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Oxidation of Aromatic Compounds by a Strain of Alcaligenes Fecalis

Jerry J. Tulis
Loyola University Chicago

OXIDATION OF AROMATIC COMPOUNDS BY A STRAIN OF *ALCALIGENES FECALIS*

by

Jerry J. Tulis

Microbiology Department

Stritch School of Medicine

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LIFE

Jerry J. Tulis was born in Cicero, Illinois, April 13, 1930.

He was graduated from J. S. Morton High School, Cicero, Illinois, in January, 1948, and from University of Illinois in February, 1953.

The author began study in the Department of Microbiology of the Stritch School of Medicine of Loyola University in September, 1953.

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CHAPTER I

INTRODUCTION

The literature describing bacterial oxidases capable of catalyzing the oxidation of aromatic amino acids to a dark brown melanin-like pigment is very sparse. Several reports have been presented on the addition of tyrosine or phenylalanine to rather complex media and the consequent elaboration of a pigment. There seem to be no reports showing the oxidation of all three aromatic amino acids by a single bacterial strain with the production of a melanin-like pigment. This report presents such evidence. Also, the aromatic amino acids are incorporated into a very simple synthetic medium and act as the sole sources of carbon, energy, and organic nitrogen. Preliminary evidence indicates that the oxidation of tyrosine and phenylalanine may follow a similar oxidative pathway but that tryptophane follows an entirely different pathway.

CHAPTER II

HISTORY AND REVIEW OF LITERATURE

The bacterial oxidation of aromatic amino acids divides itself historically into three separate fields of study. The first part deals with the oxidation of tyrosine and phenylalanine by the oxidative enzyme, tyrosinase, and other oxidative enzymes; the second part deals with the utilization of aromatic compounds as sole sources of carbon and energy for bacterial species; and the third part deals with the bacterial oxidation of tryptophane and the pathways therein. In this report the three phases of study become closely related since the end-product of the bacterial oxidation of tyrosine, phenylalanine, and tryptophane is a dark brown melanin-like pigment. Because the oxidation of these aromatic amino acids is accomplished through the medium of a single bacterial strain, Alcaligenes fecalis, an integrating relationship definitely exists.

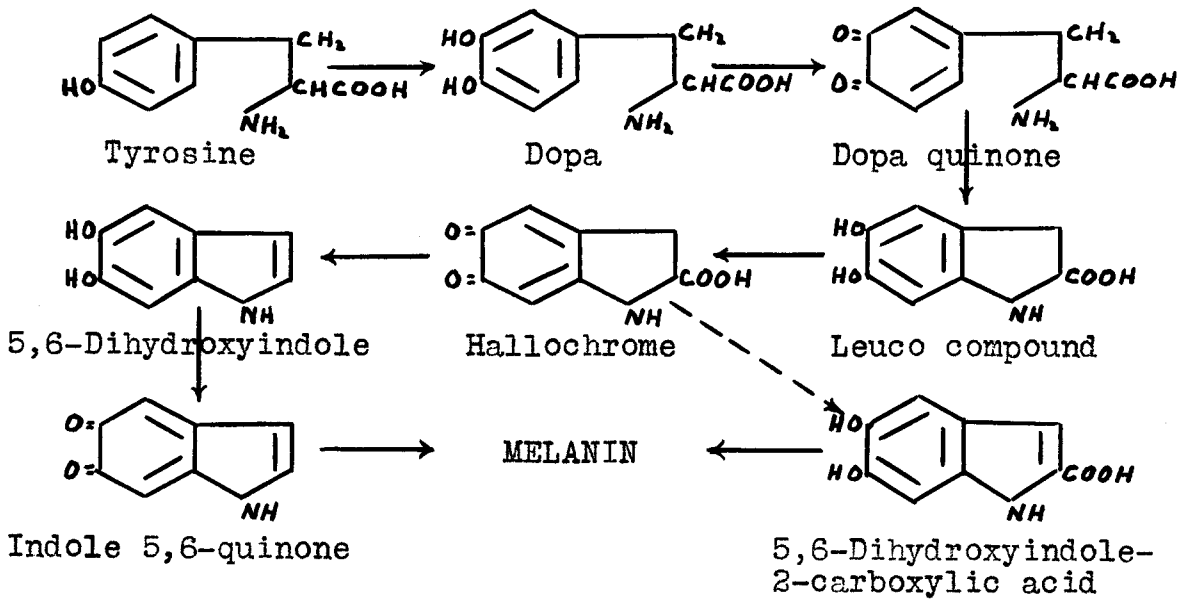
The literature that has accumulated through the years concerning the enzyme tyrosinase is vast and only a short review of the material can be presented.

In 1856 Schoenbein demonstrated the presence of an agent in the mushroom, Boletus luridus, which, under aerobic conditions, brought about the oxidation of certain material in the fungus giving rise to a blue pigment. Yoshida (1883) found the presence of an oxidative enzyme, laccase, in the latex of the lac tree. Following the study of Yoshida, Bertrand (1895) and Bourquelot and Bertrand (1896) reported a substance in the mushroom Russula nigricans which turned black as oxidation progressed. Later, Bertrand (1896) showed that the substrate being acted upon was the amino acid tyrosine and named the enzyme tyrosinase. Further experimentation by Bertrand (1907) upon the specificity of tyrosinase showed that the enzyme could catalyze the aerobic oxidation of other aromatic monohydric phenols such as p-hydroxyphenyl ethyl amine, p-hydroxyphenyl methyl amine, p-hydroxyphenyl acetic acid, p-cresol, and phenol. However, the oxidase was unable to bring about the oxidation of these compounds when the hydroxyl group was absent.

Since the work of these early investigators, many reports (Kastle, 1910; Gortner, 1910-1911) of the isolation of tyrosinase have come forth. The enzyme occurs widely in nature, in plants and in invertebrates. The presence of the oxidase has been demonstrated in mammals although difficulties have been encountered in the extraction of the active material. Gessard (1902) showed the presence of the enzyme in the ink sac of the cuttle-

fish. Onslow (1917) extracted the oxidase from the skin of rabbits, and Pinhey (1930) reported the presence of the enzyme in the blood of certain crustaceans. A good review of the isolation of tyrosinase from various sources was presented by Kastle (1910).

Raper (1928) reported the results of his experimentation upon the reaction mechanism in the conversion of tyrosine to melanin. Working with tyrosinase from plants and mealworms, he showed that in the presence of oxygen, tyrosine is converted to dihydroxyphenylalanine (dopa), and that dopa is then oxidized to dopa-quinone. Dopa-quinone then becomes converted to an indole derivative which polymerizes to form the pigment, melanin, after several complex intermediary reactions. The following diagram shows the pathway of oxidation of tyrosine, as described by Raper:



Recently, Lerner, Fitzpatrick, Calkins, and Summerson (1949) have compared the action of tyrosinase of mammaliam origin to tyrosinase obtained from plants, insects, and marine animals. Their experiments indicate that mammalian tyrosinase is much more specific in its action upon tyrosine and related substrates.

Tyrosinase for experimental purposes is usually obtained from the potato; the mushroom, Psalliota compestris; and the mealworm. Wheat bran, the Indian bean, and octopus blood have also been used. The enzyme, isolated in highly active and highly purified form, demonstrates the following activity: it can catalyze the oxidation of many o-dihydric phenols as well as the oxidation of many monohydric phenols. It, therefore, brings about two essentially different oxidations: (1) the insertion of an hydroxyl group into monohydric phenols ortho to the one already present and (2) the oxidation of o-dihydric phenols to their corresponding o-quinones.

The literature on bacterial tyrosinase is less voluminous. Stapp, in 1923, reported the presence of tyrosinase in fifteen of the seventy-six bacterial species that he studied. Ungerer (1934) demonstrated a melanin-like pigment production by Azotobacter chroococcum. In 1939 Clark and Smith showed that Bacillus niger produced a black pigment upon the addition of tyrosine to their protein medium. Griffin, Snieszko, and Friddle (1953) reported increased pigment production with the addition of

tyrosine or phenylalanine to a medium supporting the growth of Bacterium salmonicida.

Reports in the literature concerning the utilization of aromatic compounds as energy sources for bacteria are more prevalent. Stormer (1908) isolated from soil organisms capable of destroying toluene, xylene, phenol and p-cresol. Fowler, Ardern, and Lockett (1911) isolated from sewage effluent, an organism that would grow in a salt medium to which phenol (0.01 gram/100 ml. of medium) was added, and oxidize the phenol. Wagner (1914) found that substances such as phenol, cresol, phloroglucinol and toluene could serve as sources of energy for soil bacteria. Sen Gupta (1921) demonstrated the disappearance of phenols and cresols from various soils. Tausson (1928, 1929) isolated bacterial species capable of destroying naphtalene, phenanthrene, and toluene. At about the same time Gray and Thornton (1928) and Tattersfield (1928) independently isolated many bacteria capable of attacking aromatic compounds. Happold (1930) studied the oxidation of cathechol, guaicol, orcinol, phenol, p-cresol, and tyrosine by bacterial suspensions. Happold and Key (1932) isolated from sewage, a Vibrio which oxidized monohydroxyphenols. In 1942 Berheim investigated the decomposition of benzoic acid and related substances by a species of Mycobacterium. Evans (1947) isolated phenol utilizing organisms from the feces of a large variety of animals, including man. Stanier (1948) demonstrated

the utilization of aromatic compounds by twenty-two strains of pseudomonads.

Although many of the aromatic compound-utilising organisms isolated by Gray and Thornton (1928), Happold and Key (1932) and Evans (1947) could utilize tyrosine, the generally accepted tyrosine-tyrosinase color sequence leading to the formation of melanin was absent. The products of the oxidation were colorless and eventually led to the cleavage of the benzene ring.

The literature on the oxidation of tryptophane by bacterial species is so vast that only the highlights will be presented in this review. The majority of the reports are concerned with the oxidative pathways and the production of indole. In 1903 Hopkins and Cole demonstrated that tryptophane is the precursor of the indole formed by bacteria. Woods (1935, 1) using washed suspensions of Escherichia coli showed that L(-) tryptophane is quantitatively converted to indole. The unnatural isomer, D(+) tryptophane, is attacked very slowly, or not at all. Happold and Hoyle (1935) succeeded in the conversion of tryptophane to indole by the use of a non-viable alcohol precipitated culture of E. coli. Also at about the same time, Woods (1935, 2) attempted to produce indole from B-indolepropionic acid, B-indolecarboxylic acid, B-indoleacetic acid, B-indolealdehyde, and B-indoleacrylic acid. Under conditions where 100 per cent indole was formed from tryptophane none was formed from the other compounds. These

results were corroborated by Happold and Hoyle (1935). Tatum and Bonner, in 1944, suggested that the breakdown of tryptophane be the reverse process of the synthesis, namely, tryptophane splitting to give indole plus serine. In 1933 Kotake reported an oxidation of tryptophane by Bacillus subtilis resulting in the formation of kynurenic acid and anthranilic acid. Kynurenine could be substituted for tryptophane yielding the same results.

Recently, by the study of adaptive patterns, two different pathways have been proposed for the oxidative breakdown of tryptophane. Suda, Hayaishi, and Oda (1949, 1950) demonstrated the following metabolic pathway with the use of an unidentified specie of Pseudomonas as the agent promoting oxidation: L-TRYPTOPHANE⟶L-KYNURENINE⟶ANTHRANILIC ACID⟶CATECHOL. Stanier and Tsuchida (1949) also using an unidentified pseudomonad, reported the following metabolic pathway: TRYPTOPHANE⟶KYNURENINE⟶KYNURENIC ACID. The two pathways diverge at the stage of kynurenine. In 1951 Stanier, Hayaishi, and Tsuchida designated the pathway discovered by Suda, Hayaishi, and Oda as the "aromatic pathway", and the pathway of Stanier and Tsuchida as the "quinoline pathway." They further showed that the aromatic pathway continues in the following manner: CATECHOL⟶CIS-CIS MUCONIC ACID⟶B-KETO ADIPIC ACID⟶products not yet identified. The quinoline pathway was shown to continue from kynurenic acid to benzene and pyridine derivatives which also have not been identified as yet.

