg OIAA Formed per Hour
Phthalate, pH 3.0	-	-	-	-	-
Phthalate, pH 4.0	-	-	-	-	-
Phosphate, pH 5.0	0.501	1.000	0.041	0.056	1005.0
Phosphate, pH 6.0	0.664	0.440	0.038	0.048	214.5
Phosphate, pH 7.0	0.767	0.370	0.052	0.070	50.5
Citrate, pH 3.0	0.610	0.600	0.040	0.078	406.5
Citrate, pH 4.0	0.592	0.570	0.062	0.150	290.0
Phosphate, pH 5.0	0.670	0.498	0.041	0.056	276.0
Phosphate, pH 6.0	0.723	0.352	0.038	0.048	70.0
Phosphate, pH 7.0	0.788	0.352	0.052	0.070	27.5

test for protein (Gornall, Bardawell, David, 1949), and the protein released by insonation of the cells was determined spectrophotometrically. (Seaman, 1963).

Biuret Test for Protein

The biuret reagent was prepared as follows: 1.50 g of $CuSO_4 \cdot 5H_2O$ and 6.0 g of sodium potassium tartrate were dissolved in about 500 ml of H_2O in a liter volumetric flask. With constant swirling 300 ml of 10% NaOH (carbonate free) was added, and then the solution was made up to volume with water. The reagent was stored in a polyethylene bottle. This reagent gives a violet color with polypeptides of 3 or more amino acid components.

A 12 mg/ml solution of bovine serum albumin was used as the protein standard. Various aliquots of the bovine serum albumin solution were pipetted into 10 ml calibrated centrifuge tubes, and 1/4 the volume of 50% trichloro-acetic acid (TCA) was added to precipitate the protein. The precipitate was centrifuged for 15 minutes. The supernatant was carefully removed with a pipet. Eight ml of the biuret reagent was added to the centrifuge tubes, and the precipitate was dissolved in the reagent with careful stirring. The solutions were diluted to 10 ml, and the color was allowed to develop for 30 minutes before reading at 540 mμ on the Spectronic 20 colorimeter. The solutions were read against a blank which consisted of 8 ml of

biuret reagent plus 2 ml of water. The absorbance values for the various aliquots of the protein standard are tabulated in Table 13, and, the mg protein/aliquot is seen plotted against absorbance in Figure 8. Two ml. aliquots of the supernatants from freeze dried cells were analyzed by the same procedure as described for the protein standard aliquots.

Spectrophotometric Method for Protein Determination

This method for protein determination involved taking a one ml aliquot of the supernatant obtained from the insonated cells after insonation and removal of the cell debris by centrifugation. A one ml aliquot was diluted with sufficient water so that the absorbance of the solution at 280 and 260 mμ could be read on the Hitachi-Perkin-Elmer spectrophotometer.

The ratio of the absorbance value at 280 mμ to that at 260 mμ was calculated, and the concentration of protein calculated by the following equation:

$$\frac{\text{mg protein}}{\text{ml}} = F \times \text{absorbance at 280 m}\mu \times \text{dilution}$$

where F is the Warburg factor which can be obtained from Table 14 or Figure 9.

Freeze Drying of the Cells

In preparation for freeze drying, the cells were induced with NaIAA in the usual manner. The cells were collected by centrifugation

TABLE 13

BIURET DETERMINATION
OF
PROTEIN STANDARD

Ml of Bovine Serum Albumin Solution	Mg Protein	Absorbance (Duplicate #1)	Absorbance (Duplicate #2)	Avg. Absorbance
.2	2.4	0.062	0.062	0.062
.4	4.8	0.130	0.132	0.131
.8	9.6	0.272	0.270	0.271
1.2	14.4	0.389	0.385	0.387
1.6	19.2	0.520	0.510	0.515
2.0	24.0	0.610	-	0.610

