THE OXIDATION OF TYRAMINE AND RELATED COMPOUNDS BY PEROXIDASE

by

ARTHUR J. GROSS

B.A., University of Rochester
(1946)

M.D., University of Rochester
(1950)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF
PHILOSOPHY
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September, 1954

Signature of Author . . . .
Department of Biology, September 7, 1954

Certified by . . . .
Thesis Supervisor

Accepted by . . . .
Chairman, Departmental Committee
on Graduate Students

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Arthur J. Gross

Submitted to the Department of Biology on September 7, 1954, in partial fulfillment of the requirements for the degree of Ph. D.

Abstract

This study was undertaken to determine the action of peroxidase upon tyrosine. Earlier investigators had suggested that the oxidative inactivation of susceptible proteins by peroxidase could be accounted for by the direct oxidation of tyrosyl groups in the protein. The presence of peroxidase, tyrosine and H_2O_2 in many living systems, notably mammalian eosinophils, prompted attention to the possible physiological role of the reaction products.

Early in the investigation, tyramine was shown by chromatography and ultraviolet spectroscopy to be oxidized in a manner similar to tyrosine. The amine was chosen as substrate because better methods were available for its chemical manipulation and analysis.

The components of the reaction were separated by paper chromatography; extension of the technique then permitted isolation of the chief reaction product in a column using ground Whatman paper with butanol-formic acid-water solvent. This product, shown later to be dityramine, 2, 2'-dihydroxy-5, 5'-bis(β-ethylamine) diphenyl, differed from the parent compound by possessing an ϵ_{max} (base) 2.5 times greater than tyramine and by exhibiting strong blue fluorescence under ultraviolet light. λ_{max} (acid) and (base) were shifted toward longer wavelengths than those characteristic of tyramine. The melting point of the HCl salt extended over the range 210-235° with decomposition. The free base was too unstable in air and moisture to permit isolation as dry crystals.

Dityramine, (I), gave positive tests for monophenolic and primary amine groups. Elementary analysis required the empirical formula $C_8H_{11}NOCl$. Derivatives were made to establish the molecular and structural formulas.

By means of the Schotten-Baumann reaction, O, O', N, N'-tetrabenzoyl-2, 2'-dihydroxy-5, 5'-bis(β-ethylamine) diphenyl, melting point 196.5° (corr.), was prepared. Elementary analysis and molecular weight data required the formula $C_{44}H_{36}N_2O_6$, which established the molecular formula of I as $C_{16}H_{22}N_2O_2Cl_2$.

Methylation of I with dimethyl sulfate yielded a derivative, melting point 225° (corr.), with two methoxy groups and molecular formula $C_{26}H_{44}N_2S_2O_{10}$. This compound, 2, 2'-dimethoxy-5, 5'-bis (β-ethyltrimethyl-ammonium methylsulfate) diphenyl, was degraded to

2, 2'-dimethoxydiphenyl-5, 5'-dicarboxylic acid, melting point 302°, by means of potassium permanganate, the side-chains, therefore, had been oxidized to carboxyl groups. Further methylation with diazomethane converted the acid to the known 2, 2'-dimethoxy-5, 5'-dicarbomethoxydiphenyl, melting point 173° (Corr.). Melting point and elementary analysis agreed with the values reported in the literature and calculated values.

Some secondary products of the peroxidase-tyramine-H_2O_2 reaction were isolated, and, although not proven rigorously, were considered to be: a) trityramine, a compound similar to dityramine but possessing one more tyramine residue, and b) an insoluble pigment made up of condensed and polmerized di-, tri-, and poly-tyramine elements, as well as more highly oxidized substances.

Dityramine was synthesized catalytically, as adjudged by chromatographic assay, by heating tyramine hydrochloride with CuO, and by heating tyramine with $FeCl_3$ in aqueous solution.

Compounds related to tyramine, that were shown by chromatographic examination, utilizing the fluorescence of their chief reaction products, to be oxidized by peroxidase to diphenyl analogues, are: tyrosine, glycyltyrosine, tyrosine ethyl ester, N-formyltyrosine and N-formyltyrosine ethyl ester. Diiodotyrosine yielded a product which absorbed ultraviolet light, but p-methoxyphenylalanine was not oxidized at all.

Bovine fibrinogen, zinc-free insulin, trypsin and gelatin were reacted with peroxidase-tyramine, (or tyrosine), -H_2O_2 systems. Upon subjecting them to dialysis, hydrolysis and chromatography, there could be detected no in situ formation of diphenyl linkages on the proteins.

The mechanism of synthesis of diphenyl compounds by peroxidase is considered to follow the same pathway proposed by Pummerer for the conversion of β-naphthol to di-β-naphthol. The presence of a phen-oxide free radical is required, which then undergoes isomerization and dimerization.

Examples from the literature of diphenyl compounds formed as a result of peroxidase-H_2O_2 action are divanillin, di-p-cresol and tetramethylbenzidine.

Thesis Supervisor: Irwin W. Sizer

Title: Associate Professor of Biochemistry

Acknowledgment

I would like to express to all members of the Biology Department my gratitude for their kind assistance and to those, as well, in other departments who have been generous and helpful. To my fellow-students, I would like to extend my deepest thanks. For Dr. Irwin W. Sizer, who has given me much wise counsel, I reserve sincerest appreciation.

I have been generously aided, during this work, by a Fellowship from The National Foundation for Infantile Paralysis.

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I. Introduction

A. Historical Background

Despite the fact that peroxidase is one of the oldest of the known enzymes, and has been highly purified from many sources (1-4), its physiological role is not well understood today. Theories have been advanced citing a primary respiratory role for peroxidase (5, 6), others have held that, together with catalase, it serves to protect the organism from an accumulation of H_2O_2 generated by aerobic oxidizing systems (7, 8). More recently the view has been advanced that peroxidase may function to bring about important secondary oxidations in the cell of substances of physiological importance. Keilin and Hartree (9), in 1945, suggested that this may be true for catalase as well.

Peroxidase was first found in plants in 1863. Shortly thereafter, in 1868, Klebs (9a) discovered that tincture of guaiacum was stained blue by pus, this being associated with the presence of leucocytes. It was shown by Myer (9b) in 1903, that blood from patients suffering from myeloid leukemia gave a blue color with guaiacum, whereas blood from patients with lymphatic leukemia did not. Myer thought this was due to the presence of an oxidase. About this time, Linossier (10) distinguished for the first time between oxidase and peroxidase reactions, showing that the guaiacum reaction with pus would only take place in the presence of hydrogen peroxide.

Peroxidases are now known to be present in nearly all plants, in milk, in blood and tissue granulocytes, in yeast, and in the liver (11). Huszak (12) has reported a peroxidase in the adrenal medulla. Interestingly enough, he was unable to find cytochrome oxidase located there. Recently, a peroxidase has been described in the saliva of a

variety of animals, including man (13). It was found both in the sediment and in solution. Finally, the presence of peroxidase in the thyroid gland, which had been strongly suspected by Harington (14) and Keston (15), was confirmed through the studies of De Robertis and Grasso on the rat (16). Kracht and Kracht repeated this work with rabbits obtaining the same findings (17), but Glock (18) could find no true peroxidase in horse thyroid gland.

One of the chief problems arising in the detection of peroxidases in tissues, especially when histochemical techniques are employed, is the interference caused by hemoglobin and other hemin compounds, which also catalyze peroxidative reactions. However, the reactivities of these latter compounds are very low when compared with true peroxidases. Finally, the peroxidases are susceptible to isolation and further purification, in which cases their identity and uniqueness may be established.