CHAPTER III

MATERIALS AND METHODS

I. Materials

A. Organisms

The culture used throughout the study was a strain of *Alcaligenes fecalis* (ATCC 8750). In the stock culture collection of the department of Microbiology of the Stritch School of Medicine of Loyola University, the organism is designated as No. RH-135. In this report the organism shall be referred to as No. 135. Two other strains of *Alcaligenes* were used in the preliminary studies, but since they did not produce the desired pigment their use was discontinued.

Other organisms used in the comparative studies were also obtained from the stock culture collection of the Stritch School of Medicine of Loyola University.

Aerobacter aerogenes No. 7.2

Escherichia coli No. 4.1

Proteus vulgaris No. 3500

Paracolobactrum sp. No. 3131

Pseudomonas sp. No. E-13

Alcaligenes fecalis No. 135 is a gram negative rod motile by means of peritrichous flagella. Flagella were stained by the method of Leifson (1951). Utilization of carbohydrates commonly used for the identification of enteric organisms is entirely absent. Other nutritional studies conducted indicate that this organism is capable of utilizing single amino acids and other related compounds, incorporated into a basal salt medium, as sole sources of carbon and organic nitrogen. The tables on pages 42, 43, 44, and 45 give the results of the nutritional studies. Other physiological reactions are presented in the table on page 46.

B. Media

A basal salt medium was used throughout the entire study. This medium is a modification of that used by Useavage (1953).

$MgSO_4$	0.2 grams
$NH_4H_2PO_4$	1.0 grams
KH_2PO_4	1.0 grams
NaCl	5.0 grams
H_2O	1 liter

Originally, agar (1.5 %) was incorporated into the medium and plates were used to detect pigmentation. This procedure was later abandoned and liquid medium was used throughout the remainder of the study.

The basal salt medium was made in five liter amounts, adjusted to pH 7.2, with 1 N NaOH, and stored for use in the refrigerator.

The testing media were made by adding analytically weighed amounts of the desired chemical to the basal salt medium and adjusting the pH to 7.2. Unless otherwise specified, 100 mg. of the various chemicals were added to 100 ml. of the basal salt medium to give 100 mg. % solutions. Sterilization was accomplished by autoclaving the heat stable solutions (fifteen pounds for fifteen minutes), and Seitz filter sterilization of the heat labile solutions. The pH of the sterile solutions was checked and adjusted if altered by the sterilization.

Nutrient broth was used to grow the organism for some of the determinations. The basal salt medium with 0.2 % sodium citrate was used in the majority of the cases to grow large populations. Nutrient agar slants and nutrient broth were used to maintain the organism in stock.

II. Methods

A. Preparation of Inocula

The organism was grown in nutrient broth or citrate basal salt medium for twenty-four to thirty-six hours. The cultures were then dispensed into conical tubes and sedimented in the centrifuge at 3000 RPM for fifteen minutes. The supernatant was discarded and the organisms resuspended in sterile saline.

This process was repeated twice. The desired turbidity was obtained by dilution to specific turbidimetric readings on the Klett-Summerson photoelectric colorimeter, using the blue filter.

B. Shallow Layer Method

The solutions being tested were placed in shallow layers in large Erlenmeyer flasks. By allowing maximum surface area the optimum in oxidation was realized. In earlier work, the shaking machine was used, and, as the table on page 47 demonstrates proved more efficient in promoting growth and pigment formation, but this method was dropped in favor of the shallow layer method, which proved more satisfactory for the large scale experiments.

C. Colorimetric and Turbidimetric Determinations

Pigment production and turbidity were determined by placing the properly prepared test solution into previously calibrated tubes and placing these in the photoelectric colorimeter. The blue filter was used in all cases.

1. Preparation of sample for colorimetric determination

The test samples were prepared for colorimetric determinations by removing 5 ml. aliquots from the reaction flasks and placing these in the centrifuge tubes. The tubes were placed in the centrifuge and the cells sedimented at 3000 RPM for fifteen minutes. Then the tubes were removed, the supernatant

decanted into a second set of centrifuge tubes and residue discarded. This second set of tubes was again centrifuged at 3000 RPM for fifteen minutes. This time the supernatant was decanted into calibrated colorimeter tubes and readings made.

2. Preparation of sample for turbidimetric determination

The test samples for turbidimetric determinations were prepared by removing 5 ml. aliquots from the reaction flasks and placing these in the centrifuge tubes. The tubes were placed in the centrifuge and the cells sedimented at 3000 RPM for twenty-five minutes. Then the tubes were removed, the supernatant discarded and the residual organism resuspended in physiological saline and placed into calibrated colorimeter tubes for determinations of turbidity.

3. Dilution of pigment

Highly pigmented samples were serially diluted to determine what effect dilution would have on the pigment and whether or not a straight line relationship could be established on graph paper. The diluent that was used was the stock salt solution and the dilution factor was two-fold.

D. pH Studies

Using the shallow layer method 100 ml. of 100 mg. % amino acid medium was placed in 1000 ml. Erlenmeyer flasks. This amount of medium proved sufficient to allow the removal of 5 ml.

aliquots for colorimetric determinations. For each comparative study, flasks having pH levels 5.0, 6.0, 7.0, 8.0, and 9.0 were prepared by the addition of either 1 N NaOH or 1 N HCl. Accurate pH readings were made by the use of the Beckman pH-meter. The flasks were sterilized by placing them in the autoclave at fifteen pounds for fifteen minutes. The pH was again checked and adjusted whenever necessary by the addition of sterile acid or base. After proper cooling, the inoculum (2 ml. turbid suspension washed cells; 900 on Klett) was added and the flasks were placed in the 30°C incubator. Periodic readings (twelve-hour intervals) were made on the colorimeter by removing 5 ml. aliquots.

E. Temperature Studies

Again using the shallow layer method 100 ml. of 100 mg. % amino acid medium was placed in 1000 ml. Erlenmeyer flasks. Preliminary studies, presented in the table on page 48, indicated that growth and pigment formation does not take place at 10°C and 45°C. Due to these observations the more extensive experiments determining quantitative pigment formation were conducted at 20°C, 30°C, and 37°C. After proper sterilization the flasks were inoculated (2 ml. turbid suspension washed cells; 900 on Klett) and placed in the various temperature incubators. Aliquots (5 ml.) were removed at twelve-hour intervals and, after proper preparation of the sample, colorimetric determinations were made.

F. Inoculum Studies

Using 100 ml. of 100 mg. % tryptophane medium in shallow layers the effect of size of inoculum was determined. Duplicate sets of flasks were inoculated with 0.1 ml., 0.5 ml., and 1.0 ml. of a turbid suspension of washed cells (900 on Klett) respectively. Aliquots (5 ml.) were removed at four-hour intervals and colorimetric determinations made. Since the effect of size of inoculum did not appear very significant in these studies the other two aromatic amino acids were not tested in relation to inoculum size.

G. Inhibitor Studies

Various chemicals were added to 100 ml. medium containing 100 mg. % of the respective aromatic amino acids. These chemicals were added in 10 mg. % and 50 mg. % amounts. The controls contained only 100 mg. % amino acid. The following chemicals were used: thiouracil, thiourea, glutathione, cysteine, and potassium cyanide. After proper pH adjustments were made the solutions were sterilized and inoculated with 1 ml. of a slightly turbid suspension of washed cells (200 on Klett). The flasks were placed in the 37°C incubator and checked at twenty-four intervals for possible inhibition of growth and pigment formation.

H. Serial Transfers

In order to determine the possibility of a carry-over of nutrient from the nutrient broth or citrate media serial

transfers were performed using 35 ml. of basal salt medium plus 100 mg. % of the respective aromatic amino acids as the serial transfer medium. The transfers were carried out over a ten-day period by transferring 1 ml. each day to the next flask. Also each day 1 ml. was transferred into sterile nutrient broth to insure that the cells were viable.

I. Growth Curve Studies

Comparative studies were made to determine whether the organisms grow as well in the various aromatic amino acids as they do in the sodium citrate medium. Flasks containing 100 ml. basal salt medium plus either 100 mg. % of one of the aromatic amino acids or 100 mg. % sodium citrate were inoculated with 2 ml. of a turbid suspension (900 on Klett) and placed in the 30°C incubator. Aliquots (5 ml.) were removed at twelve-hour intervals and, after proper preparation of the sample, turbidimetric determinations made. In the case of tryptophane, difficulties were encountered in making correct determinations after about seventy-two hours since it become impossible to entirely separate the pigment from the bacterial cells.

J. Nutritional Studies

Determinations were made to establish the general nutritional requirements of the organisms. Carbohydrates, alcohols, and amino acids and compounds related to amino acids were incorporated into the basal salt medium as sole sources of carbon and

organic nitrogen. This assay was performed by using 25 ml. of the test medium per flask and inoculations of 0.1 ml. washed cells (350 on Klett) per flask. Sterilization was generally performed in the autoclave except in the case of heat labile compounds, which were sterilized by the use of the Seitz filter. In all cases 100 mg. % of the compounds were used except in the cases of the following compounds, which were also used at the 50 mg. % level: phenol, p-cresol, indole, anthranilic acid, catechol, kynurenic acid, glutathione, thiourea, thiouracil, cysteine, and indoleacetic acid. Utilization of the compounds was determined by the appearance of turbidity in the inoculated flasks as compared to the uninoculated controls.

K. Adaptation Studies

Preliminary studies were conducted to determine the possibility of the existence of adaptive enzymes responsible for the oxidation of these aromatic amino acids. Since the formation of pigment through the oxidation of tryptophane proceeds at a much faster rate than phenylalanine or tyrosine, it was chosen as the substrate for these experiments. Washed organisms were inoculated in equal amounts into 100 ml. of 100 mg. % sodium citrate-basal salt medium and into 100 mg. % tryptophane-basal salt medium. After twenty-four hours incubation in the 30°C incubator the organisms were treated as described under preparation of inoculum. The actual experiment was set up in duplicate using 100

mg. % tryptophane in all cases as the substrate. Fifteen 250 ml. flasks containing 10 ml. each of the medium were inoculated with 0.5 ml. of a slightly turbid suspension (250 on Klett) of the tryptophane-grown and sodium citrate-grown organisms respectively. Also, 1000 ml. flasks containing 160 ml. of the medium were inoculated with 8 ml. of the same suspensions of tryptophane-grown and sodium citrate-grown organisms. All flasks were placed in the 30°C incubator. Colorimetric readings were made every two hours over a twenty-four hour period. At each reading 10 ml. aliquots were removed from the 1000 ml. flasks for determinations while the entire contents of the 250 ml. flasks were used. Before each determination the sample was treated as described under preparation of sample for colorimetric determination.

L. Aromatic Amino Acid Utilization by Related Organisms

Experiments were conducted to determine whether related gram negative organisms would be capable of utilizing these amino acids as sole sources of carbon and organic nitrogen and whether a pigment was formed. Shallow layer flasks containing 100 ml. each of 100 mg. % and 200 mg. % of the respective amino acids were prepared. The organisms to be tested were grown for a twenty-four hour period in nutrient broth at 37°C. Inocula were prepared as recorded under preparation of inoculum. Alcaligenes fecalis No. 135 was used as the control of growth and pigment

production. Inoculations were as follows: 1 ml. of a slightly turbid suspension (250 on Klett) of the respective organisms was inoculated into 100 mg. % and 300 mg. % solutions of the three aromatic amino acids and incubated at 37°C. Readings were made at twenty-four, forth-eight, seventy-two, and ninety-six hours for the appearance of growth and/or pigment production.

M. Comparative Utilization of D and L-Phenylalanine

Since all the data had been compiled using the natural isomers of the amino acids, it was decided that a comparative study of the unnatural isomer should be made. These preliminary studies were conducted only with the use of the unnatural isomer of phenylalanine and are by no means an indication that the unnatural isomers of tyrosine and tryptophane behave in the same manner.