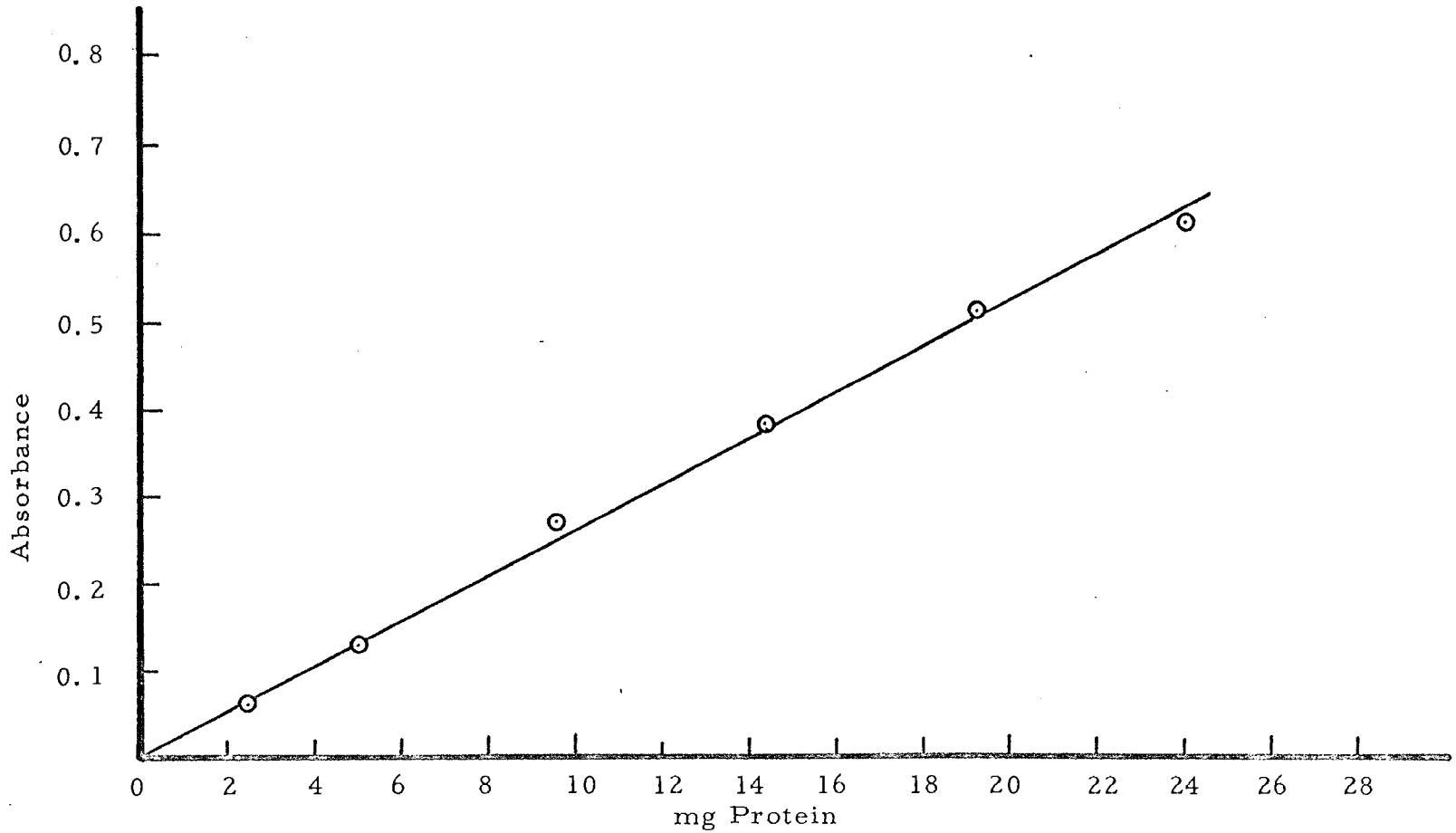


Figure 8. STANDARD CURVE FOR BIURET DETERMINATION

TABLE 14 *

NUCLEIC ACID CONCENTRATIONS AND F VALUES AT VARIOUS $\frac{280}{260}$ RATIOS

$\frac{A_{280}}{A_{260}}$	% Nucleic Acid	F
1.75	0.00	1.116
1.63	0.25	1.081
1.40	0.75	1.023
1.30	1.25	0.970
1.16	2.00	0.899
1.09	2.50	0.852
0.979	3.50	0.776
0.874	5.00	0.682
0.822	6.00	0.632
0.804	6.50	0.607
0.767	7.50	0.565
0.730	9.00	0.508
0.671	12.00	0.422
0.615	17.00	0.322
0.595	20.00	0.278

* Taken from Seaman, G.R., (1963)