It is useful, for purposes of presentation, to classify the peroxidases. On the basis of literature reports (2, 11, 13, 19-21) describing the substrate specificaties of the various peroxidases, a division may be made into two categories: wide-spectrum and narrow-spectrum peroxidases. The classification is as follows:

A. Wide-spectrum Peroxidases
   1. Horseradish, and all plant peroxidases
   2. Lactoperoxidase
   3. Myeloperoxidase
   4. Thyroid peroxidase

B. Narrow-spectrum Peroxidases
   1. Tryptophane (liver) peroxidase
   2. Cytochrome c peroxidase

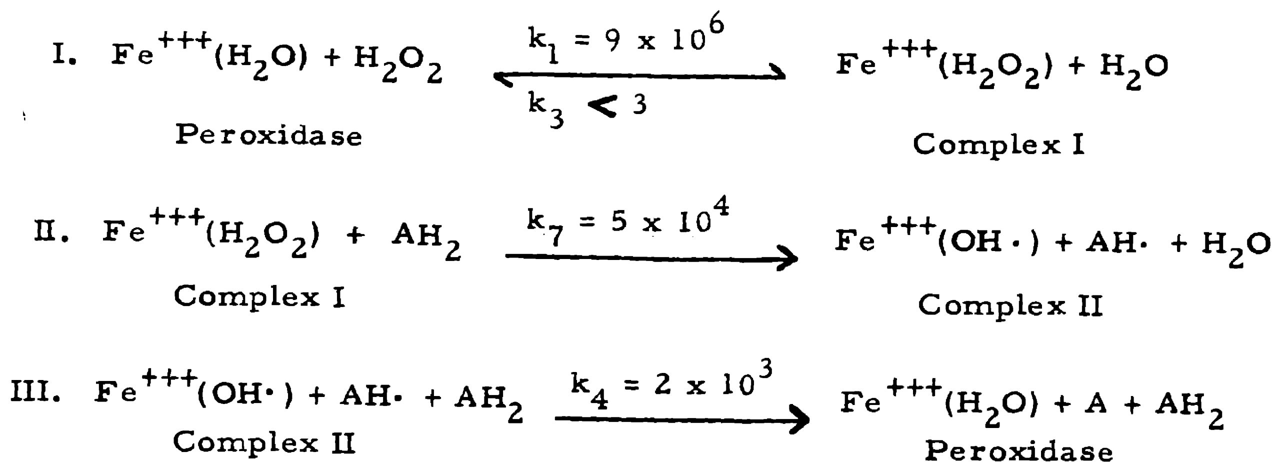
Salivary and adrenal medullary peroxidases have not been purified or characterized sufficiently to determine with certainty the category into which they fall. However, both of them are capable of oxidizing several substrates: salivary peroxidase oxidizes guaiacol, p-cresol and pyrogallol (13); adrenal medulla peroxidase oxidizes cytochrome c, p-phenylenediamine and "different phenolic substances" (12). Even the case of cytochrome c peroxidase is not clear, since, in a recent article (22), Chance refers to unpublished experiments which reveal that this enzyme oxidizes p-phenylenediamine and related substances.

Perhaps then, the classification is not revealing except to distinguish tryptophane peroxidase. This enzyme is wholly specific for tryptophane and catalyzes its conversion to formylkynurenine (20). The fact remains, however, that the wide-spectrum peroxidases, which are found throughout the plant and animal kingdoms in sizeable concentrations, are capable of catalyzing the oxidation of many diverse substances which share no characteristic chemical properties.

The differences in chemical structure of the various peroxidases have been revealed through molecular weight studies (23, 24), determination of sedimentation and diffusion constants (25); examination for copper content (26, 27), and more elegantly, by comparing their ultraviolet absorption spectra, as well as the visible spectra characterized by the Soret and neighboring bands (3, 21, 27-30).

As a result of these spectrophotometric studies, largely through the work of Chance (31-33) and George (34, 35), the mechanism of peroxidase enzyme-substrate compound formation, transition and decomposition has been elaborated. These steps are outlined in the following reaction sequence, the kinetic rate constants are given for

horseradish peroxidase and p-aminobenzoic acid (36). Units are liters per mole-second.



$$\text{I.}\quad \underset{\text{Peroxidase}}{Fe^{+++}(H_2O) + H_2O_2} \underset{k_3 < 3}{\overset{k_1 = 9 \times 10^6}{\rightleftharpoons}} \underset{\text{Complex I}}{Fe^{+++}(H_2O_2) + H_2O}$$

$$\text{II.}\quad \underset{\text{Complex I}}{Fe^{+++}(H_2O_2) + AH_2} \xrightarrow{k_7 = 5 \times 10^4} \underset{\text{Complex II}}{Fe^{+++}(OH\cdot) + AH\cdot + H_2O}$$

$$\text{III.}\quad \underset{\text{Complex II}}{Fe^{+++}(OH\cdot) + AH\cdot + AH_2} \xrightarrow{k_4 = 2 \times 10^3} \underset{\text{Peroxidase}}{Fe^{+++}(H_2O) + A + AH_2}$$

The question has been raised concerning the location of the half-oxidized donor molecule, AH•, of equation II.; does it remain attached to the enzyme until reaction occurs with the second donor molecule as in III., or does it become liberated into solution?

It is recognized from the equations above that oxidation occurs in two distinct steps: Complex I loses one oxidation equivalent in reaction II, undergoing conversion to Complex II, whereupon Complex II loses its remaining oxidation equivalent in reaction III, as peroxidase is regenerated.

B. Action of Peroxidase on Amino Acids, Proteins, and Relevant Compounds.

Horseradish peroxidase, the prototype of the wide-spectrum peroxidases, is known to catalyze the oxidation of the following compounds or classes of compounds: monoamines, diamines, phenols, diphenols, aromatic acids, leuco dyes, flavones, adrenaline, ferro-cytochrome c, ascorbic acid, bilirubin, iodides, nitrites, and others (21). Furthermore, horseradish peroxidase and myeloperoxidase possess the remarkable capacity of oxidizing dihydroxymaleic acid in the absence of H_2O_2, utilizing molecular oxygen directly.

The action of peroxidase upon amino acids and proteins has been reviewed recently by Sizer (37). Tyrosine, tryptophane and cystine are oxidized to unknown end-products. Related compounds such as dopa, tyramine and diiodotyrosine also serve as substrates. Adrenaline is oxidized to red adrenochrome and thence to a black melanin which precipitates from solution. Peroxidase oxidizes substances such as hydroquinone and uric acid which behave as cofactors, or intermediate carriers of oxidation, and shuttle back and forth between enzyme and final substrate, effecting a coupled system.

The mode of action of peroxidase upon tyrosine was thought to be similar to the tyrosinase catalyzed reaction (37), i.e., through a hallochrome intermediate to an eventual melanin pigment. An orange color does form initially in the peroxidase-tyrosine reaction, but later changes to brown.

The identification of the reaction product, or products, formed in the peroxidase reaction constituted the point of departure of the present work; if the products were similar to those formed by tyrosinase, chemical proof has yet to be obtained. The importance of determining their structures resides in the fact that peroxidase is capable of inactivating certain plant and animal proteins, thought to be chiefly the result of attack upon the protein tyrosine residues.

The following proteins are inactivated by peroxidase with either complete or partial loss of their biological activities: tetanus toxin (38), crude diphtheria toxin (39), hypertensin and pepsitensin (40), Rh and A, B antibodies (41), β-amylase (42) and invertase (43). In the presence of uric acid, purified diphtheria toxin is completely inactivated (39), while hypertensin and pepsitensin, in the presence of KI_3, suffer extensive inactivation (40). Not all proteins are susceptible to peroxidase

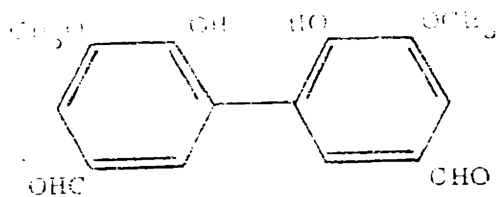
action; for instance, pepsin, trypsin and chymotrypsin are not inactivated (37).

Fibrinogen, casein and β-globulin are oxidized by peroxidase, as indicated by peroxide utilization, ultraviolet absorption changes, and disappearance of a fraction of the tyrosyl groups present (44). Further implication of tyrosine as the substrate in these protein effects is contributed by Wagley et al. (41) and Sizer (37), who have shown that similar ultraviolet absorption changes take place in proteins, as in tyrosine alone, when oxidized by peroxidase. Furthermore, tests for changes in tryptophane and cystine content have proved negative. But there is wanting final chemical evidence to signify whether or not tyrosine is involved in peroxidase-protein inactivation, and if so, the nature of the chemical involvement.

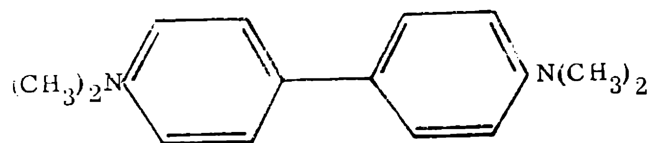
The compounds to be described in this section are products of peroxidase action bearing configurations similar to the reaction products identified in this study (see Fig. I; the Roman numerals in the following paragraph refer to this figure exclusively).