Flasks containing 100 ml. of 100 mg. % of the two isomers were prepared and inoculated with washed organisms. The inocula were prepared as described previously and the inoculum consisted of 2 ml. of a turbid suspension (700 on Klett) per 100 ml. medium. Incubation was at 37°C and readings of turbidity and pigment production were made at twenty-four hour intervals as described under colorimetric and turbidimetric determinations.

CHAPTER IV

EXPERIMENTAL DATA

As noted in the table on page 42 the experimental organism does not utilize any of the carbohydrates of alcohols tested except ethanol, under the conditions tested. The organism grows very well with ethanol as the carbon and energy source and exhibits heavy turbidity in a twenty-four hour culture. Within seventy-two hours the pH of the medium has changed from 7.2 to 5.0.

Utilization of the amino acids as sole sources of carbon and organic nitrogen, as shown in the table on page 43, is generally very good with a few exceptions. Norvaline, threonine, serine, hydroxyproline, arginine, and lysine cannot be utilized by the organism. Utilization of cystine, cysteine, and histidine results in the formation of a water-soluble pigment. Cystine medium becomes yellow-orange at twenty-four hours incubation and turns a dark orange within forty-eight hours. Cysteine medium turns a slight yellow color at twenty-four hours incubation and by forty-eight hours has turned a yellow-orange. Histidine medium becomes slightly yellow at forty-eight hours incubation

and at seventy-two hours incubation has turned yellow-orange. None of the other amino acids, except the aromatic amino acids, produce any color change while under the influence of bacterial oxidation.

The tables on pages 44 and 45 give the results of the utilization of some related aromatic compounds and suspected degradation products of the oxidation of tryptophane. Anthranilic acid is utilized very well by the organism and also produces a water-soluble pigment, which appears at twenty-four hours incubation as a yellow discoloration of the medium. No pigment was formed from any of the other compounds.

The graphs on pages 52, 53, and 54 give the results of the pH studies determining pigment production, using the three different aromatic amino acids as substrate. A rather drastic difference in pH optimum for the appearance of the pigment is noted between tryptophane and the other two amino acids, tyrosine and phenylalanine. The pH optimum for tryptophane appears between 6.0 and 7.0 while the pH optimum for the other amino acids appears between 8.0 and 9.0. Also, the formation of pigment with tryptophane as substrate appears gradually over a forty-eight hour period while the formation of a pigment with phenylalanine as substrate appears rapidly after ninety-six to one hundred twenty hours incubation. The graph on page 55 illustrates the difference between the time and intensity of pigment produc-

tion when using the three aromatic amino acids as substrate at pH 7.0.

Since the preliminary studies, as indicated by the table on page 48, showed that growth and pigment production did not take place at 10°C or 45°C, the graphs on pages 56, 57, and 58 give the results of pigment production at 20°C, 30°C, and 37°C. With tryptophane as substrate the optimum temperature for pigment production is between 30°C and 37°C, although at 20°C pigment production proceeds very well except not as rapidly. On the other hand, when phenylalanine or tyrosine are used as substrate the optimum remains the same (30°C--37°C), but there is only a trace of pigment production at 20°C.

The graph on page 59 shows the results of the effect of inoculum on pigment production. The readings were made every four hours over a twenty-four hour period; and as the graph illustrates the difference between 0.5 ml. inoculum and 1.0 ml. inoculum is not significant although when 0.1 ml. is used as inoculum, the pigment production proceeds at a slower rate.

The table on page 49 gives the results of the inhibitor studies. None of the compounds added, except thiouracil, showed any inhibitory effects. Glutathione, cysteine, and potassium cyanide stimulated growth.

As the table on page 51 indicates the organisms are definitely capable of utilizing the aromatic amino acids as sole

sources of carbon and organic nitrogen. The turbidity present within twenty-four hours in the first day flasks is comparable to that formed within twenty-four hours in the tenth day flasks. Also, the intensity of the pigment formation has a tendency to become more pronounced as the serial transfers continue, that is, the time for pigment to appear is shortened.

The graph on page 60 gives the growth curves of the organism in various media (sodium citrate, tryptophane, tyrosine, and phenylalanine). As the graph shows the optimum population, when the organisms are grown in sodium citrate, is reached at about thirty-six hours incubation. This situation differs from that encountered when the aromatic amino acids are used as substrate, since in these cases there appears to be more of a lag phase present and the optimum is reached at about one hundred twenty hours incubation. Also, the population reached is higher in the case of the amino acids. A difficulty was encountered when the turbidity of organisms grown in tryptophane was being established since it became impossible to entirely separate the bacterial cells from the pigment, and even after washing the organisms five times they still retained a brown discoloration.

The table on page 50 gives the results of the comparative study using related gram negative organisms to determine whether they could utilize the aromatic amino acids as sole sources of carbon and organic nitrogen. As the table shows none

of the organisms produced any pigment nor was growth very evident. Escheriohia coli gave a positive indole test after twenty-four hours incubation when using tryptophane as substrate.

The graph on page 61 indicates that adapted organisms did not utilize the substrate to which they were adapted any faster, according to the methods used, than unadapted organisms. This graph also shows that oxidation proceeds more rapidly in the samples that are taken from the individual flasks than in the large flasks from which aliquots are removed.

Since the unnatural isomer phenylalanine was available a comparative study was conducted between the two isomers. As the graph on page 62 shows the D form is utilized by the organism, but to a much lesser extent than the natural L form. The pigment produced when using the D form never goes beyond a very slight discoloration of the medium, while the L form reaches its maximum (dark brown color) in about one hundred twenty hours.

On page 63 is the graph showing the results of dilution of the pigment with basal salt solution. The graph shows that it is possible to obtain almost a straight line relationship on dilution of the pigment although at the higher concentrations of pigment the line has a tendency to curve.

CHAPTER V

DISCUSSION AND CONCLUSIONS

For the sake of coherence the discussion shall be separated into three sections: (I) Relationship of the aromatic amino acid oxidase of Alcaligenes fecalis No. 135 to tyrosinase, (II) Physical-chemical characteristics of pigment formation, and (III) Discussion of the oxidative pathways of tryptophane.

I. Relationship of the Aromatic Amino Acid Oxidase of A. fecalis No. 135 to Tyrosinase.

Within the last hundred years the enzyme tyrosinase has been isolated from a great variety of plant and animal sources. The specificity has varied considerably depending on its source; the general concept being that the enzyme isolated from the higher animals possesses a higher degree of specificity than that isolated from the lower animals and plants. In contrast to this is the case of the enzyme being produced by No. 135. The oxidase seems to possess a rather high degree of specificity, not attacking many of the substances attacked by tyrosinases described in the literature. Cresol and catechol, used as indicators of tyrosinase activity by many investigators (Miller, Mallette,

Roth, and Dawson, 1944; Mallette and Dawson, 1947), are not oxidized by No. 135. This observation is supported by Griffin et al. (1953) who reported that a pathogen of salmonoid fishes, Bacterium salmonicida, produced a deep amber color on nutrient agar when either tyrosine or phenylalanine were added. No pigment production was observed by these investigators when phenol, cresol, or catechol were incorporated into the nutrient agar.

Raper and coworkers (Raper and Wormall, 1933; Raper and Speakman, 1926) conducted very extensive experiments on the pathway of oxidation of tyrosine to the high molecular weight polymerized pigment, which is non-specifically called melanin. The diagram on page 4 illustrates their experimental results. These workers demonstrated that the following three compounds are formed during the oxidative process: (1) dihydroxyphenylalanine (dopa), (2) 5,6-dihydroxyindole, and (3) 5,6-dihydroxyindole-3-carboxylic acid. They showed that the enzymatic oxidation of dopa would result in the formation of the other two compounds and therefore postulated that dopa was probably the first oxidation product of tyrosine.

The following facts make the oxidation of dopa to dopaquinone appear possible: (1) o-dihydroxyphenyl compounds are readily oxidized to the corresponding orthoquinones (2) substances that react with orthoquinones inhibit melanin formation in the dopa-tyrosinase reaction (3) plant tyrosinase has been

shown to catalyze the oxidation of catechol to orthobenzoquinone and the oxidation of dopa to dopa-quinone appears similar.

Raper (1927) has further shown that a red substance (hallochrome) was one of the products formed in the oxidative mechanism. Reduction of this red compound resulted in the formation of 5,6-dihydroxydihydroindole-2-carboxylic acid (leuco compound) and the subsequent oxidation of this leuco compound again gave the red compound. Oxidation of the red compound resulted in the formation of the indole compounds, 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid. Spectrophotometric data (Mason, 1948a) indicate that the 5,6-dihydroxyindole is rapidly oxidized to the corresponding quinone which then polymerizes to give melanin.

As yet no degradation products of the oxidation of tyrosine or phenylalanine by No. 135 have been identified, but since the oxidative scheme of Raper is generally accepted by investigators working with tyrosinase from various sources it is worthy of mention.

When No. 135 is grown in the presence of tyrosine or phenylalanine there is a period of growth before any pigment formation is evident. This lag phase has also been described by Griffin et al. (1953) and may be similar to the induction phase described by Lerner et al. (1949). The presence of a lag phase is also reported by Sussman and Markert (1953), working with

mutants of Glomerella cingulata. There is no evidence of a lag phase when tryptophane is oxidized by No. 135, but instead the formation of pigment proceeds along with the increase in bacterial population. Only when tyrosine and phenylalanine are used as substrate is a lag phase evident and the appearance of the pigment with phenylalanine as substrate is precipitated very rapidly after ninety-six to one hundred twenty hours incubation.

Lerner et al. (1949) encountered the presence of an induction period when they reacted tyrosine in the presence of tyrosinase and oxygen. These investigators showed that this induction period could be shortened by the addition of trace amounts of dopa, which possessed catalytic effects. Griffin et al. (1953) showed that the addition of dopa to the nutrient agar containing tyrosine or phenylalanine and seeded with Bacterium salmonicida did not catalyze the production of pigment. When dopa was added to the reaction flasks containing either tyrosine or phenylalanine and inoculated with No. 135, no catalytic effects were observed.

Sussman and Markert (1953) reported the development of tyrosinase activity in induced mutants of Glomerella cingulata. They induced mutations by using as mutagenic agents ultraviolet light, x-rays and neutron bombardment. They found that the conidia possessed little tyrosinase activity and upon germination the mycelial mass completely lost this activity. After one

hundred twenty hours growth tyrosinase activity began to show up and increased with the end of growth of the mycelium and the beginning of autolysis. These authors suggest that possibly the production of tyrosinase is induced by some substance accumulated during growth and released during autolysis. The observation is similar to the oxidation of tyrosine and phenylalanine by No. 135, where active pigment production (especially when phenylalanine is used as substrate) does not take place until the culture is in the stationary or death phase of the growth curve.

Since the conversion of phenylalanine to tyrosine has been demonstrated by many investigators (Moss and Schoenheimer, 1940; Bernheim and Bernheim, 1944; Lein and Greenberg, 1952; Udenfriend and Cooper, 1952) one might consider that the oxidation of phenylalanine proceeds in this manner. This idea is suggested in the report of Griffin et al. (1953) and the fact that the oxidation of phenylalanine proceeds more slowly than tyrosine in their observations might account for this conversion. This theory is contraindicated in the experimentation performed with No. 135 since the oxidation of phenylalanine proceeds at a much faster rate than the oxidation of tyrosine. Also the pigment produced by the bacterial oxidation of tyrosine never reaches the intensity of that produced by the bacterial oxidation of phenylalanine. Therefore, the possibility that alternate pathways exist for the oxidation of these two closely related amino acids

must be considered. Dagley, Fewster, and Happold (1953), working with vibrio organisms, reported that the bacterial oxidation of phenylalanine has been observed to follow two alternate metabolic pathways. These pathways are as follows: (1) PHENYLALANINE⟶ PHENYLPYRUVIC ACID⟶PHENYLACETIC ACID⟶HOMOGENTISIC ACID (2) PHENYLALANINE⟶TYROSINE⟶p-HYDROXYPHENYLPYRUVIC ACID⟶ HOMOGENTISIC ACID. These workers stated that the former pathway has been confirmed as the proper oxidative sequence. Their results are based on adaptive pattern experiments and the isolation of the phenylhydrazone of phenylpyruvic acid from the metabolic fluids. Simmonds, Tatum, and Fruton (1947) and Davis (1950) demonstrated that independant routes of tyrosine and phenylalanine synthesis take place and that phenylalanine is not converted to tyrosine. This has also been demonstrated by Gilvarg and Bloch (1951) in yeast cells.