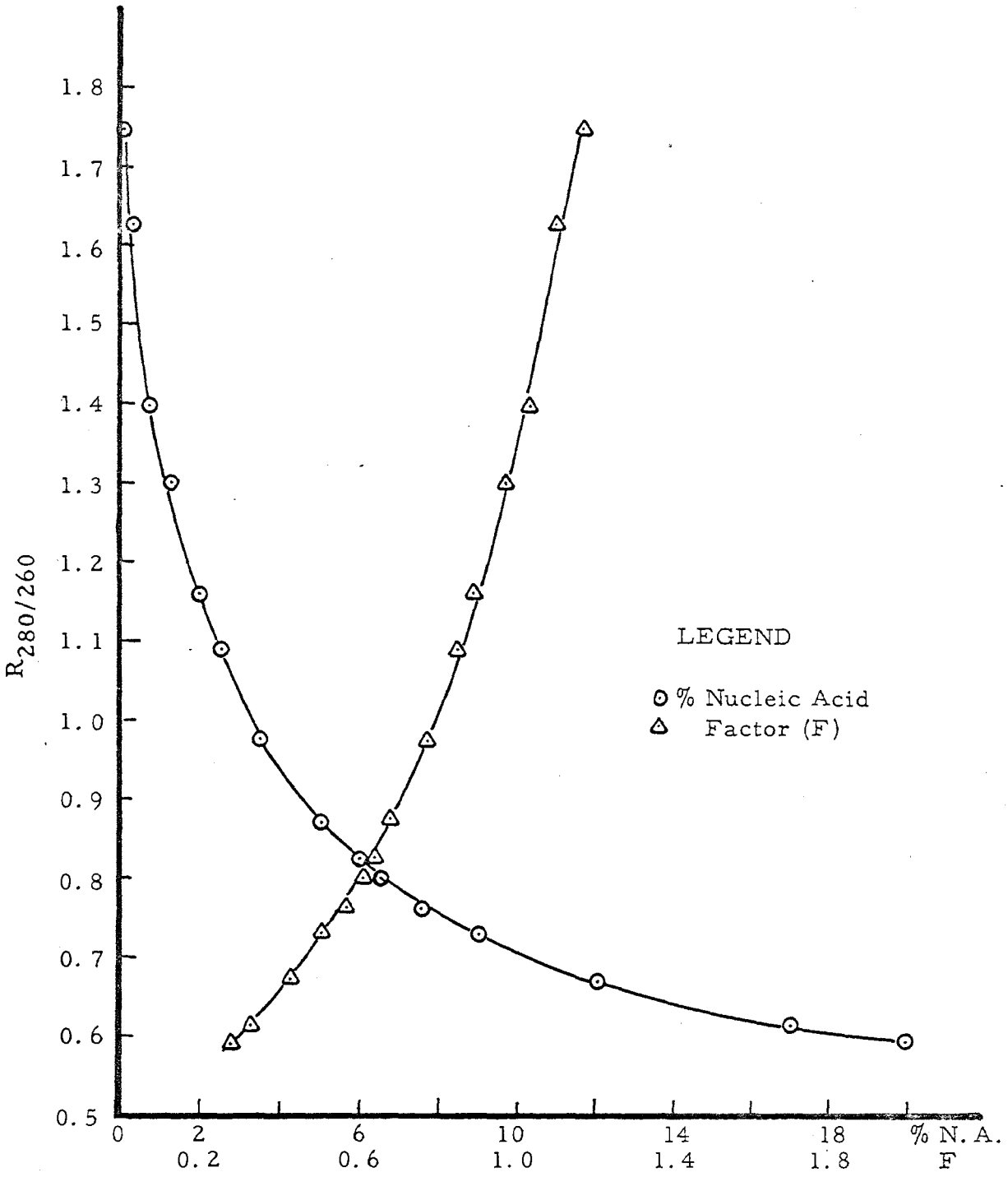


Figure 9. NUCLEIC ACID CONCENTRATIONS AND F VALUES AT VARIOUS 280/260 RATIOS

and were washed twice with distilled water. The washed cells were suspended in approximately 10 ml of water and transferred to a ground glass, pear-shaped flask. The cells were frozen in a thin layer on the sides of the flask by swirling the flask in a dry ice-acetone bath. The flask was placed on the freeze drying apparatus, and the cells were dried in a vacuum of 30-50 microns of mercury for approximately four hours or until all moisture was removed. The moisture which was removed from the cells during the freeze drying was condensed in a trap cooled by a dry ice-acetone mixture.

The dried cells were divided into two portions, and each portion was hydrated with ice cold water. A portion of the cells was ground with chilled alumina in a chilled mortar and pestle for five minutes. The ground cells were washed into a cold centrifuge tube, and the volume was made up to 15 ml. The second portion of cells was also suspended in 15 ml of water. The suspensions were centrifuged for 15 minutes at 6000 rpm, and the residues washed twice with 5 ml of water. Two ml of each of the supernatants were saved for protein analysis by the biuret test. The supernatants and residues from both of the samples were each transferred to separate flasks containing 10 ml of 0.282 mg/ml Na IAA solution, and the volumes were made up to 50 ml. The incubation was carried out for two hours at 25^{o}C on the rotary shaker. The samples containing the residues

were centrifuged and were washed twice with water. The supernatants and the other two samples were acidified with 5% HCl to a pH of 3. The solutions were saturated with NaCl and were extracted with ether and Na_2CO_3 solution as described in Enzyme Assay under Method 1. Extraction of the IAA and OIAA was necessary in this experiment since the protein released by the cells would interfere with the spectrophotometric analysis. The neutralized Na_2CO_3 extracts were made up to a volume of 100 ml and were then filtered through the Gelman multipore filter to remove any remaining turbidity. The samples were scanned on the Beckman DK-2A. For comparison purposes a sample of whole cells was also assayed. The data and results for this experiment are given in Table 15 as Run 1. A similar experiment was also conducted in which the cells were suspended in a 10^{-3} M solution of reduced glutathione instead of distilled water before freeze drying. The results of this experiment are shown in Table 15 A as Run 2.