In 1904, Bourquelot and Marchadier (45) isolated divanillin (I) as a product of the reaction between peroxidase and vanillin. They were able to synthesize this compound by treating vanillin with $FeCl_2$. In part V of a series of papers by Saunders and co-authors on the isolation and characterization of products of aromatic amines with peroxidase (see review: 46), Naylor and Saunders showed that dimethylaniline becomes oxidized initially to tetramethylbenzidine (II) before further oxidation to blue quinonoid products (47). These authors discuss free radical mechanisms for the reactions. In 1942, Westerfeld and Lowe (48) showed that peroxidase yielded the same reaction products (III-V) in its action upon p-cresol as Pummerer et al. (49) had obtained with

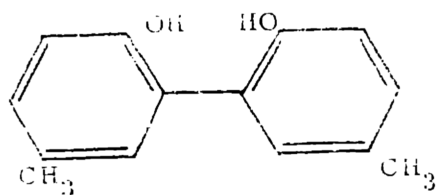
Figure I - PEROXIDASE OXIDATION PRODUCTS



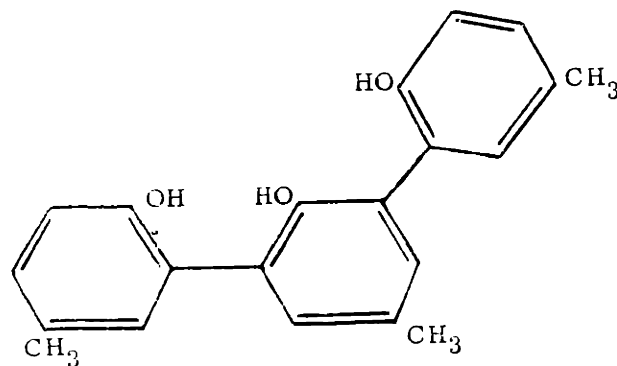
I



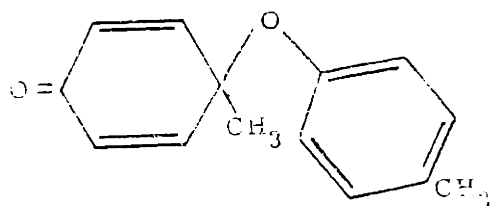
II



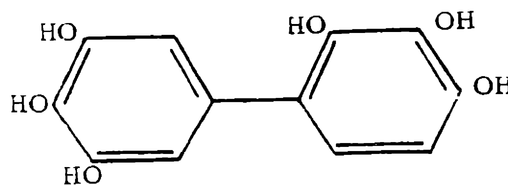
III



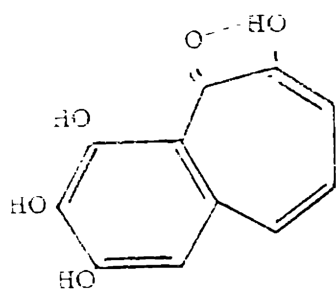
IV



V



VI



VII

potassium ferricyanide. The presence of tri-p-cresol (IV) was not proven rigorously, however. Finally, it is interesting to note that Willstatter and Heiss (50) postulate a diphenyl intermediate (VI) in the peroxidase oxidation of pyrogallol to purpurogallin (VII). Haworth et al. (51) concur with this theory although they advocate modifications to take into account the newly proposed structure for purpurogallin (VII).

C. Statement of Problem. Selection of Tyramine

This investigation was undertaken in order to: a) establish a basis for the known action of peroxidase upon proteins; and b) to gain understanding of the physiological significance of peroxidases in plants and animals. Since there were signs that tyrosine was the physiological substrate involved in these systems, the decision was made to identify the chief products of the peroxidase-tyrosine reactions in vitro. It was observed early in the investigation that tyramine behaved similarly to tyrosine in the reaction as indicated by ultraviolet light absorption, chemical and analytical data. For two reasons, tyramine was selected as substrate rather than tyrosine: a), only the tyramine pigment was insoluble and easily separated from the reaction mixture; and b) synthesis of products of the tyramine reaction promised to be easier due to the presence of fewer functional groups.

Tyramine is found in nature in comparatively few organisms. It has been isolated from the salivary glands of cephalopods (e.g., the octopus), from broom plant pods (Sarothamnus scoparius) (53), and from the grain parasite Glaviceps purpurea, the agent in the grain borne disease known as ergot (54). Tyramine is synthesized as a result of bacterial decomposition of proteins; specifically, an amino acid decarboxylase is responsible. This enzyme has been purified from cultures of Streptococcus faecalis (55).

II. The Oxidation of Tyramine by Peroxidase

A. Materials

The horseradish peroxidase used in these experiments was a 1947 powder preparation made by Eli Lilly Company which was kept under refrigeration. The purpurogallin number was checked at the outset of this work, using the method of Sumner and Gjessing (56), and found to be 71.6 (mgms. of purpurogallin formed per mgm. enzyme preparation employed).

L(-) tyrosine was obtained from Eastman Kodak, recrystallized twice, yielding a final m.p. of 312°d. Tyramine was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, in the form of the monohydrochloride salt, m.p. 269°.

The hydrogen peroxide 30% stock solution was made by Merck and Co., Rahway, New Jersey. N-butanol (technical grade) was supplied by Howe and French, Inc., Boston, Mass. All other chemicals employed were reagent or analytical grade unless otherwise specified.

B. Experimental Procedures and Results

It had been found by Mann (57) that the optimum hydrogen peroxide concentration in reaction mixtures with small amounts of peroxidase was of the order of .001 molar. This figure was used as a basis for determining the optimum mixture of the tyramine reaction. The following mixture was adopted:

Tyramine HCl (or Tyrosine)	100 mgm. (5.8×10^{-4} mole)
Distilled Water	91 ml.
Hydrogen Peroxide	5 ml. 0.1% sol'n (1.5×10^{-4} mole
Peroxidase	4 mgms. in 4 ml. dist. water

The pH was brought to 9.2 with 6N NaOH. Since the substrate

itself acted as a weak buffer, no external source was necessary. It was also desirable to keep salt concentration low. However, the pH usually fell 0.5 unit during the course of the reaction. The reaction mixture was incubated at 37° for 8-12 hours in a water bath.

It will be noted that an excess of substrate is present. This was found necessary to prevent the substrate from being completely oxidized to pigment instead of accumulating at an intermediate stage. From now on, unless tyrosine is specified as the substrate, discussion will concern the peroxidase-tyramine reaction only.

In both reactions, the same color changes are noted, namely, the rapid formation of an orange color which gradually deepens to brown. A brown pigment precipitates out in the tyramine reaction if the pH is kept between 8.5 and 10.5.

Now that the optimum reaction mixture has been described, it is necessary to define what is meant by optimum here. The first product of the reaction to be noted is the brown pigment. However, upon spectroscopic examination of the solution, a new absorption band not characteristic of tyramine appears at 316 mμ. Chromatographic work showed later that this band was due to the accumulation of a new soluble product. The term optimum refers to the production of a maximum amount of this product.

The changes in ultraviolet light absorption, as the reaction progressed, were measured in a Beckman model DU spectrophotometer using a 1 cm. quartz cell. Fig. II shows the ultraviolet light absorption spectrum of tyramine at different pH's. Lemon (58) has studied the effect of pH upon phenols and states that the shift of λ_{max} to longer wave-lengths, and the increase in intensity in alkaline solution is due to the enhanced resonance of the phenoxide ion. For tyramine, λ_{max} (acid) = 275 mμ, with ϵ_{max} (acid) = 1490 liters/mol. cm.;