The literature emphasizes that the most distinguishing feature of tyrosinase is its ability to catalyze two characteristically different oxidations: (1) the oxidation of monohydric phenols (2) the oxidation of dihydric phenols; to give a dark brown melanin-like pigment. The literature further states that the existing evidence indicates that phenylalanine may be oxidized via tyrosine. There is no indication in the literature that tyrosinase oxidizes tryptophane to melanin. Lerner and Fitzpatrick (1950) state that although there are differences among the tyro-

sinases obtained from various sources they all have three characteristics in common: (1) all catalyze the oxidation of tyrosine to melanin (2) the enzymatic reaction with the monohydroxyphenol compound is catalyzed by some o-dihydroxyphenol compound (3) copper is associated with the activity of the enzyme. Comparing the oxidase of No. 135 to the standards proposed for tyrosinase by previous investigators, one observes some striking differences. The oxidase of No. 135 catalyzes the oxidation of tyrosine to give pigment which never reaches a dark brown state but rather remains yellow-brown. Other monohydric phenols tested are not oxidized and neither are dihydric phenols. Phenylalanine and tryptophane both become oxidized by No. 135 to give a dark brown pigment. The first premise of the common characteristics of tyrosinases as elaborated by Lerner and Fitzpatrick (1950) appears satisfied. The second premise may possibly also be satisfied even though experimental results do not indicate that dopa catalyzes the reaction. The methods used may not be sensitive enough to detect a possible catalysis. Whether the third premise is satisfied has not yet been experimentally determined.

Therefore, in conclusion, one may state that the oxidase of Alcaligenes fecalis No. 135 belongs to the realm of tyrosinases since tyrosine is oxidized to give pigment production. Also, and probably more indicative, is the realization that No. 135 possesses three different oxidases, namely, tyrosinase,

phenylalaninase, and tryptophanase. Each of these aromatic amino acid oxidases is, of course, supported in the oxidative process by a host of other enzymes.

II. Physical-chemical Characteristics of Pigment Formation

The pigment produced from the aromatic amino acids by bacterial oxidation falls into the general class of melanins. The pigment is water soluble and colloidal in nature. The color varies according to the substrate. Oxidation of tyrosine produces a yellow-brown pigment whereas oxidation of phenylalanine and tryptophane results in the formation of a dark brown pigment.

Melanin is a non-specific name for a group of very widely distributed pigments varying in color from black, brown, and buff to blue. Mason (1953) presents the following definition of melanins:

> These substances are pigments of high molecular weight formed by the enzymatic oxidation of phenols. These melanins, which are obtained as the result of oxidative processes, cannot be simple substances homogenous with respect to a repeating unit, but are likely to contain structural elements in various states of oxidation, condensation, and degradation.

Many properties have been used by investigators to characterize melanin. Of primary consideration has been the production of color. Bloch (1927) described the color range from bright yellow and red yellow to light and dark brown to deep black. Attempts to characterize melanin in terms of absorption

spectra have been complicated by the colloidal state of these pigments, and by the presence of concommitants with which they are known to be associated. Some investigators have reserved the term melanin only to pigments formed in vivo whereas others have used the term to describe in vitro formation of pigment (Bloch and Schaaf, 1925). Other properties that have been used to describe melanin are solubility, reactivity, and chemical composition. Bloch and Schaaf (1925) have reported that natural (in vivo) and synthetic (in vitro) melanins are soluble in alcohol and pyridine. Greenstein, Turner, and Jenrette (1940) have shown that melanin from a mouse melanoma may be dissolved in neutral buffers and water. In 1945 Lee found that both natural and synthetic melanins were soluble in ethylenediamine and ethylenechlorohydrin solutions. Melanin is generally considered as being soluble in acid and alkali. Figge (1939) reported that melanin may be bleached by permanganate and bichromate, and Sachs (1944) stated that it may be bleached by hydrogen. A review of the composition of melanin was presented by Bloch (1937) and Waelsch (1933). Generally, the presence of carbon, hydrogen, nitrogen, and sulfur have been reported.

Mason (1948b), in a conference concerned with the meaning of the term "melanin," presented the following classification of melanins:

A. Natural Melanins--formed in vivo

1. Native melanins--forms in which natural melanins occur

a. Animal melanins--skin, hair, melanomas, etc.

b. Plant melanins--mushrooms, etc.

2. Derived melanins--extracted native proteins

a. Simple melanins--uncombined pigments

b. Conjugate melanins--melanoproteins, melanolipoids, and melanopolypeptides

B. Synthetic Melanins--in vitro oxidation of benzenoid amino acids and phenolic amines and amino acids

1. Melanins from photochemical oxidations--(Speigel-Adolph, 1937; Rothman, 1940)

2. Melanins from chemical oxidations and autoxidations

3. Melanins from enzymatic oxidations

According to the classification of Mason the pigment produced by No. 135 is "a synthetic melanin produced by enzymatic oxidation."

Pigment formation by No. 135 takes place over a wide range of pH values. Production of pigment is most pronounced in the slightly alkaline pH range when using tyrosine or phenylalanine as substrate whereas tryptophane shows better pigment production at neutral or slightly acid pH values. Production of pigment at acid pH ranges (5.0 and 6.0), when using tyrosine or phenylalanine as substrate, is almost entirely absent.

Production of pigment at various temperature levels coincides with the optimum temperature for the growth of the organisms. This is indicated by the complete absence of pigment when the reaction flasks are placed at 10°C or 45°C, whereas optimum pigment production is observed in the temperature range of 30°C to 37°C. The optimum growth temperature of Alcaligenes fecalis is 37°C (Bergey, 1948). Formation of pigment takes place at 20°C when using tryptophane as substrate whereas only traces of pigment are produced at this temperature level when using tyrosine or phenylalanine. Lerner and Fitzpatrick (1950) reported that the rate of enzymatic oxidation of tyrosine to melanin is more rapid above 30°C. Griffin et al. (1953) indicated that actively growing cells of B. salmonicida can produce pigment at 22°C but are devoid of pigment production above 32°C. Horowitz and Shen (1952) stated that tyrosinase production in Neurospora is dependent on the temperature of cultivation and that above 35°C enzymatic activity is almost entirely absent.

Various inhibitors of pigment production have been described by investigators (Lerner and Fitzpatrick, 1950). Among these are the organic sulfur-containing compounds and cyanide compounds which bind or remove the copper ions by forming weakly dissociable complexes with the copper. Of these inhibitors the following were used to determine possible inhibitive effects: cysteine, glutathione, thiourea, thiouracil, and potassium cya-

nide. Only thiouracil showed inhibitive effects, as compared to the control, and these were only slight. Reaction flasks containing glutathione and cysteine showed stimulation of growth and as further experimentation indicated could serve as sole sources of carbon and energy for the organisms. Thiourea had no effect and potassium cyanide actually stimulated growth slightly. In view of these results the presence of copper ions for activity of the oxidative enzymes in the case of No. 135 is not indicated.

Attempts to draw any definite conclusions as to the physical-chemical characteristics of pigment production by No. 135 are not justified at present. By definition the pigment produced is a melanin-type pigment. Preliminary experiments conducted with the use of cell-free filtrates indicate that the enzymes are of intracellular nature. Therefore, future experiments will have to be conducted with the use of cell-extract filtrates with subsequent purification of the active material.

III. Discussion of the Oxidative Pathways of Tryptophane

Experimentation entailing the pathway of oxidation of tryptophane was limited because of the unavailability of the majority of degradation products described by investigators. Indole, indoleacetic acid, kynurenic acid, and anthranilic acid were available and therefore were tested. Of these compounds only anthranilic acid could be utilized by No. 135.

The oxidation of tryptophane may follow various pathways, each leading to different degradation products. Four possible pathways, based on reports in the literature, are as follows:

(1) Reversal of the synthetic mechanism involving primary cleavage to indole and serine with subsequent oxidation of these fragments, anthranilic acid occurring as a degradation product of indole.

(2) Breakdown of the tryptophane molecule resulting in production of indole, ammonia, and pyruvic acid. Indole and pyruvic acid undergo further oxidation.

(3) Oxidative deamination of the aliphatic side chain resulting in indolepyruvic acid with subsequent oxidation to indoleacetic acid.

(4) Primary attack on the indole nucleus, with elimination of the second carbon atom in the five-membered ring giving kynurenine and by oxidative deamination and ring closure giving the formation of kynurenic acid.

Stanier and coworkers (Stanier, Hayaishi, and Tsuchida, 1951) conducted rather extensive experiments on the bacterial oxidation of tryptophane. They used an unidentified species of Pseudomonas as the source of oxidative enzymes. These workers concluded that the oxidative degradation of tryptophane follows

pathway No. 4. They came to this conclusion since the bacterial cells could be adapted to oxidize the degradation products of this pathway as well as the original substrate, tryptophane.

These investigators based their experimentation on the general theory of enzymatic adaptivity which was proposed by Karstrom in 1938 and is stated as follows:

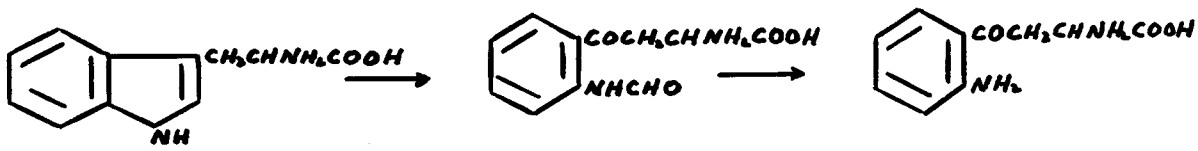
> Cells adapted to attack the primary substrate should be adapted simultaneously to attack all the intermediate formed during the oxidation of that substrate.

Suda, Hayaishi and Oda, in 1949 and 1950, formulated the concept that exposure to a primary substrate activates the adaptive enzymes which act upon the subsequent metabolic intermediates and named the phenomenon "successive adaptation." These investigators further studied the metabolic pathway of tryptophane which was oxidized by an unidentified Pseudomonas sp., and presented the following metabolic sequence: L-TRYPTOPHANE ⟶ L-KYNURENINE ⟶ ANTHRANILIC ACID ⟶ CATECHOL.

Stanier and Tsuchida (1949) made independant studies of the oxidation of tryptophane by another unidentified specie of Pseudomonas, also using the method of adaptive patterns. The phenomenon of subsequent adaptation was developed by Stanier, in 1947, under the name of "simultaneous adaptation." These investigators found that the oxidation of tryptophane proceeded in the following manner: TRYPTOPHANE ⟶ KYNURENINE ⟶ KYNURENIC ACID. In this pathway both isomers of tryptophane could be oxidized

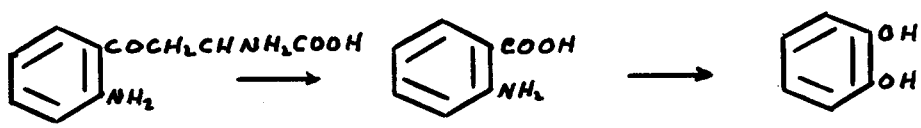
whereas in the metabolic pathway of Suda, Hayaishi and Oda only the natural or L form of tryptophane could be oxidized.