Insonation of the Cells

In preparation for insonation, the cells were grown and induced with NaIAA in the normal manner, only in larger quantities (5 ml of inoculum per 100 ml of malt medium in a 500 ml flask). The cells were harvested by centrifugation and were washed twice with 10^{-2} M reduced glutathione in saline solution. The cells were suspended in 45 ml of the glutathione solution. Fifteen ml of the cell

TABLE 15
ENZYME ACTIVITY OF FREEZE-DRIED CELLS
FOR RUN 1

Sample	Absorbance at 280 mμ	Absorbance at 250 mμ	μg OIAA Formed per Hour	Protein Released $\frac{mg}{ml}$
Supernatant From Rehydrated Cells	0.750	0.300	44	0.5
Rehydrated Cells	0.759	0.318	58.5	-
Supernatant From Cell Debris	0.753	0.300	43	0.1
Cell Debris	0.708	0.274	25	-
Control Cells	0.463	0.474	396	-

TABLE 15 A

ENZYME ACTIVITY OF FREEZE-DRIED CELLS
FOR RUN 2

Sample	Absorbance at 280 mμ	Absorbance at 250 mμ	μg OIAA Formed per Hour	Protein Released $\frac{mg}{ml}$
Supernatant From Rehydrated Cells	0.718	0.256	- 2	-
Rehydrated Cells	0.713	0.243	-16	-
Supernatant From Cell Debris	0.749	0.306	46.5	-
Cell Debris	0.719	0.270	15	-
Control Cells	0.436	0.556	513	-

suspension were saved for use as a control. The remaining 30 ml of the cell suspension were insonated with the Bronson Sonifier for seven minutes at 6 amps. The cell suspension was cooled during insonation by placing the vessel containing the suspension in an ice-water bath. Following insonation the suspension was centrifuged for 1/2 hour at 15,000 xg, and the cell debris was washed with 10 ml of the glutathione solution. The supernatant and the cell debris were transferred to separate flasks containing 10 ml of 0.282 mg/ml NaIAA solution, and the volume was made up to 50 ml. These flasks and the flask containing the control cells plus NaIAA were incubated for two hours at 25°C on the rotary shaker. The control cells and the cell debris samples were centrifuged, and the residues washed with the glutathione solution. The supernatant from the cell debris and the sample containing the supernatant from the insonated cells were acidified, saturated with NaCl, and extracted with ether and then with the 2% Na_2CO_3 solution. The Na_2CO_3 extracts were neutralized and made up to 100 ml. These solutions and the supernatant from the control cells were filtered through the Gelman multipore filter and scanned on the Beckman DK-2A. The results of this experiment are given as Run 1 in Table 16.

The experiment reported as Run 2 in Table 16 was conducted as the experiment described above except the cells were suspended in a 10^{-2} M reduced glutathione in 0.9 M sucrose solution for insonation.

TABLE 16

ENZYME ACTIVITY OF INSONATED CELLS

	Sample	Absorbance at 280 mμ	Absorbance at 250 mμ	μg OIAA Formed per Hour	Protein Released $\frac{mg}{ml}$
Run 1	Supernatant From Insonated Cells	0.859	0.541	198	-
	Cell Debris	0.670	0.365	159.5	-
	Control Cells	0.320	1.15	1330 *	-
Run 2	Supernatant From Insonated Cells	0.839	0.405	133	0.69
	Cell Debris	-	-	-	-
	Control Cells	0.390	1.18	1335 *	-
Run 3	Supernatant From Insonated Cells	0.922	0.452	155	2.53
	Cell Debris	0.632	0.379	195	-
	Control Cells	0.650	0.729	635 *	-

* Not Corrected for Glutathione Absorption

In Run 3 the cells were washed with the glutathione in sucrose solution as well as being suspended in it during insonation. The cells were insonated for 10 minutes at 5 amp. The cell debris and the control cells were suspended in the glutathione-sucrose solution during the assay incubation.

IV. DISCUSSION

Preparation of the Sodium Salt of Indole Acetic Acid

Since indole acetic acid is only slightly soluble in water (it was difficult to dissolve 25 mg in 100 ml), there was reason to believe that this limited solubility might have hindered its effectiveness as an inducer. The more soluble sodium salt of IAA was, therefore, prepared and was found to be a much more effective inducer than IAA as the data in Table 1 shows.

Enzyme Assay

Three methods of harvesting the cells in preparation for the enzyme assay were tried. One method involved collecting the cells by filtration through a Büchner funnel. In a second method the cells were removed from suspension by filtration through a Gelman 2 Micron multipore filter. These methods proved to be very slow and unsatisfactory. The third method tried was centrifugation of the cells. This method was a much quicker and a more satisfactory way of harvesting the cells. Centrifugation was used for collecting the cells in most of the experiments.