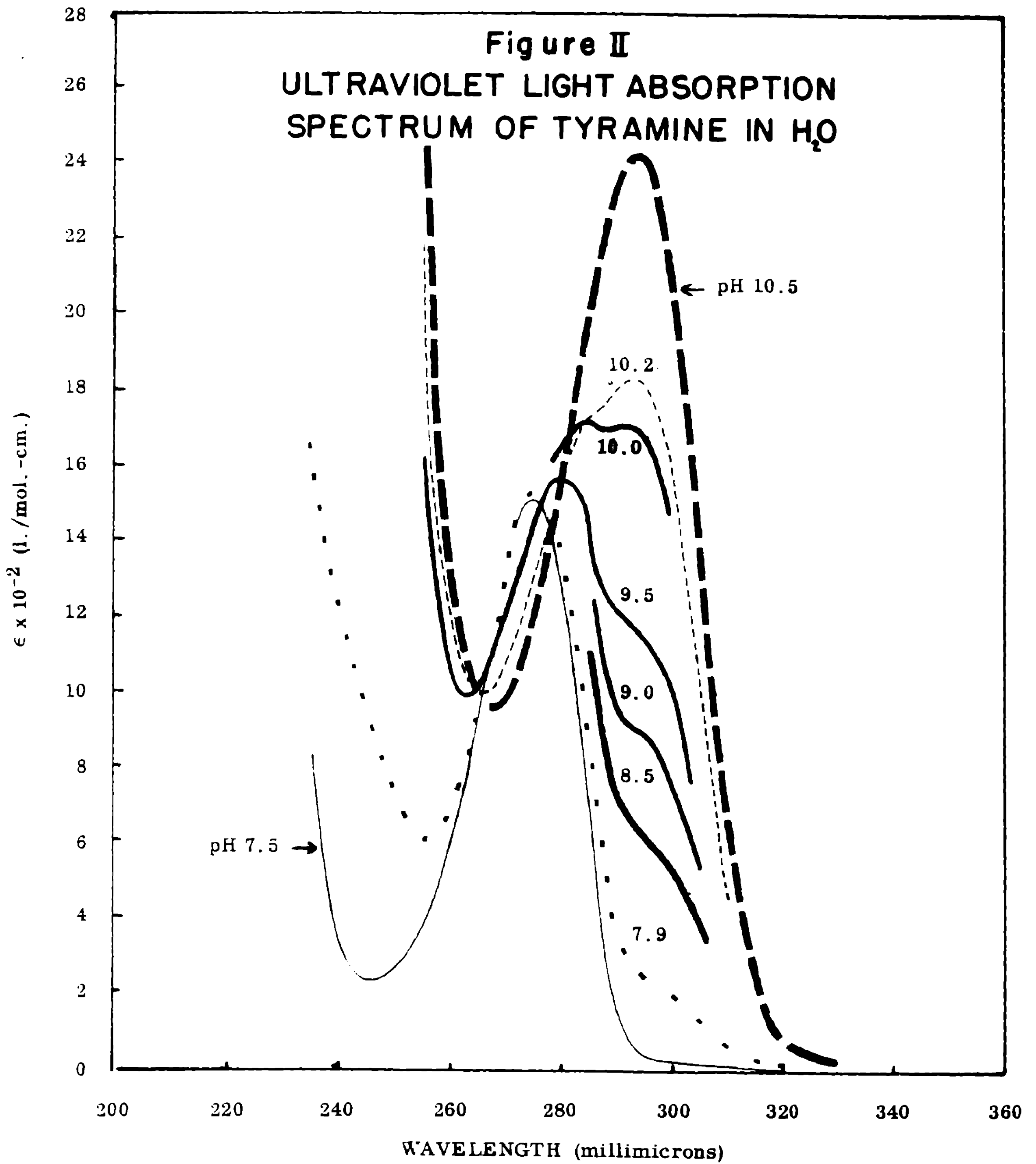


Figure II
ULTRAVIOLET LIGHT ABSORPTION
SPECTRUM OF TYRAMINE IN H2O
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
ϵ x 10-2 (l./mol.-cm.)
pH 10.5
10.2
10.0
9.5
9.0
8.5
7.9
pH 7.5
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

λ_{max} (basic) = 294 mμ, ϵ_{max} (basic) = 2480 liters/mol cm. The curves for tyrosine are practically identical. One may note that at intermediate pH's there is overlapping of acidic and basic species; at pH 7.5 all the tyramine is in the acidic form.

Aliquots of solution from the standard reaction mixture were examined under ultraviolet light. The mixture was incubated purposely at room temperature to slow down the reaction. Each aliquot was brought to pH 7.5 before being analyzed in order to assure freedom from tyramine phenoxide ion absorption. The results may be seen in Fig. III.

In Fig. IV are plotted the kinetics of the appearance of the 316 mμ peak. Little change was noted after 12 hours. The limiting factor in the reaction was the depletion of enzyme; further addition of H_2O_2 did not change the picture, but further addition of enzyme did result in increased production of 316 mμ compound. Upon acidification of the solution, the 316 mμ peak disappeared indicating a phenolic compound was probably responsible.

The next curve, Fig. V, shows the pH optimum of the reaction with respect to the appearance of the 316 mμ peak. It is at pH 8.6. It will be recalled that the reaction is usually started at pH 9.2. This assures precipitation of the brown pigment which appears later in the reaction. Also, as stated earlier, the pH falls 0.5 pH unit by the end of the reaction.

The same ultraviolet absorption changes that have been described for the tyramine reaction take place for tyrosine as well, particularly the appearance of a new absorption peak at 316 mμ.

It was desirable next to ascertain the ultraviolet absorption picture of the brown pigment. The precipitated tyramine pigment was too insoluble to examine, so recourse was made to tyrosine pigment. It

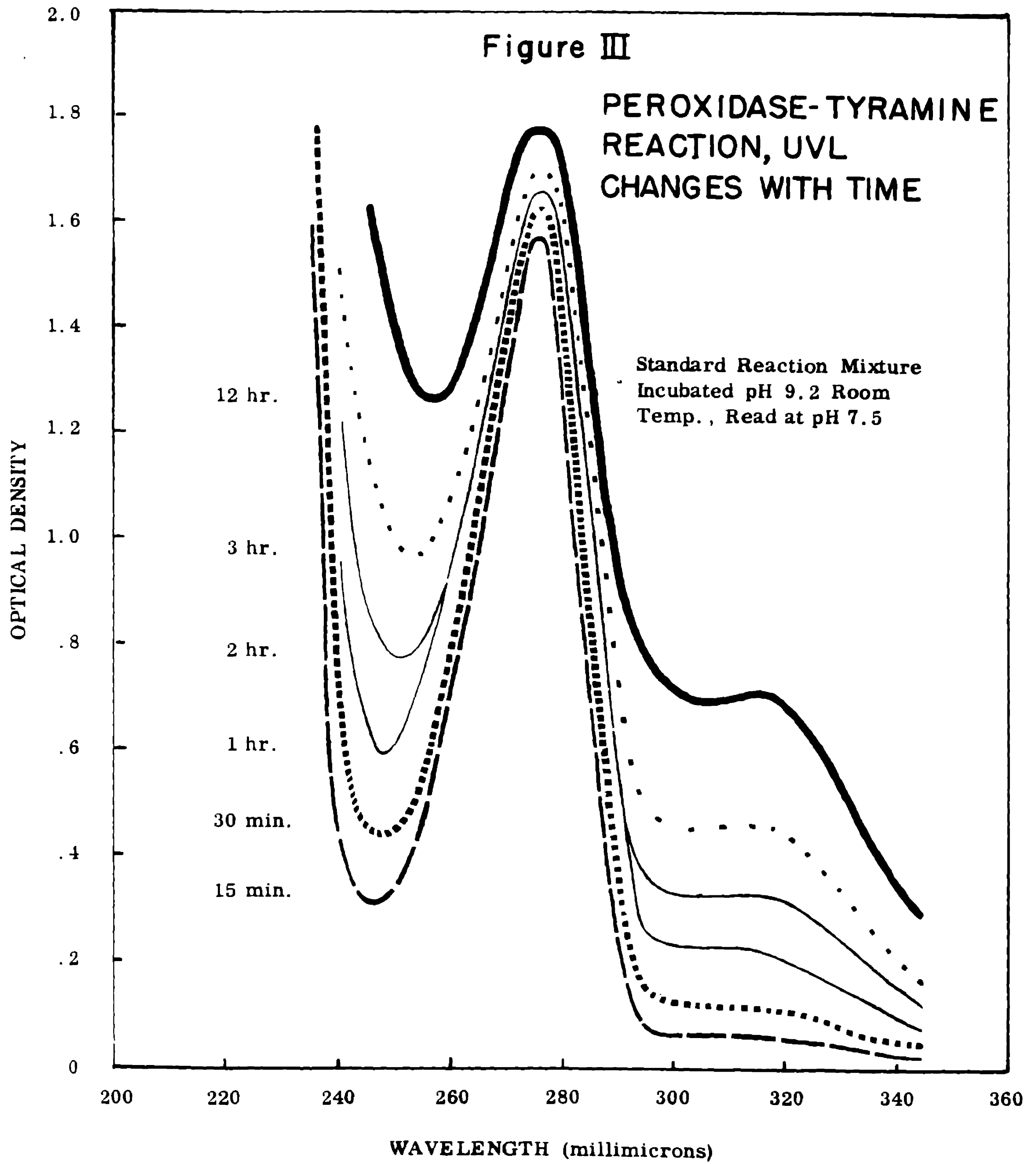


Figure III
PEROXIDASE-TYRAMINE REACTION, UVL CHANGES WITH TIME
Standard Reaction Mixture Incubated pH 9.2 Room Temp., Read at pH 7.5
12 hr.
3 hr.
2 hr.
1 hr.
30 min.
15 min.
OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