In 1951 Stanier, Hayaishi, and Tsuchida designated the pathway discovered by Suda, Hayaishi, and Oda as the "aromatic pathway" and that discovered by Stanier and Tsuchida as the "quinoline pathway." They further reported that all strains tested showed the following initial pathway:

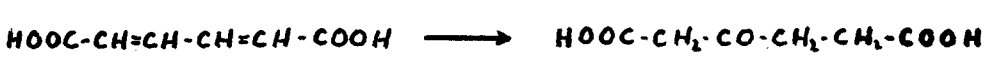


Tryptophane Formylkynurenine Kynurenine

Beyond kynurenine there exists a strict dichotomy in the pathway of oxidation following either the aromatic or the quinoline pathway. Further degradation products of the aromatic pathway have been identified and the metabolic sequence continues as follows:



Kynurenine Anthranilic Acid Catechol



Cis-cis-muconic Acid B-ketoadipic Acid

Oxidative products beyond B-ketoadipic acid have not been identified. The quinoline pathway beyond kynurenic acid has not been investigated sufficiently to warrant the establishment of further degradation products.

Since No. 135 cannot utilize indole, serine, or indoleacetic acid and since indole is not produced as a degradation product the first, second and third oxidative pathways presented in the beginning of the discussion may be disregarded. This leaves the aromatic pathway of Suda, Hayaishi, and Oda and the quinoline pathway of Stanier and Tsuchida. Due to the incompleteness of the studies concerning the utilization of the degradation products of these two pathways by No. 135 it becomes difficult to definitely establish which of these pathways the oxidation follows. Preliminary evidence indicates that the aromatic pathway is indicated since No. 135 is able to utilize anthranilic acid very well and is completely unable to utilize kynurenic acid. The fact that No. 135 is unable to utilize catechol may indicate that the aromatic pathway is not followed directly but that the pathway diverges at some point. Further experimentation with the use of cell-extract filtrates of the active enzyme, performed with the use of the Warburg, should resolve the problem of the metabolic sequence in the oxidation of tryptophane by Alcaligenes fecalis No. 135.

SUMMARY

1. Oxidation of tyrosine, phenylalanine, and tryptophane by Alcaligenes fecalis No. 135 results in the formation of a melanin-like pigment.

2. Preliminary evidence indicates that the pathway of oxidation is different for each of the aromatic amino acids.

3. The oxidation of tryptophane may follow the "aromatic pathway" described by Suda, Hayaishi, and Oda

TABLE I

UTILIZATION OF CARBOHYDRATES AND ALCOHOLS BY ALCALIGENES FECALIS NO. 135

Dextrose	(-)
Lactose	(-)
Sucrose	(-)
Maltose	(-)
Mannitol	(-)
Xylose	(-)
Arabinose	(-)
Erythritol	(-)
Adonitol	(-)
Sorbitol	(-)
Dulcitol	(-)
Inositol	(-)
Glycerol	(-)
Methanol	(-)
Ethanol	(+)

(+)- appearance of turbidity within 48 hours

(-)- no appearance of turbidity within 48 hours

TABLE II

(-) - no appearance of turbidity

(/) - slightly turbid

(//) - turbid

(///) - heavy turbidity

(////) - very heavy turbidity

TABLE II

UTILIZATION OF AMINO ACIDS BY ALCALIGENES FECALIS NO. 135

	24 hrs.	48 hrs.	72 hrs.
glycine	+	+	+
DL-alanine	++	+++	+++
DL-valine	-	+	++
DL-norvaline	-	-	-
DL-threonine	-	-	-
DL-serine	-	-	-
DL-leucine	+	++	+++
DL-isoleucine	+	+++	+++
DL-norleucine	+	+++	+++
DL-phenylalnine	+	++	+++
DL-tyrosine	+	++	+++
L-cystine	+	++	++
DL-cysteine	+	+	+
DL-methionine	+	++	++
DL-tryptophane	+	+++	+++
L-proline	+++	+++	+++
L-hydroxyproline	-	-	-
L-glutamic acid	+++	+++	+++
DL-aspartic acid	++	+++	+++
L-histidine	-	+	++
L-arginine	-	-	-
DL-lysine	-	-	-

TABLE III

(-) - no appearance of turbidity

(/) - slightly turbid

(//) - turbid

(///) - heavy turbidity

(////) - very heavy turbidity

TABLE III

UTILIZATION OF RELATED COMPOUNDS BY
ALCALIGENES FECALIS NO. 135

	24 hrs.	48 hrs.	72 hrs.
tyrosine ethyl ester	+++	+++	++++
tyramine hydrochloride	++	++	+++
3,5 diiodo-L-tyrosine	-	+	++
phenol	-	-	-
p-methoxyphenol	-	-	-
p-aminophenol	-	-	-
p-cresol	-	-	-
pyrogallol	-	-	-
epinephrine	-	-	-
phenylacetic acid	++	+++	++++
p-nitrophenylacetic acid	-	-	-
3,4 dimethoxyphenylacetic acid	-	-	-
benzoic acid	-	-	+
p-aminobenzoic acid	-	-	-
p-hydroxybenzoic acid	-	-	-
glutathione	+	++	+++
thiourea	-	-	-
thiouracil	-	-	-

TABLE IV

UTILIZATION OF DEGRADATION PRODUCTS OF TRYPTOPHANE OXIDATION BY *ALCALIGENES FECALIS* NO. 135

indoleacetic acid.....(-)

kynurenic acid.....(-)

anthranilic acid.....(+)

indole.....(-)

catechol.....(-)

kynurenine.....(not available)

cis-cis-muconic acid.....(not available)

B-ketoadipic acid.....(not available)

indolepyruvic acid.....(not available)

(+)- appearance of turbidity within 48 hours

(-)- no appearance of turbidity within 48 hours

TABLE V

GENERAL PHYSIOLOGICAL REACTIONS OF *ALCALIGENES FECALIS* NO. 135

indole.....(-)

methyl red.....(-)

Voges-Proskauer.....(-)

urea.....(-)

nitrate.....(-)

gelatin-liquefaction.....(-)

hydrogen sulfide.....(-)

citrate.....(+)

blood agar....alpha hemolysis

TABLE VI

COMPARATIVE STIMULATION OF OXIDATION
BY SHAKING MACHINE AND
SHALLOW LAYER METHOD

	Growth	Pigment
L-tyrosine		
shaking machine	18-24 hrs.	48-72 hrs.
shallow layer method	24-48 hrs.	96-144 hrs.
L-phenylalanine		
shaking machine	18-24 hrs.	24-48 hrs.
shallow layer method	24-48 hrs.	72-96 hrs.
L-tryptophane		
shaking machine	18-24 hrs.	6-12 hrs.
shallow layer method	24-48 hrs.	6-12 hrs.

TABLE VII

PRELIMINARY TEMPERATURE STUDIES

Temperature	L-tyrosine	L-phenylalanine	L-tryptophane
10° C	(-)	(-)	(-)
20° C	(+)	(+)	(+)P
30° C	(++)P	(++)P	(++)P
37° C	(++)P	(++)P	(++)P
45° C	(-)	(-)	(-)

(-) - absence of turbidity
(+) - slightly turbid
(++) - turbid
P - presence of pigment

TABLE VIII

EFFECT OF INHIBITORS ON PIGMENT FORMATION

L-tyrosine

	24 hrs.	48 hrs.	72 hrs.
thiouracil	(+)	(++)	(++)
thiourea	(++)	(+++)	(+++)
glutathione	(+++)	(++++)	(++++)P
cysteine	(+++)P*	(++++)P	(++++)P
potassium cyanide	(+++)	(++++)	(++++)P
control	(++)	(+++)	(+++)

L-phenylalanine

	24 hrs.	48 hrs.	72 hrs.
thiouracil	(+)	(++)	(++)
thiourea	(++)	(+++)	(+++)
glutathione	(+++)	(+++)	(++++)P
cysteine	(+++)P*	(+++)P	(++++)P
potassium cyanide	(+++)	(+++)P	(++++)P
control	(++)	(+++)	(+++)

L-tryptophane

	24 hrs.	48 hrs.	72 hrs.
thiouracil	(+)	(++)P	(++)P
thiourea	(++)P	(+++)P	(+++)P
glutathione	(+++)P	(++++)P	(++++)P
cysteine	(+++)P	(+++)P	(++++)P
potassium cyanide	(+++)P	(+++)P	(++++)P
control	(++)P	(+++)P	(+++)P

*pigment due to oxidation of cysteine

P - appearance of pigment
(+) - slight turbidity
(++) - turbid
(+++) - heavy turbidity
(++++)- very heavy turbidity

TABLE IX

UTILIZATION OF AROMATIC AMINO ACIDS BY RELATED ORGANISMS

	Ty	Ph	Tr
Aerobacter aerogenes No. 7.2	(-)	(-)	(-)
Escherichia coli No. 4.1	(-)	(-)	(-)*
Proteus vulgaris No. 3500	(-)	(-)	(-)
Paracolobactrum sp. No. 3131	(-)	(-)	(-)
Pseudomonas sp. No. E-13	(-)	(-)	(-)
Alcaligenes fecalis No. 135	(+)P	(+)P	(+)P

*positive indole test

Ty - L-tyrosine
Ph - L-phenylalanine
Tr - L-tryptophane
(-)- no appearance of turbidity
(+)- appearance of turbidity
P - appearance of pigment

TABLE X

SERIAL TRANSFER EXPERIMENT

Day	L-tyrosine	L-phenylalanine	L-tryptophane
1	≠(120)	≠(96)	≠(24)
2	≠(120)	≠(96)	≠(24)
3	≠(120)	≠(72)	≠(24)
4	≠(96)	≠(72)	≠(24)
5	≠(96)	≠(72)	≠(24)
6	≠(96)	≠(72)	≠(24)
7	≠(96)	≠(72)	≠(24)
8	≠(96)	≠(72)	≠(24)
9	≠(72)	≠(48)	≠(24)
10	≠(72)	≠(72)	≠(24)