In the first experiments a substrate level of 25 mg of IAA was used during the assay incubation. In order to read the absorbance of the IAA on the spectrophotometer the assay sample had to be diluted 1 to 1000. This large dilution made it impossible to detect

the OIAA, which was present in much smaller amounts than the IAA. By reducing the substrate level to 2.5 mg of IAA or 2.82 mg of NaIAA and diluting the sample only 100 fold the presence of the OIAA was more evident.

Another reason for the difficulty in detecting OIAA in early experiments was that OIAA was lost when the sample was extracted with ether. It was later found that the OIAA recovery was greatly improved if the sample was saturated with NaCl before extraction. In most of the experiments, however, extraction of the samples was unnecessary and, therefore, very little loss of OIAA occurred.

In order to evaluate the precision of the enzyme assay the standard deviations were calculated for the absorbance values at 250 and 280 mμ of the standards used in twenty five experiments (Appendix 2, Table 2A). Twice the standard deviation (2σ) for the absorbance values of the IAA standard at 280 mμ was ± 0.014, and the 2σ deviation for the absorbance values at 250 mμ was ± 0.020. By substituting the 2σ standard deviations of the absorbances at 250 and 280 mμ into equation (2) (page 35) the 2σ deviation in the concentration of OIAA was calculated to be $\pm 0.064 \times 10^{-2} \frac{mg}{ml}$ or ± 64 μg in the total sample (Appendix 2).

Rate of Growth of H. Conicus and Formation of the Enzyme

The great variation of cell weights in the triplicate samples as

seen in Table 5 may be explained by the fact that some of the malt solids, which precipitated during autoclaving, were pipetted into some of the culture flasks. Great care was taken to transfer only the clear medium, but it was impossible to keep from stirring up the solids and to keep from pipetting some of the solids with the clear medium. The solid material possibly contained vitamins, co-factors, or protein which aid in the growth of the cells. It has been observed that when the malt solids are left in the medium they disappear after the culture has grown for approximately 48 hours. Apparently the cells use them in their growth. A possible means of avoiding this problem with the malt solids is to sterilize the medium by filtration through a Seitz filter instead of by autoclaving. The drawback to this method is that it is very slow and tedious.

Figure 6 shows that H. conicus grown in the presence of the inducer has what is considered a normal growth curve for fungi (Cochrane, 1958). The cells, after transfer into the medium containing the inducer, had a lag period of about 6 hours. From 12-36 hours the growth was rapid and apparently logarithmic. The logarithmic period was followed by the usual stationary period.

As can be seen from Tables 6 and 7, the total amount of OIAA produced (column 5) during the first 18 hours fell within the range of the 2σ deviation calculated (Appendix 2). The OIAA produced

during this time can, therefore, be considered insignificant.

The plot of the specific enzyme activity as a function of time as shown in Figure 7 was rather unusual when compared with that for bacteria studied. In most bacteria in which enzyme induction has been studied the maximum specific activity occurs during the lag period of growth (Stanier, Doudoroff, Adelberg, 1957). In H. conicus the maximum specific enzyme activity occurred approximately twelve hours after the end of the logarithmic period of growth.

Another unusual observation was that after 48 hours the total production of OIAA (Column 5, Table 6 and 7) actually decreased. The three following explanations could account for this observation: 1) the enzyme, IAA oxidase, was not stable and was destroyed after 48 hours, 2) the cells which were induced during the first 48 hours began to die, and the level of NaIAA after 48 hours was not great enough to cause induction of the enzyme in the new cells, and 3) OIAA induces an enzyme in H. conicus which destroys OIAA. The first two possibilities could account for part of the decrease in the OIAA produced. However, the induction of an enzyme which will destroy OIAA was indicated by the results shown in Table 6 and 7. The IAA in the 60 and 72 hour samples was still being converted, but the OIAA levels in these samples was lower than those in the 48 hour samples. The third possibility would account for these two

seemingly contradictory observations since the OIAA produced by the enzymatic conversion of IAA could induce an enzyme which would destroy the OIAA. A possible reason for the sudden appearance of the OIAA destroying enzyme is that the concentration of OIAA was not great enough to act as an effective inducer until after 48 hours, and perhaps the high concentration of NaIAA up to that time inhibited its formation.

OIAA Induced Enzyme

In order to investigate further the evidence that OIAA induces an enzyme which destroys OIAA, the experiment described in the Experimental Section (page 40) was conducted. The result for sample 6 (see Tables 9 and 10) supports the evidence that OIAA induces an enzyme which destroys OIAA. Sample 2 was a control sample, in which NaIAA was used as the inducer and the substrate. The result of sample 3 shows that after 48 hours of induction with IAA the cells destroy a small amount of the substrate, OIAA. This suggests that some of the OIAA destroying enzyme is present even after 48 hours of incubation of the cells with NaIAA. The result of sample 5 falls within the 2σ deviation of the assay method; therefore, it can be concluded that the cells which were grown in the presence of OIAA as an inducer do not attack IAA. In other words, the OIAA does not act as an inducer for IAA oxidase.