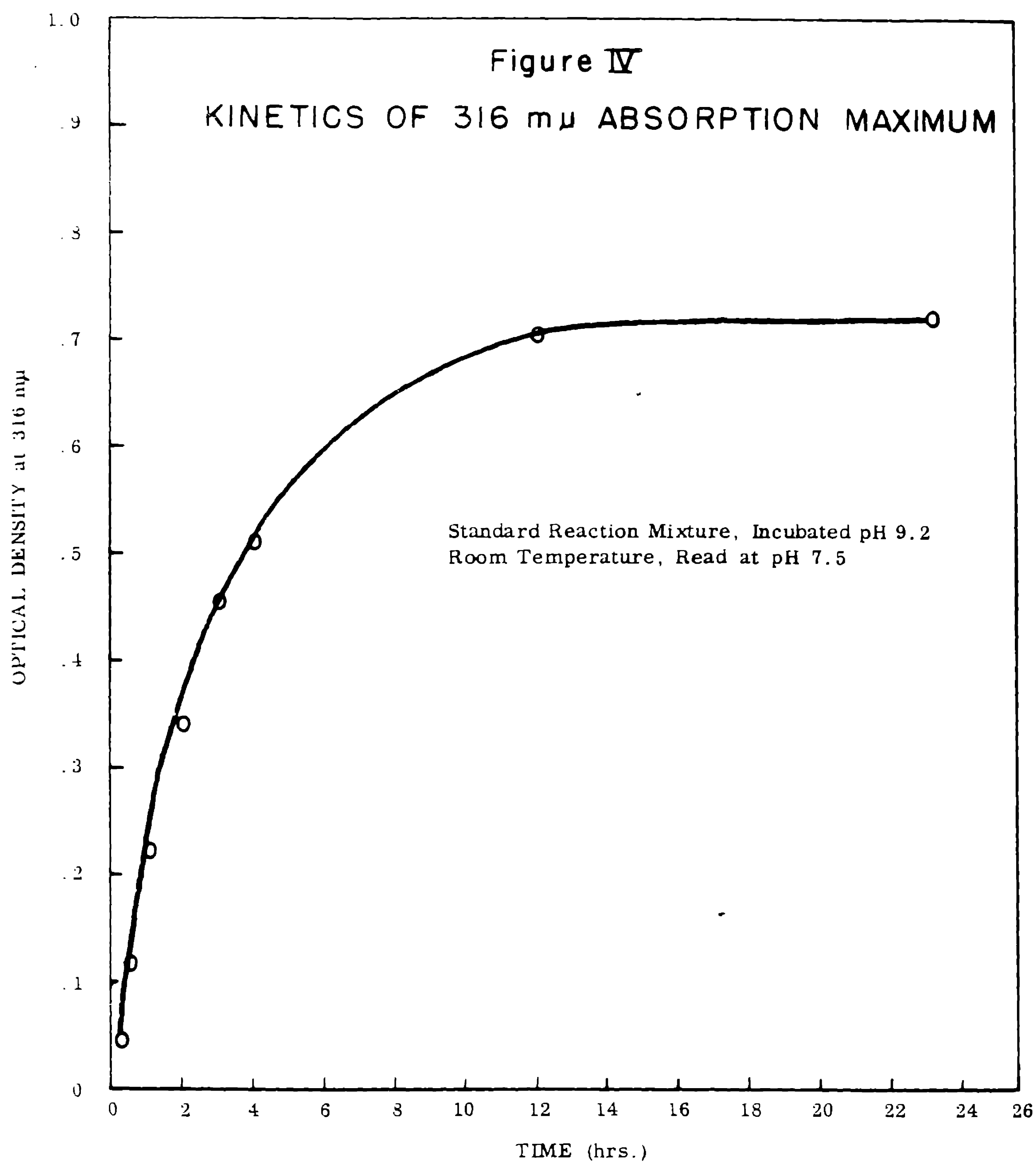


Figure IV
KINETICS OF 316 mμ ABSORPTION MAXIMUM
Standard Reaction Mixture, Incubated pH 9.2
Room Temperature, Read at pH 7.5
OPTICAL DENSITY at 316 mμ
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME (hrs.)

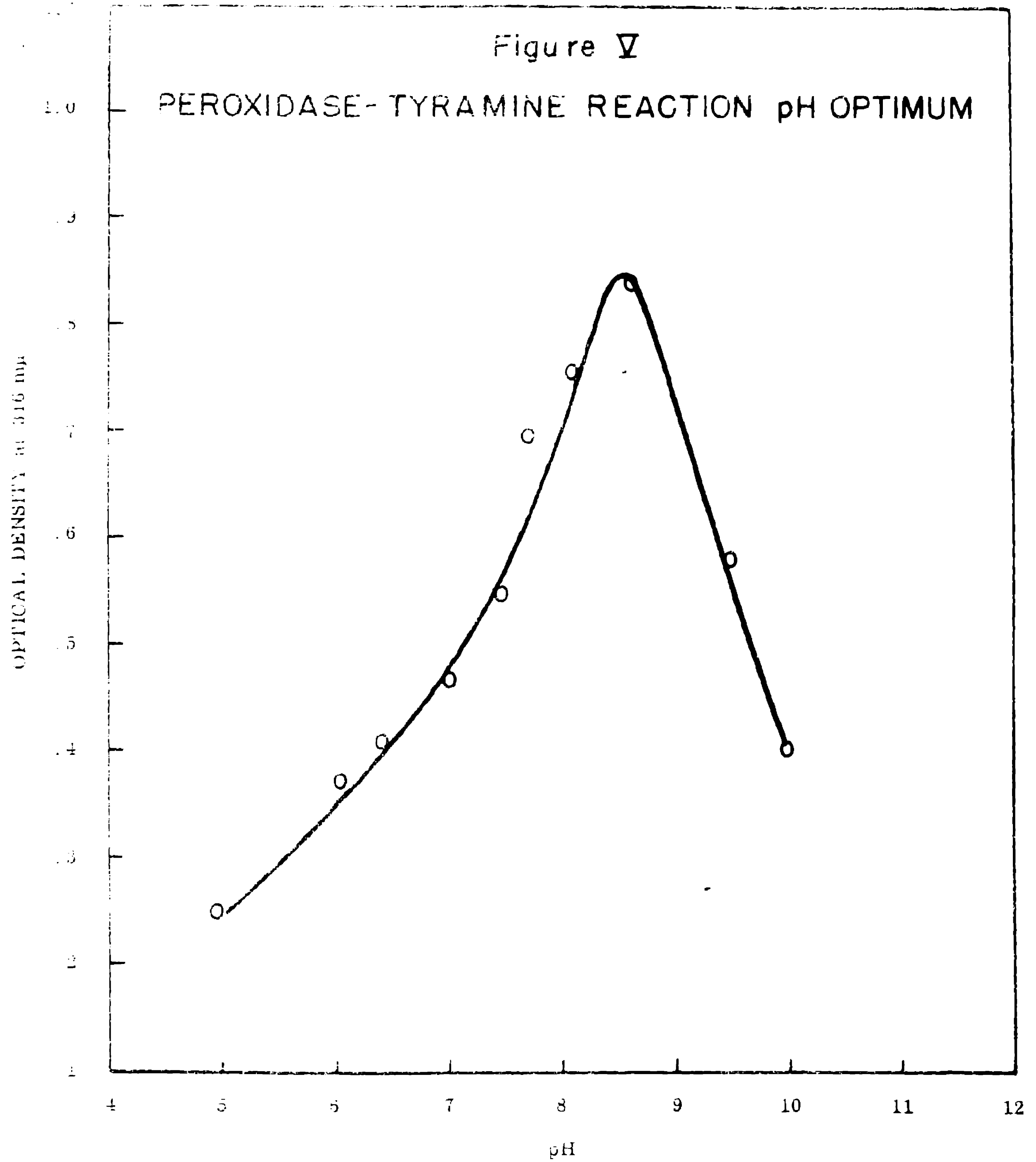


Figure V
PEROXIDASE-TYRAMINE REACTION pH OPTIMUM
OPTICAL DENSITY at 316 mμ
pH
4
5
6
7
8
9
10
11
12

was isolated by dialyzing the whole tyrosine reaction mixture for 5 days and examining the non-dialyzable sac contents. Fig. VI shows the spectrum so obtained. Some pH variability is present.

The effect of initial enzyme concentration was tested next using appearance of the 316 mμ peak as criterion. The curve thus plotted may be seen in Fig. VII. The reactions were stopped after five minutes by the addition of catalase (crystalline, beef, Worthington Biochemical Sales Co., Freehold, New Jersey). On the same diagram it may be seen that after 12 hours incubation, there were no differences in the amounts of 316 mμ compound produced. Evidently there is reached a steady state concentration of this compound, where, as fast as it is produced, it is removed by further oxidation. Later studies confirmed this assumption.