≠ - appearance of turbidity within 24 hours

( ) - hours within which pigment appears

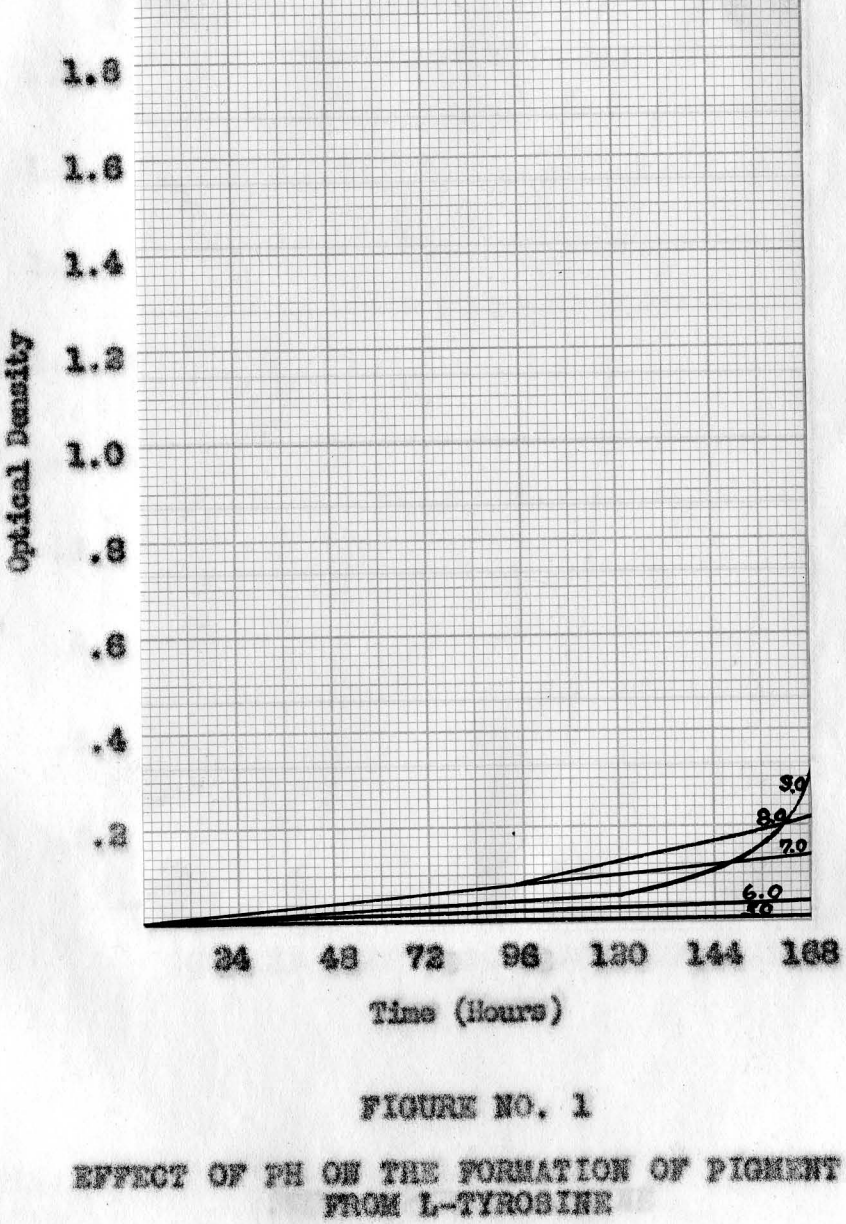


FIGURE NO. 1

EFFECT OF PH ON THE FORMATION OF PIGMENT FROM L-TYROSINE

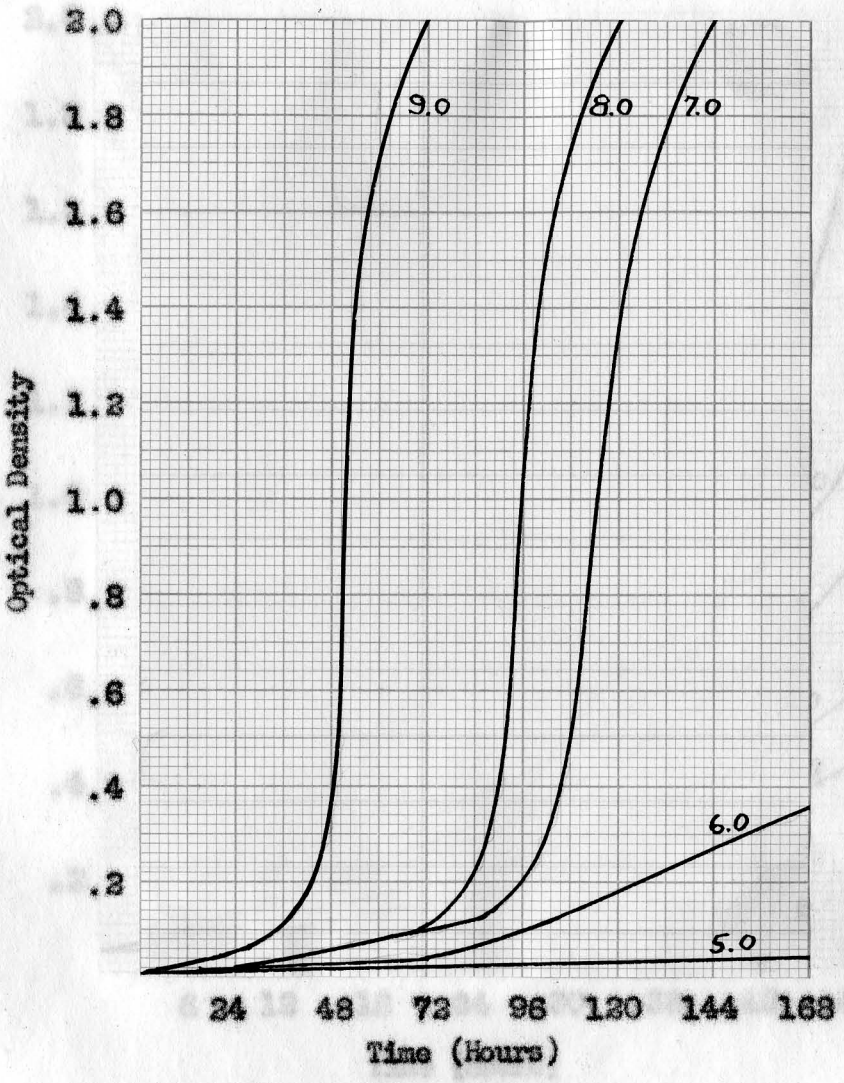


FIGURE NO. 2

EFFECT OF PH ON THE FORMATION OF PIGMENT FROM L-PHENYLALANINE

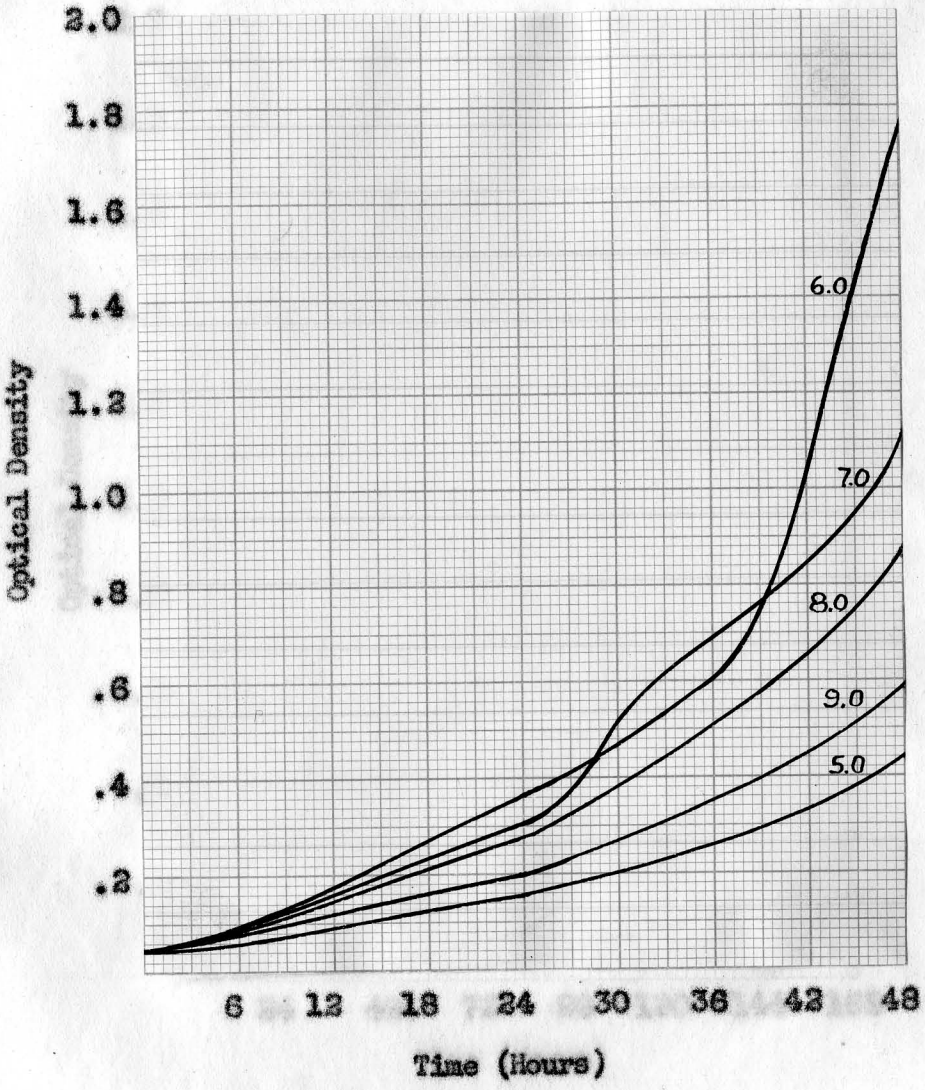


FIGURE NO. 3

EFFECT OF PH ON THE FORMATION OF PIGMENT
FROM L-TRYPTOPHANE

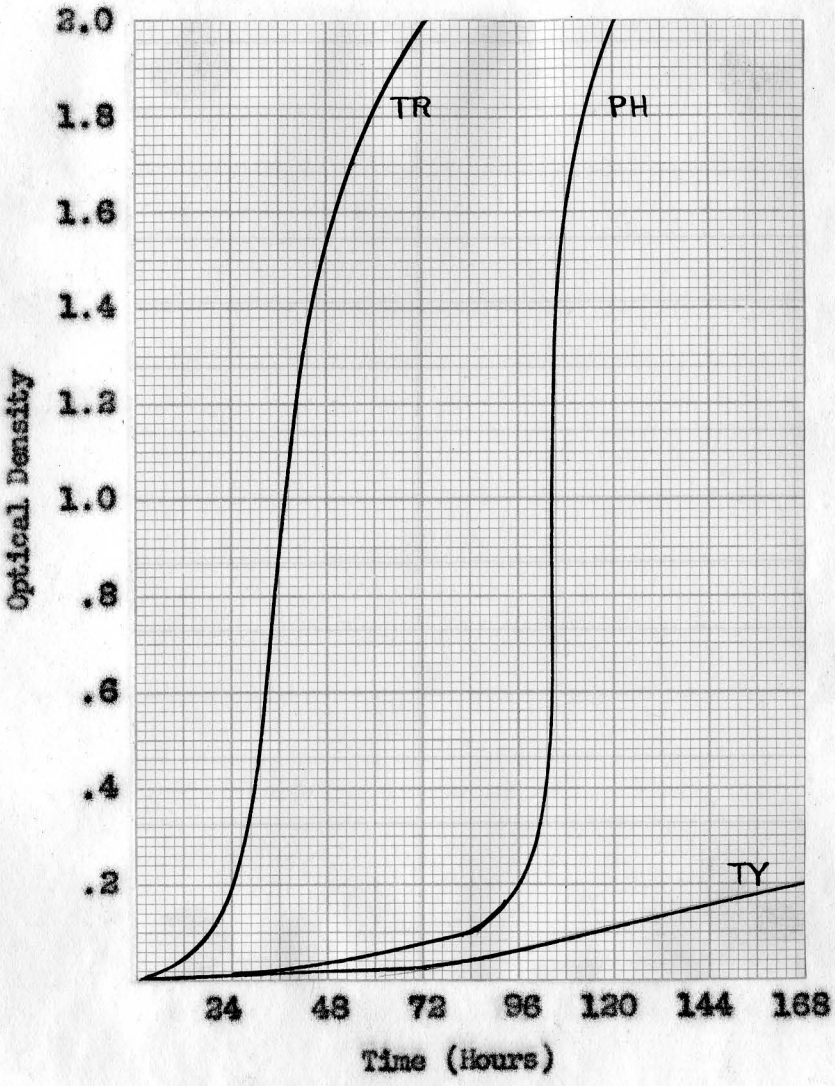


FIGURE NO. 4

PIGMENT FORMATION FROM L-TYROSINE, L-PHENYLALANINE, AND L-TRYPTOPHANE AT PH 7.0

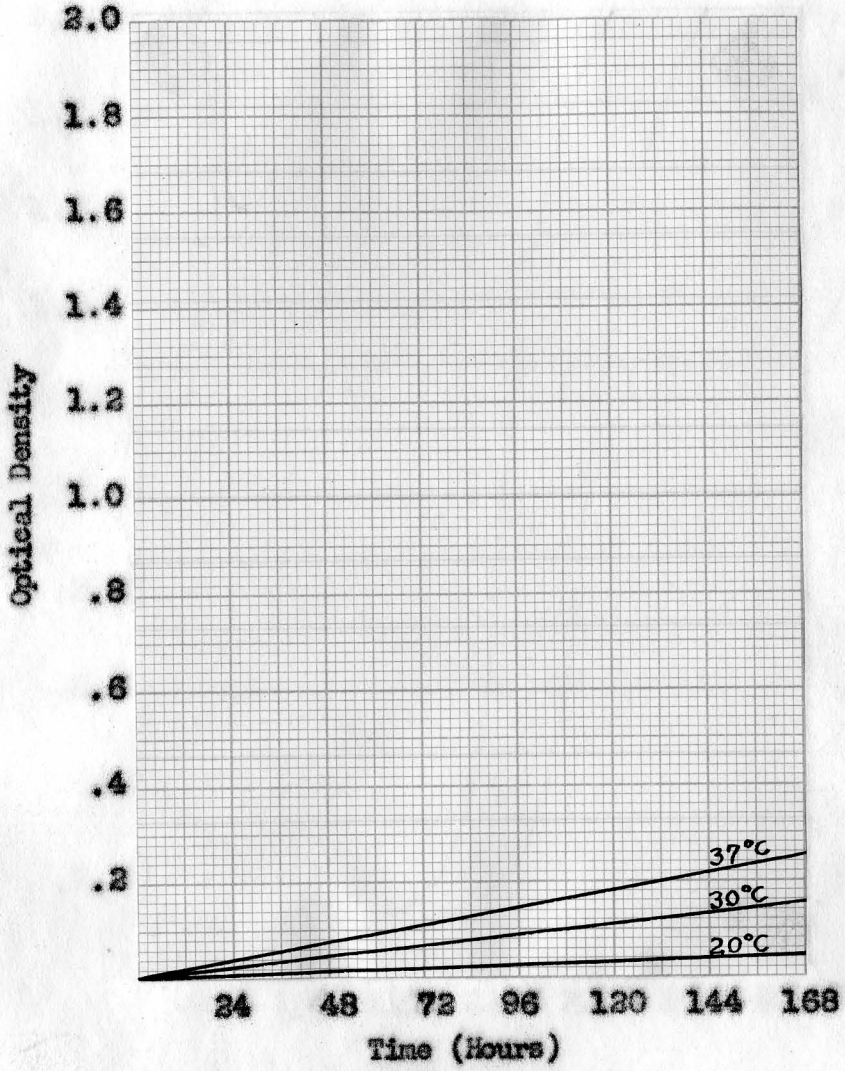