The results of the paper chromatograms developed with the Pauly and Ehrlich reagents suggest that the products of the enzymatic destruction of OIAA are neither indoles nor phenols. No further attempt was made to isolate or to identify the products. NaIAA which contains an isotopic labeled carbon atom in the indole nucleus could be used in an experiment to supply information concerning the fate of the OIAA and the products of its destruction.

Effect of pH on Enzyme Activity

The results of the experiment using various buffers (Table 12) indicate that the enzyme activity is greatest between pH 3.0 and pH 5.0.

The results obtained using the citrate buffer are questionable since the citrate may have been metabolized by the cells, even though no noticeable change in the pH of the buffer was observed after the assay incubation. A change in the citrate concentration in the sample would cause error in the spectrophotometric readings since the sample is compared to a reference citrate solution.

Another problem incurred with this experiment was finding a buffer which did not interfere with the spectrophotometric analysis. As mentioned before the phthalate buffer absorbed strongly in U.V. It has an absorptivity of 8 $\frac{ml \cdot cm}{mg}$ at 278 mμ (Swann, Adams, and Esposito, 1955). The sodium citrate buffer also exhibited some end

absorption starting at 250 mμ (Buck, Singhadeja, Rogus, 1954), but the absorption was not great enough to interfere with the analysis.

In order to obtain accurate results on the effect of pH on the activity of IAA oxidase the cell-free enzyme should be used.

A possible explanation for the great difference in the amount of OIAA produced per hour in comparable samples of Run 1 and 2 is that the H. conicus cultures used inthe experiments were obtained from two different slants. It should be mentioned here that some of the cultures of H. conicus underwent a morphological change. Although the mycelia from all submerged cultures appeared to be identical under the microscope, their appearances were different when grown on solid medium. The different forms still had the ability to produce IAA oxidase but apparently in varying degrees.

Preparation of Cell-Free Enzyme

Spectrophotometric Method for Protein Determination

The basis for the spectrophotometric determination of protein is that the protein released from the cell absorbs strongly at 280 mμ. The nucleic acids released also absorb in this region, but they absorb more strongly at 260 mμ. Warburg and Christian (Seaman, 1963) calculated the effect of the interference of the nucleic acids in the determination of protein by measuring the absorbance at 260 and 280 mμ and then applying a correction factor based on the ratio of

absorbances at the two wavelengths. The relationship between the ratio of absorbances at the two wavelengths and the Warburg correction factor is shown in Figure 9 and Table 14.

If the nucleic acid content is above 15%, as it sometimes is in crude microbial extracts, the calculations made by the Warburg and Christian method give an excessively low value for the protein. The Layne equation (Seaman, 1963) is useful in cases where the nucleic acid exceeds 15%. The equation is:

$$\frac{\text{mg}}{\text{ml}} \text{ protein} = (1.55)(\text{Absorbance}_{280}) - 0.76(\text{Absorbance}_{280})$$

The Warburg and Christian equation was used in all the calculations of the protein released by insonation of the H. conicus cells.

Freeze Drying of the Cells

The results of Run 1 in Table 15 connote that the enzyme activity is lost during freeze drying of the cells. It was thought that this loss of activity was the result of oxidation of an active site on the enzyme surface by air. Reduced glutathione was, therefore, added before freeze drying in order to protect the enzyme from oxidation, but it did not prevent the destruction of enzyme activity as the results of Run 2 show. It was later discovered that Lion and Bergmann (1961) had found that reduced glutathione increased the adverse effect of air on freeze-dried cells of Escherichia coli. This loss of enzyme activity

may possibly be avoided by addition of some of the protective agents which Lion and Bergmann (1961) found effective in their system.

Insonation of the Cells

The results in Table 16 indicate that there was some enzyme activity in cell-free extracts from the insonated cells. The activity seen in the cell debris was probably caused by the presence of a few unruptured cells.

The exceptionally high enzyme activity of the control cells was caused by the U.V. absorption of the glutathione. At the time of the experiments which were run it was not known that glutathione has an absorptivity of 2500 $\frac{l \cdot cm}{mole}$ (8.14 $\frac{ml \cdot cm}{mg}$) at 260 mμ (Konigsburg and Craig, 1958). The cell-free extract (supernatant) and cell debris samples, however, should not be affected by the presence of glutathione since they were extracted with ether and then with Na_2CO_3 solution before spectrophotometric analysis. Glutathione is insoluble in ether and, therefore, should have remained in the aqueous phase.

V. CONCLUSIONS

The sodium salt of IAA was prepared and found to be a more effective inducer of IAA oxidase in H. conicus than IAA.

A spectrophotometric analysis was developed for determining the concentrations of OIAA and IAA in mixtures of the two substances. Simultaneous analysis for these two substances was possible since OIAA and IAA absorb at two different wavelengths and because the mixtures conformed to Beer's Law.