Paper chromatography was employed next to establish correspondence between the 316 mμ compound and a resolved chromatographic spot. Block's manual (59) was used as a guide. The paper employed was Whatman No. 1 and the solvents were as indicated in Fig. VIII.

A word must be mentioned about paper chromatography, especially with reference to R_F's reported. (R_F is the ratio the distance the spot has moved to the distance the solvent front has moved.) First, the movement of a compound is influenced to a great degree by the presence of other compounds; its R_F may be doubled if it is chromatographed in the presence of large concentrations of fast-moving substances. Secondly, salts may retard or accelerate the movement of a spot, but in any event produce smearing if the organic and salt spots run close to one another. (It is noted at this time that salts usually fluoresce under ultraviolet light.) Thirdly, descending chromatograms inevitably produce higher R_F's than are found on ascending chromatograms. Finally, the age of the solvent influences the R_F. Volatile substances are lost by

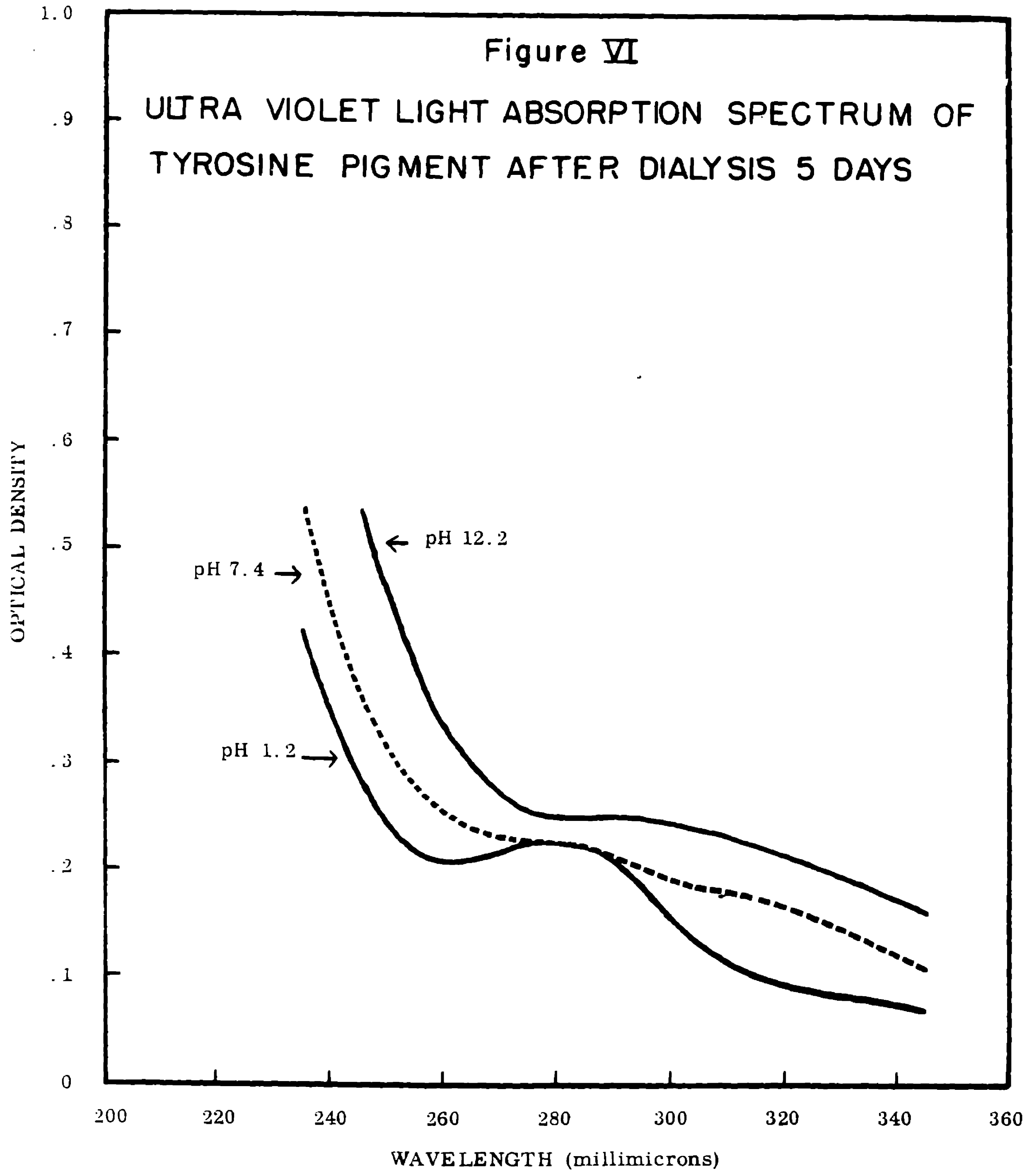


Figure VI
ULTRA VIOLET LIGHT ABSORPTION SPECTRUM OF TYROSINE PIGMENT AFTER DIALYSIS 5 DAYS
OPTICAL DENSITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
pH 12.2
pH 7.4
pH 1.2
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

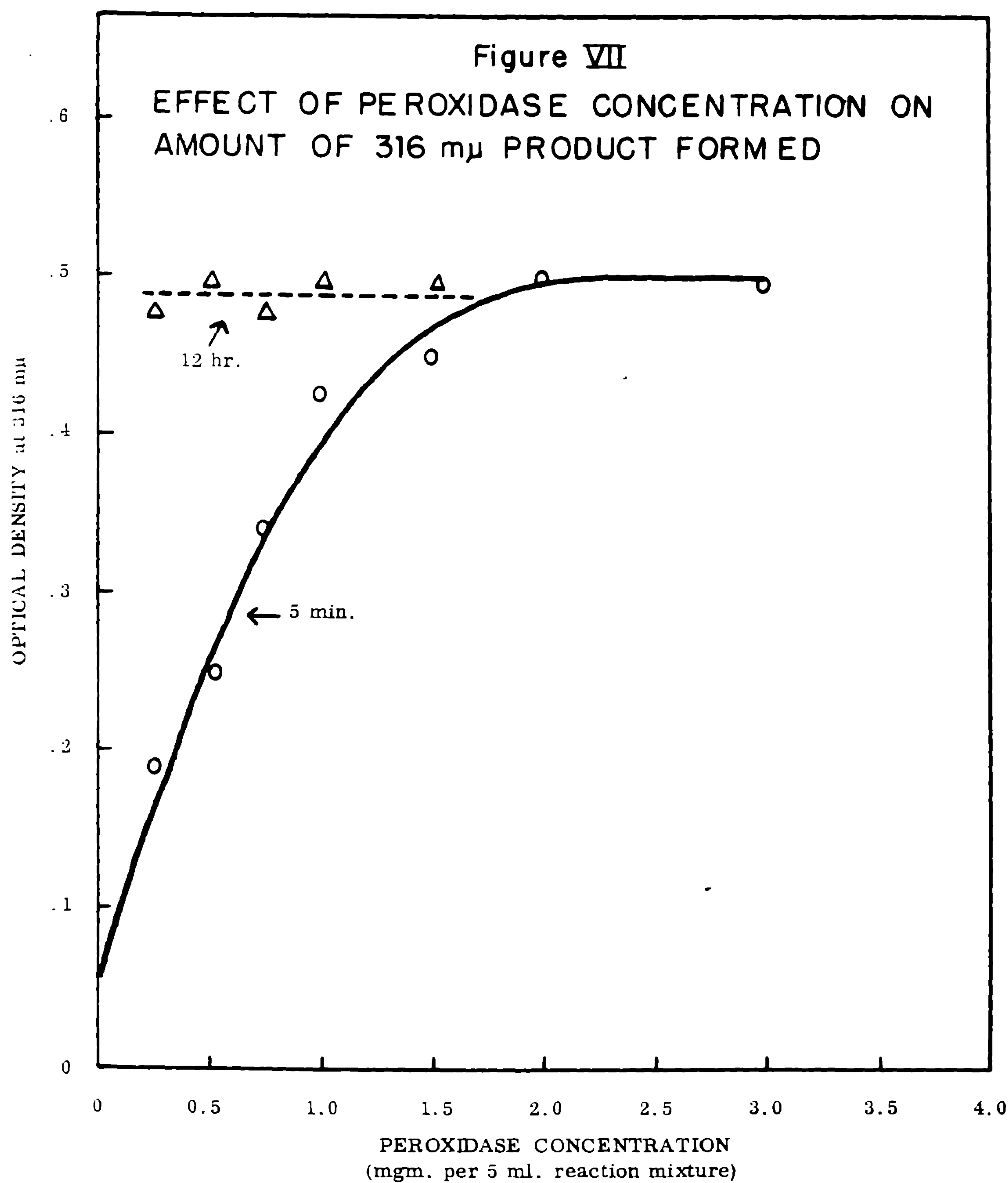


Figure VII
EFFECT OF PEROXIDASE CONCENTRATION ON AMOUNT OF 316 mμ PRODUCT FORMED
12 hr.
5 min.
OPTICAL DENSITY at 316 mμ
.6
.5
.4
.3
.2
.1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
PEROXIDASE CONCENTRATION
(mgm. per 5 ml. reaction mixture)

evaporation; and, proceeding on a time basis, ester formation takes place between the alcoholic and acidic substances commonly present.