FIGURE NO. 5

EFFECT OF TEMPERATURE ON THE FORMATION OF PIGMENT FROM L-TYROSINE

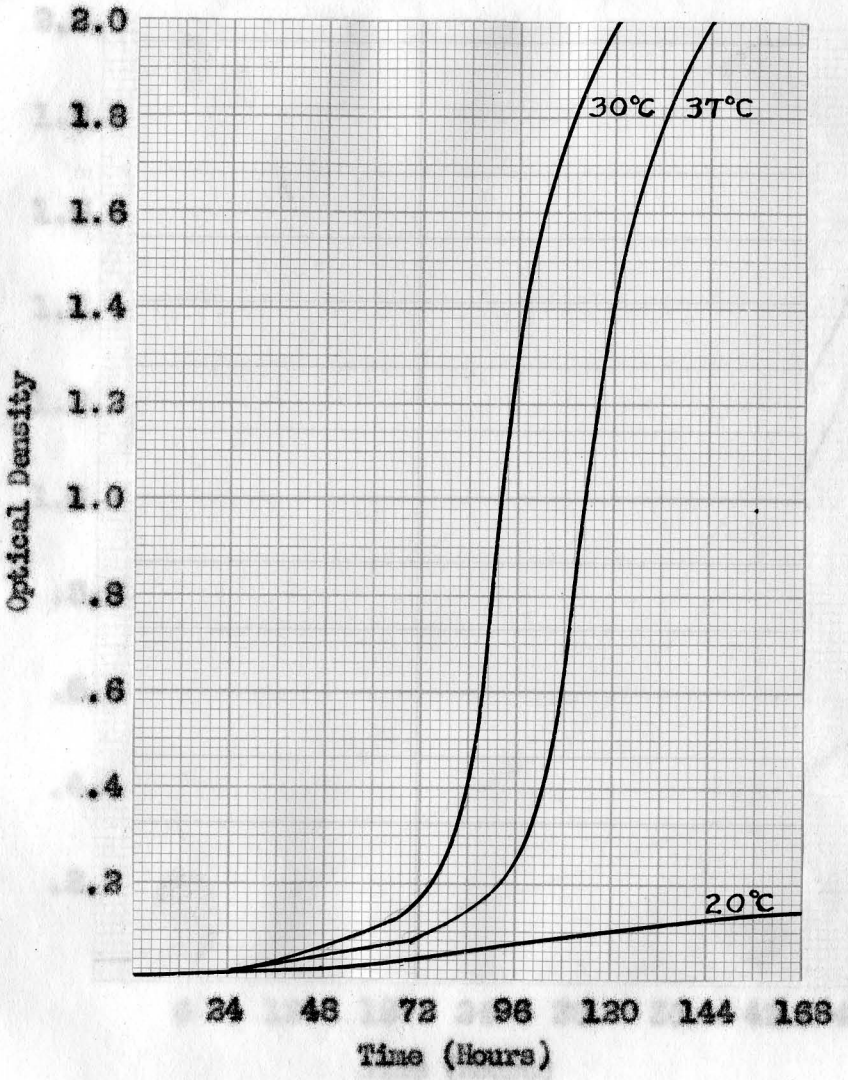


FIGURE NO. 6

EFFECT OF TEMPERATURE ON THE FORMATION OF PIGMENT FROM L-PHENYLALANINE

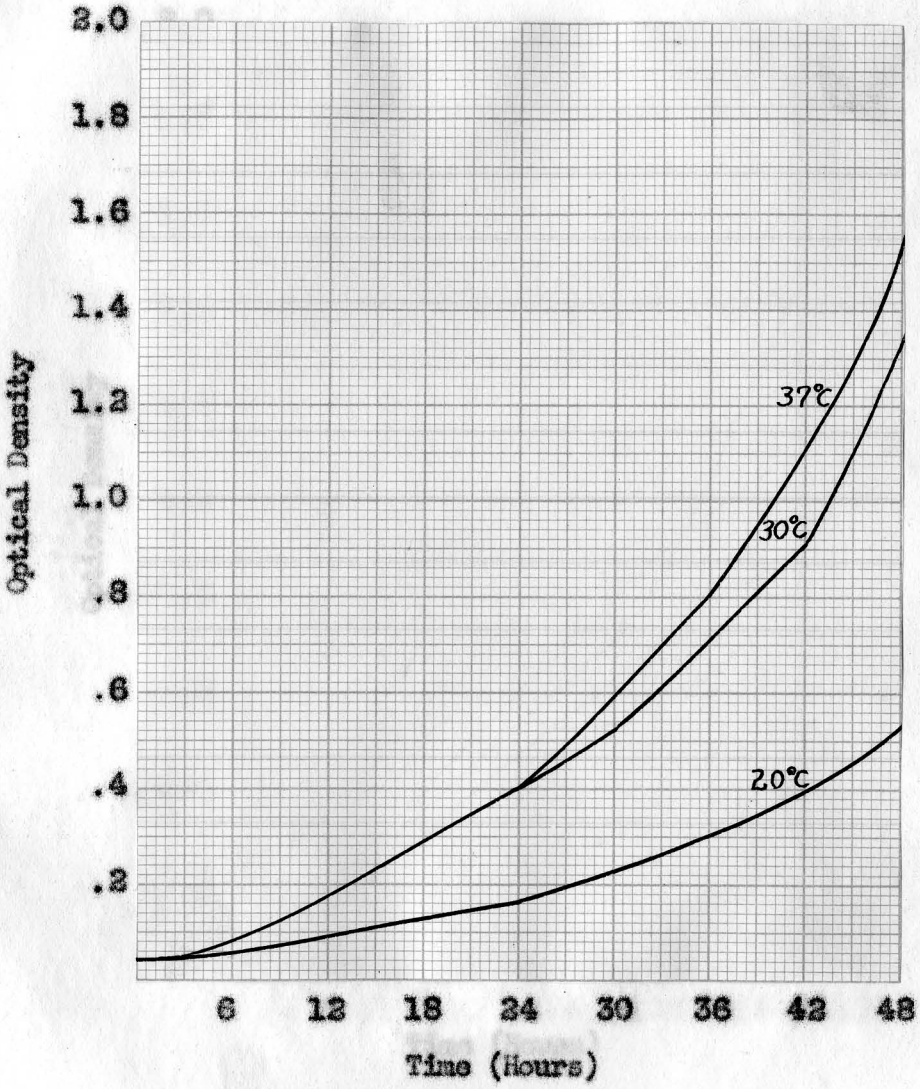


FIGURE NO. 7

EFFECT OF TEMPERATURE ON THE FORMATION OF PIGMENT FROM L-TRYPTOPHANE

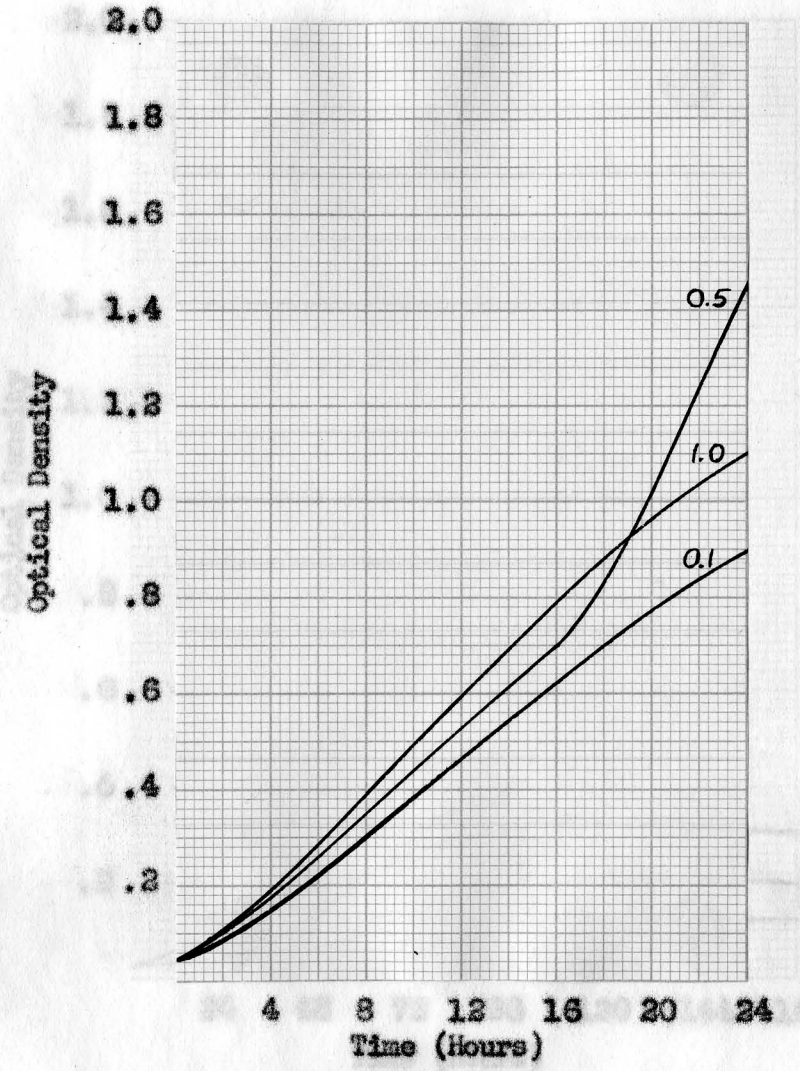


FIGURE NO. 8

EFFECT OF SIZE OF INOCULUM ON THE FORMATION OF PIGMENT FROM L-TRYPTOPHANE

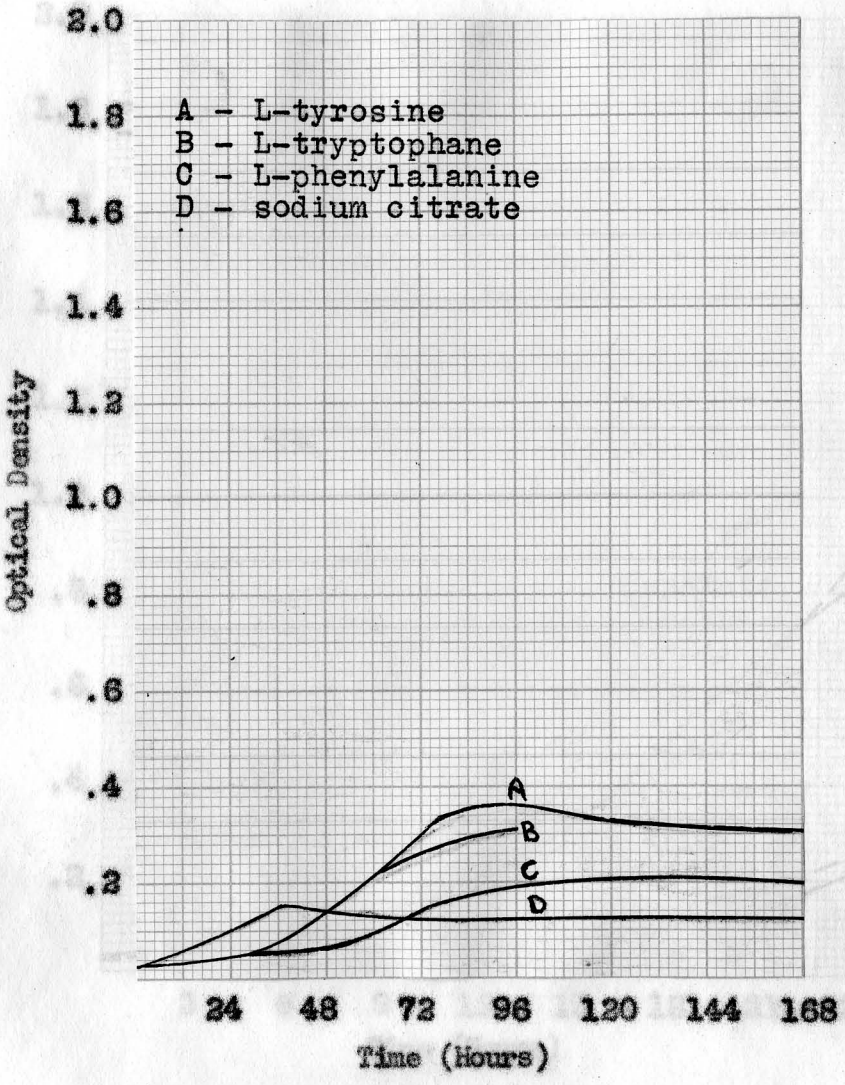


FIGURE NO. 9

GROWTH CURVE OF ALCALIGENES FECALIS NO. 135 IN VARIOUS MEDIA

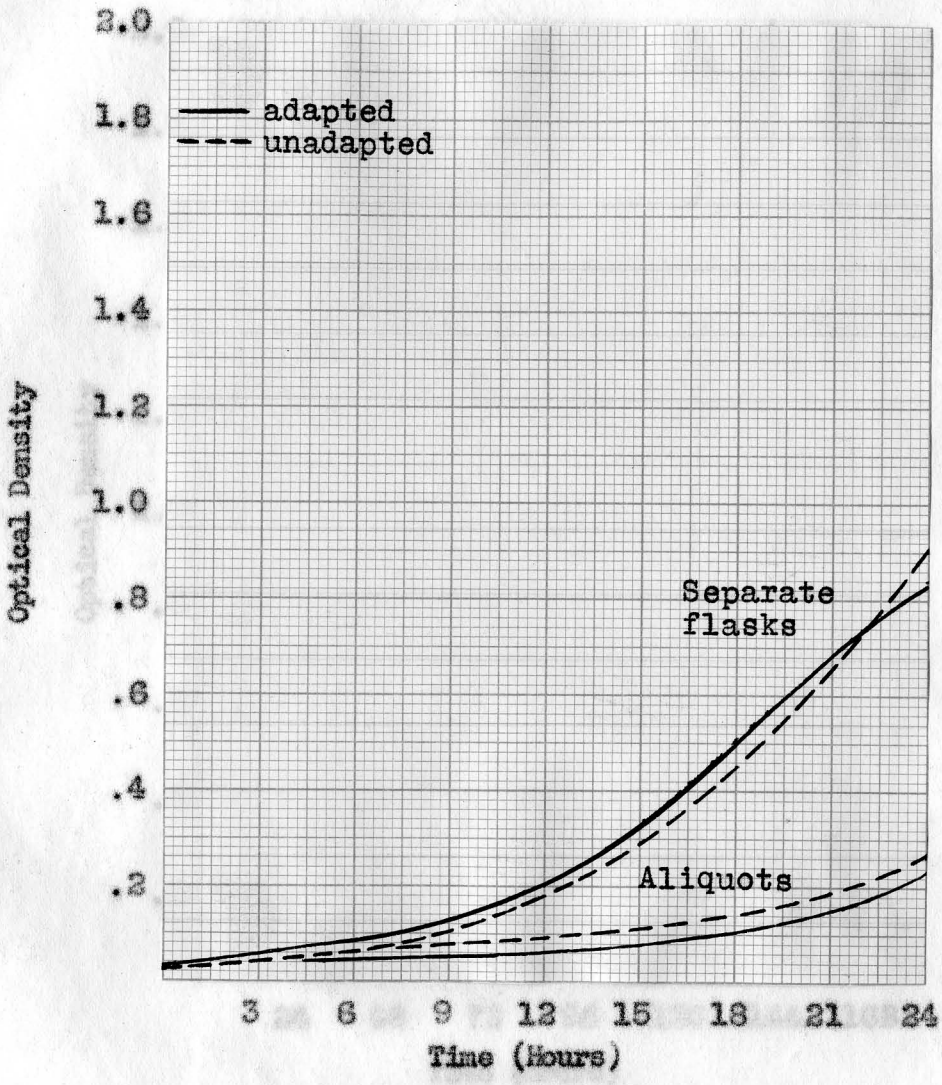