The growth of H. conicus was found to be similar to that of other fungi, but the production of the enzyme was unusual as compared to induced enzyme synthesis in bacteria.

Oxindole acetic acid was also found to induce an enzyme in H. conicus which destroyed OIAA. The products of the destruction of the OIAA were not identified.

The enzyme activity was found to be greatest between pH 3.0 and pH 5.0.

All attempts to prepare cell-free enzyme by freeze drying and by insonation of the mycelia resulted in loss of enzyme activity.

VI RECOMMENDATIONS

The following is a list of suggestions for the further study of the indole acetic acid oxidase of H. conicus.

1) Determine the temperature effect on enzyme activity.

2) Investigate the various compounds suggested by Lion and Bergmann (1961) as protective agents for the enzyme during the preparation of cell-free extracts of the enzyme.

3) Investigate the effect of Mn^{++}, phenols and H_2O_2 on the enzyme activity.

4) Determine the effect of various levels of the inducer, NaIAA, on the enzyme activity.

5) Determine H. conicus growth and the rate of enzyme production using Seitz filtered malt medium.

6) Develop an artificial medium of known composition for growth of H. conicus during induction of the enzyme.

7) Use IAA labeled with C^{14} in the indole nucleus and attempt to identify the product formed by the OIAA induced enzyme.

8) Determine with the aid of O^{18} the source of the oxygen in the oxindole acetic acid.

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IX VITA

Barbara Ruth Patterson, nee Lay, was born on March 10, 1939 in Pittsburgh, Pennsylvania. She received her primary education in Dallas, Texas, St. Louis, Missouri, and Rolla, Missouri. Her secondary education was obtained in Rolla, Missouri. She received her Bachelor of Science degree in Chemistry from Missouri School of Mines and Metallurgy, Rolla, Missouri, in January, 1961.

She was employed by the University of Michigan, Ann Arbor, Michigan as a laboratory technician in the Department of Biological Chemistry from February, 1961 until September, 1961. She was also employed by Louisiana State University, Baton Rouge, Louisiana from July, 1962 to August, 1963 as a research associate in the Animal Science Department.

She entered graduate school at Missouri School of Mines and Metallurgy in September, 1963. She served as a research assistant for the 1963-64 school year, the summer session of 1964 and the fall semester of 1964-65.

APPENDIX 1

Since the solutions of IAA and OIAA conform to Beer's law, the following equations should hold for mixtures of IAA and OIAA (Lingane and Collat, 1950):

$$A_{280} = {}^{280}a_{IAA}\ C_{IAA} + {}^{280}a_{OIAA}\ C_{OIAA} \quad (1)$$

$$A_{250} = {}^{250}a_{IAA}\ C_{IAA} + {}^{250}a_{OIAA}\ C_{OIAA} \quad (2)$$

where A is the absorbance at the designated wavelength

a is the absorptivity in $\frac{ml \cdot cm}{mg}$

C is the concentration in $\frac{mg}{ml}$

Solving both of the above equations for C_{IAA} gives

$$C_{IAA} = \frac{A_{280} - {}^{280}a_{OIAA}\ C_{OIAA}}{{}^{280}a_{IAA}} \quad (3)$$

$$C_{IAA} = \frac{A_{250} - {}^{250}a_{OIAA}\ C_{OIAA}}{{}^{280}a_{IAA}} \quad (4)$$

Substituting equation (3) into equation (4) and solving for C_{OIAA} gives:

$$C_{OIAA} = \frac{{}^{250}a_{IAA}\ A_{280} - {}^{280}a_{IAA}\ A_{250}}{{}^{250}a_{IAA}\ {}^{280}a_{OIAA} - {}^{280}a_{IAA}\ {}^{250}a_{OIAA}} \quad (5)$$

Solving equations (1) and (2) for C_{OIAA} gives:

$$C_{OIAA} = \frac{A_{280} - {}^{280}a_{IAA}\, C_{IAA}}{{}^{280}a_{OIAA}} \qquad (6)$$

$$C_{OIAA} = \frac{A_{250} - {}^{250}a_{IAA}\, C_{IAA}}{{}^{250}a_{OIAA}} \qquad (7)$$

Substituting equation (6) into (7) and solving for C_{IAA} gives:

$$C_{IAA} = \frac{{}^{250}a_{OIAA}\, A_{280} - {}^{280}a_{OIAA}\, A_{250}}{{}^{250}a_{OIAA}\, {}^{280}a_{IAA} - {}^{280}a_{OIAA}\, {}^{250}a_{IAA}} \qquad (8)$$

The absorptivity (a) may be calculated from the relationship:

$A = (a)(C)(1)$

where A = absorbance

C = concentration

1 = length of the light path which is equal to 1 cm

Table 1A shows the absorptivities for OIAA and IAA calculated from several experiments.