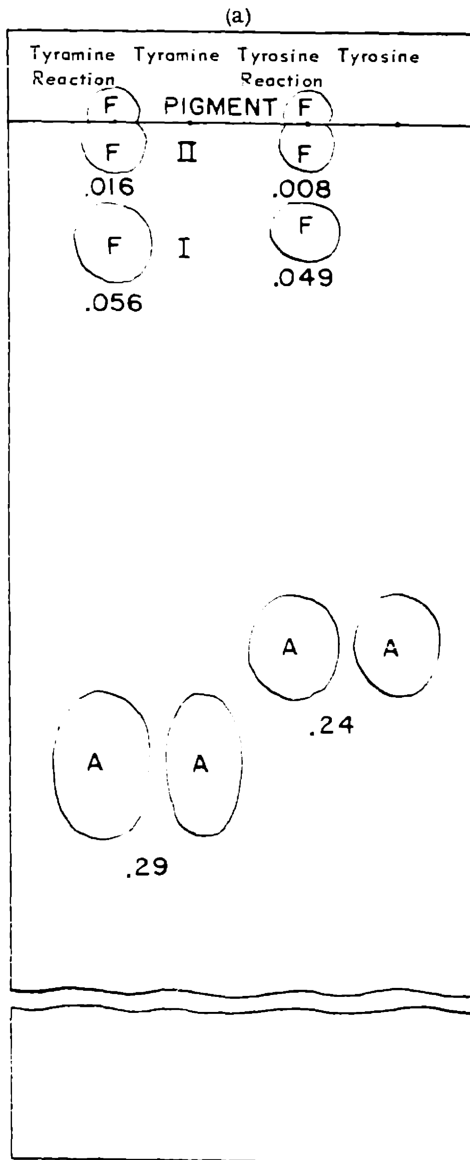
The reaction mixture is incubated for 8 - 12 hours. (The reaction has usually stopped after 3-4 hours; however, the residual H_2O_2 present protects against bacterial contamination.) It is then pipetted in sufficient quantity onto the origin of a paper chromatogram. It was found that evaporating the reaction mixture to dryness before reconstituting with a few drops of water did not alter the chromatographic picture.

Fig. VIII shows that the reaction mixture may be resolved into a number of ninhydrin positive spots. There are no additional spots detectable with the use of a bromine chamber or direct ultraviolet light. The R_F's are presented and indication is made as to whether or not the spot fluoresces or absorbs under ultraviolet light before spraying with ninhydrin.

The first essential feature of Fig. VIII to be noted is that tyrosine and tyramine behave almost identically. There is an excellent parallelism when their reaction product spots are compared. Secondly, all the new spots display a light-blue fluorescence under ultraviolet light. The fluorescence may be highly intensified by spraying with dilute NaOH solution. The spot at the origin, marked pigment, fluoresces with the same brilliance as the others. The pigment particles actually constitute the spot. Their chromatographic inertness is due in large measure to the large molecular size of the pigment. The third point may be made apparent by comparing chromatograms a) and b). The latter was developed with a more polar solvent. It is seen that another spot, III, has been eluted from the pigment spot. This indicates that the pigment is not homogeneous. Other spots appear revealing the presence of other compounds in much lower concentrations when the chromatogram

Figure VIII - PAPER CHROMATOGRAPHY OF PEROXIDASE-TYRAMINE AND PEROXIDASE - TYROSINE REACTIONS

(a)



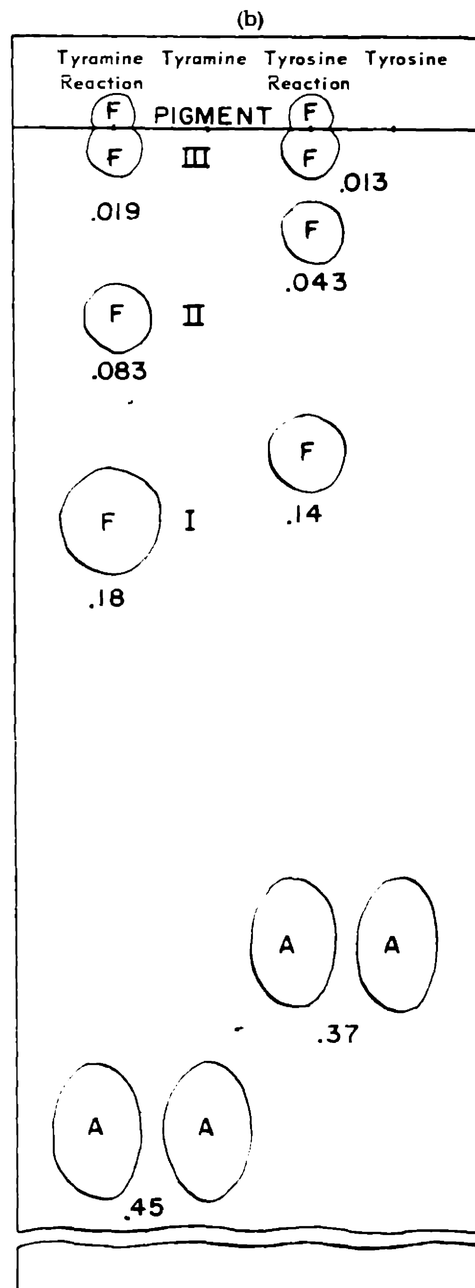
Solvent: 75 ml. n-butanol
10 ml. formic acid (88%)
15 ml. water

A absorbs } under UVL
F fluoresces }

Figures indicate R_Ps of spots

Both descending, ninhydrin developed.

(b)



Solvent: 55 ml. n-butanol
15 ml. glacial acetic acid
30 ml. water

is permitted to develop for a longer period of time.

The spot marked I was eluted with water for both the tyrosine and tyramine reactions and the ultraviolet absorption curves taken. Fig. IX gives the results so obtained. (Interfering substances in the paper were responsible for a small amount of base line absorption in control strips.)

The absorption spectra of the material from these spots from the chromatogram differ considerably from those of the reactants, tyrosine and tyramine (compare Figs. II and IX). There are, for example, absorption maxima at 285 mμ (acidic) and 318 mμ (basic). The absorption maxima shift to 318 mμ in basic solution is indicative of the presence of a phenolic compound and agrees with the basic absorption peak of 316 mμ found previously.

Compound II (see Fig. XX) was present in these incubation mixtures in concentrations too dilute to analyze accurately by this technique, but was later found to have an absorption spectrum very similar to that of Compound I.

Chromatography had thus made possible the isolation of compounds presumed to be intermediate products in the formation of pigments from tyramine and tyrosine by peroxidase action. This presumption was borne out later, after Compound I had been isolated in good yield, by showing that it could be oxidized further by peroxidase to Compound III and pigment. Compound II was not formed by peroxidase action on Compound I. The fact that the pigment contained I, II and III could be shown by hydrolysis and chromatography. I, II and III thus isolated by chromatography showed characteristic fluorescence. The pigment was very resistant to hydrolysis.