FIGURE NO. 10

COMPARATIVE PIGMENT FORMATION FROM L-TRYPTOPHANE BY ADAPTED AND UNADAPTED ORGANISMS

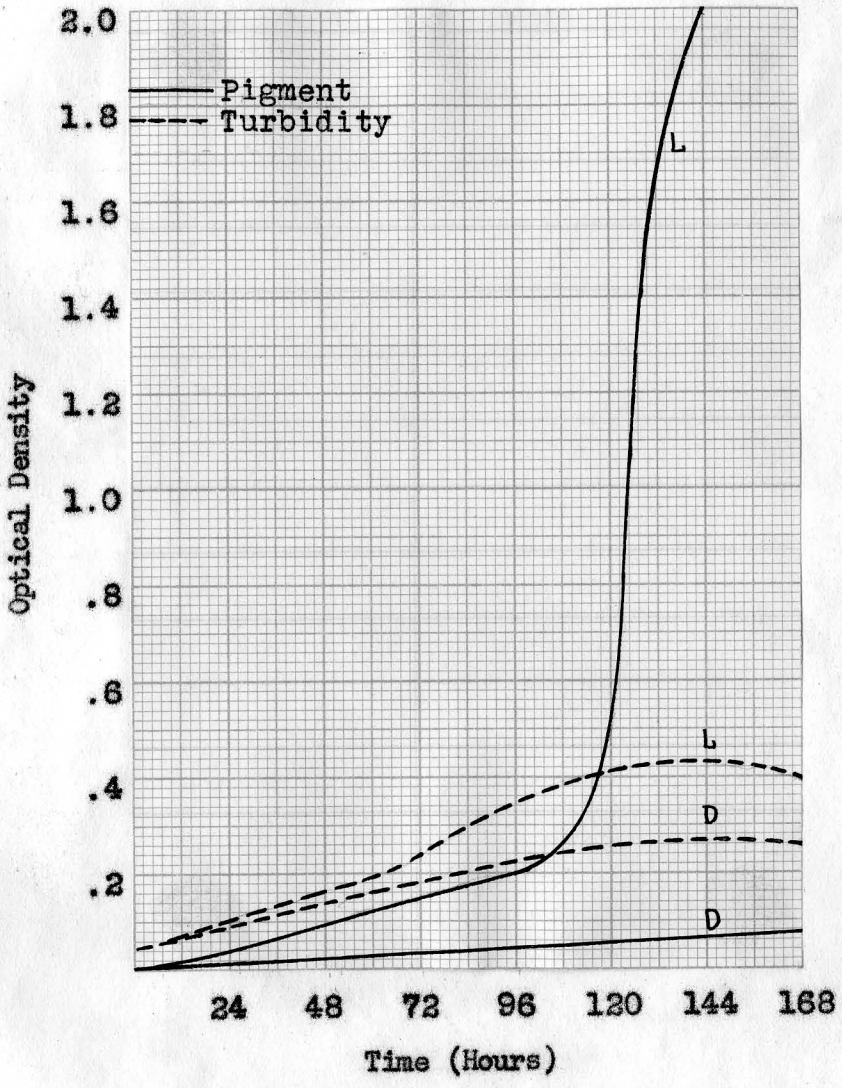


FIGURE NO. 11

COMPARATIVE OXIDATION OF D AND L-PHENYLALANINE

FIGURE NO. 12

100 - undiluted sample

50 - 1:2 dilution

25 - 1:4 dilution

12.5 - 1:8 dilution

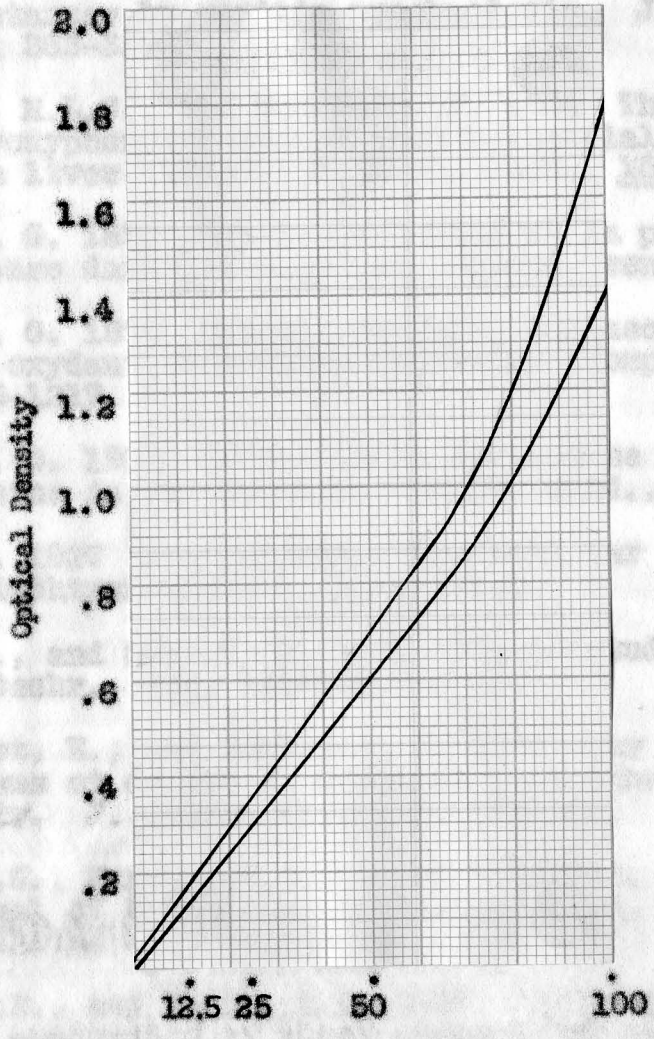


FIGURE NO. 12

DILUTION OF HIGHLY PIGMENTED SAMPLES

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APPROVAL SHEET

The thesis submitted by Jerry J. Tulis has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 24, 1955
Date

Signature of Adviser