Substituting the numerical values for the absorptivities into equation 5 and 8, the equations become:

$$C_{IAA}\left(\frac{mg}{ml}\right) = \frac{42.1\, A_{280} - 8.46\, A_{250}}{(42.1)(31.4) - 8.46\,(11.31)}$$

$$= 3.43 \times 10^{-2}\, A_{280} - 6.91 \times 10^{-3}\, A_{250} \qquad (9)$$

$$C_{OIAA}\left(\frac{mg}{ml}\right) = \frac{11.31A_{280} - 31.4\,A_{250}}{(11.31)(8.36) - (31.4)(42.1)}$$

$$= 2.57 \times 10^{-2}\,A_{250} - 9.24 \times 10^{-3}\,A_{280} \quad (10)$$

TABLE 1-A

ABSORPTIVITIES OF IAA AND OIAA

at 250 and 280 mμ

Experiment	a_{280}IAA $\frac{ml}{mg \cdot cm}$	a_{250}IAA $\frac{ml}{mg \cdot cm}$	a_{280}OIAA $\frac{ml}{mg \cdot cm}$	a_{250}OIAA $\frac{ml}{mg \cdot cm}$
1	31.7	11.75	8.23	42.3
2	31.5	11.17	8.55	41.9
3	31.6	11.12	8.62	42.2
4	31.2	11.20		
5	31.3			
Avg.	31.4	11.31	8.46	42.1

APPENDIX 2

The standard deviation of the absorbances at 250 and 280 mμ of the IAA were calculated by the equation:

$$\sigma = \sqrt{\frac{\Sigma D^2}{n - 1}}$$

where σ is the standard deviation

D = deviation from the mean

n = number of data

From the calculations tabulated in Table 2A

$$\sigma_{280} = \pm \sqrt{\frac{13.29 \times 10^{-4}}{24}} = \pm 0.007$$

$$2\sigma_{280} = \pm 0.014$$

$$\sigma_{250} = \pm \sqrt{\frac{21.80 \times 10^{-4}}{24}} = \pm 0.010$$

$$2\sigma_{250} = \pm 0.020$$

The 2σ deviation in the concentration of OIAA is calculated as follows using equation (10) from page 83:

$$C_{OIAA}\left(\frac{mg}{ml}\right) = 2.57 \times 10^{-2} (A_{250}) - 9.24 \times 10^{-3} (A_{280})$$

$$= 2.57 \times 10^{-2} (\pm 0.020) - 9.24 \times 10^{-3} (\pm 0.014)$$

$$C_{OIAA} = \pm 0.064 \times 10^{-2} \frac{mg}{ml} = \pm 64 \ \mu g/sample$$

TABLE 2-A

THE ABSORBANCE VALUES AT 250
AND 280 mμ FOR IAA STANDARDS

Date Standard Run	A_{280}	Deviation of A_{280} From Mean	(Deviation)^2 x 10^{-4}	A_{250}	Deviation of A_{250} From Mean	(Deviation)^2 x 10^{-4}
2/10/64	0.738	-0.009	0.81	0.272	-0.004	0.16
2/13	0.755	+0.008	0.64	0.291	+0.015	2.25
3/5	0.745	-0.002	0.04	0.274	-0.002	0.04
3/5	0.741	-0.006	0.36	0.273	-0.003	0.09
6/5	0.752	+0.005	0.25	0.282	+0.006	0.36
6/11	0.758	+0.011	1.21	0.288	+0.012	1.44
6/12	0.749	-0.002	0.04	0.279	+0.003	0.09
6/17	0.738	-0.009	0.81	0.281	+0.005	0.25
6/19	0.745	+0.002	0.04	0.296	+0.020	4.00
6/23	0.759	+0.012	1.44	0.279	+0.003	0.09
6/23	0.760	-0.013	1.69	0.282	+0.006	0.36
6/24	0.742	-0.005	0.25	0.278	+0.002	0.04
6/25	0.740	+0.007	0.49	0.272	-0.004	0.16
6/26	0.750	+0.003	0.09	0.281	+0.005	0.25
7/16	0.758	+0.011	1.21	0.288	+0.012	1.44
7/22	0.748	-0.001	0.01	0.269	-0.007	0.49
7/28	0.742	-0.005	0.25	0.272	-0.004	0.16
7/29	0.745	-0.002	0.04	0.271	-0.005	0.25
7/31	0.738	+0.009	0.81	0.268	-0.008	0.64
8/3	0.749	-0.002	0.04	0.269	-0.007	0.49
8/6	0.740	-0.007	0.49	0.250	-0.026	6.76
8/12	0.739	-0.008	0.64	0.269	-0.007	0.49
9/25	0.739	+0.008	0.64	0.265	-0.011	1.21
10/2	0.755	+0.008	0.64	0.271	-0.005	0.25
11/20	0.753	+0.006	0.36	0.278	+0.002	0.04
	18.678		13.29	6.898		21.80

$$\text{Mean} = \frac{18.678}{25} = 0.747 \qquad \text{Mean} = \frac{6.898}{25} = 0.276$$