C. Summary and Discussion

Upon incubating both tyramine and tyrosine in the presence of

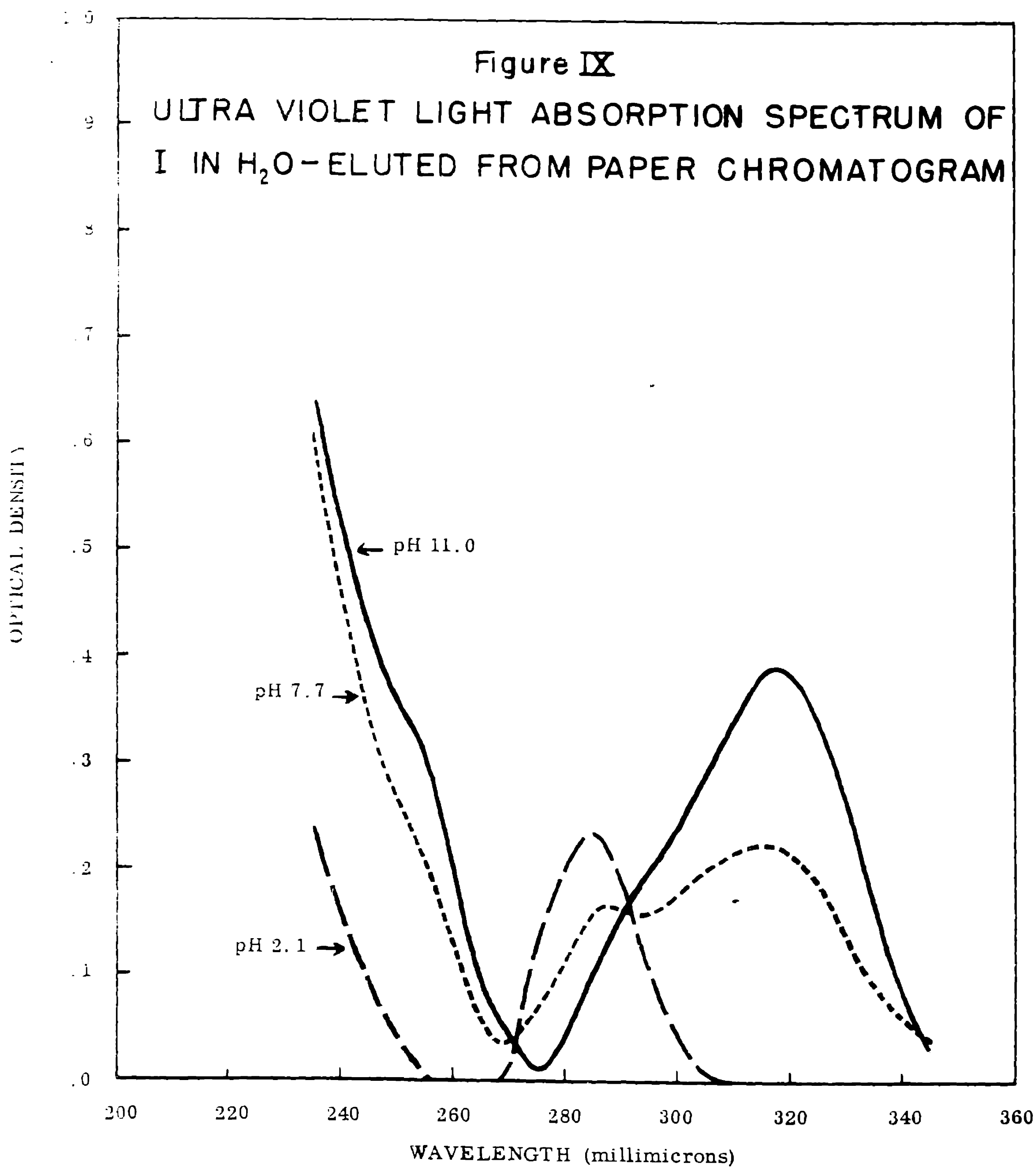
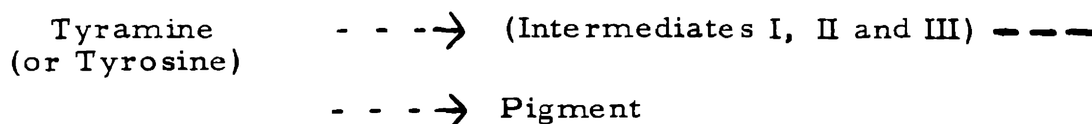


Figure IX
ULTRA VIOLET LIGHT ABSORPTION SPECTRUM OF I IN H_2O-ELUTED FROM PAPER CHROMATOGRAM
pH 11.0
pH 7.7
pH 2.1
.7
.6
.5
.4
.3
.2
.1
.0
OPTICAL DENSITY
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

H_2O_2 and peroxidase an orange-colored solution results which gradually deepens in color. In the case of tyramine, a brown insoluble precipitate is formed. A new compound with λ_{max} (basic) of 316 mμ appears. The pH optimum of the reaction with respect to the formation of this compound is 8.6. The addition of high concentrations of peroxidase to the reaction mixture does not increase the yield of I. This may be explained by the fact that I is also a substrate for peroxidase and is removed as fast as it is formed. Thus a steady state concentration is reached. Pigment may be hydrolyzed to Compounds I, II and III. We may now write the equation for the reaction:



Tyramine (or Tyrosine) - - -→ (Intermediates I, II and III) ——— - - -→ Pigment

I is formed in higher amounts than II or III. Its slower chromatographic mobility, relative to tyramine, indicates that it is larger or has more polar groups. Ultraviolet light absorption signifies that it is aromatic and the presence of both amine and phenolic functions is shown by a positive ninhydrin reaction and ultraviolet light absorption variability with changes in pH. It is not a ketone since spraying the paper chromatogram with 2,4-dinitrophenylhydrazine did not bring out a colored spot. The compound is more acidic than tyramine since it has a lower phenolic pK value. This value may be taken as the pH of solution where ultraviolet light absorption shows the presence of equal amounts of the acidic and basic species. Finally, it is more highly conjugated than tyramine since it fluoresces and λ_{max} is shifted to longer wave lengths.

It may be concluded finally that peroxidase affects tyramine and tyrosine chemically in a similar manner.

III. Isolation of Compound I

A. Materials

Finely ground Whatman No. 1 paper (standard grade cellulose powder, Fisher Scientific Co., New York) was employed as the inert material for packing the chromatographic columns. The solvent consisted of a homogeneous mixture of:

80 parts per volume technical n-butanol

10 parts per volume 88% formic acid

10 parts per volume distilled water .

Darco G-60 was used as the decolorizing charcoal (Atlas Powder Co., New York).

B. Experimental Procedures and Results

After unsuccessful attempts to separate I on the basis of solubility characteristics from other components of the reaction mixture, (largely unchanged tyramine), resort was made to partition column chromatography. It yielded excellent results but required considerable trial and error. No previously described procedure was found applicable. The following procedure was adopted:

1. Preparation of column

A 65 x 1 cm. (inside diameter) pyrex tube with a constriction at the neck was packed with a medium-thick slurry of Whatman paper suspended in solvent mixture after introducing a plug of glass wool into the bottom of the column. In order to assure evenness in packing of the column, application was made alternately of compressed air at the top of the column and suction from a water pump at the bottom. At no time was air permitted to enter the column. Finally, gentle pressure with a well-fitting glass rod tamped the column to the correct consistency

of packing. The final dimensions of the column were 41 cm. long and 1 cm. in diameter.

2. Preparation of material for column chromatography

A 1000 ml. reaction mixture was incubated 8 - 12 hours and the 200 mgms. of pigment formed were filtered off from a clear yellow solution. The fluid was brought to pH 6.0 with 6N HCl, divided into two portions, and evaporated down to a combined total of 150 - 200 ml. over a boiling water bath. The portions were recombined, filtered to remove any residue, and while still hot, treated with one gram of decolorizing charcoal. Next, the solution was filtered and refiltered through the same charcoal pad. The solution which became almost colorless was reevaporated down to dryness. The material was scraped off the evaporating dish, weighed, and combined with three times its weight of cellulose powder. These components were intimately mixed, wetted with a little distilled water, and redried for the last time over a boiling water bath. The material, finely ground in a mortar, could be stored indefinitely in a stoppered bottle.

3. Column operation. Assay of fractions

Two hundred milligrams of combined reaction powder were placed on top of a column of Whatman paper described above from which excess solvent had been decanted. The powder was shaken level by tapping and a plug of glass wool was placed on top. The solvent reservoir consisted of a cork-stoppered 250 ml, Erlenmeyer flask with a short piece of tubing inserted. The flask was filled with 150 ml. of solvent and inverted over the column. The column filled up to the level of the introduced tubing. It was thus a self-feeding, adjustable, gravity-operated column. When 150 ml. had passed through, the column was stoppered from the bottom and thus could be used repeatedly upon

reapplication of 200 mgms. of reaction powder. As the reaction powder, which had been exhaustively extracted, accumulated on the column to a significant extent, it was removed. No air was permitted to enter the column.

Usually four columns were run simultaneously in order to yield a sizeable quantity of material. One large column was not employed because the height-area relationship would have required an unwieldy 165 cm. column.

The fractions were collected in a time-governed fraction collector at the rate of 6 ml. every half-hour. Approximately 12 - 13 hours were required to complete a run. The fractions were examined in a spectrophotometer at 275 mμ. Solvent was used as the control. Fig. X shows the elution curve so obtained.

C. Summary and Discussion

It has been shown above that partition-column chromatographic separation of the components of the peroxidase-tyramine reaction is feasible. In the operation of the column, an attempt was made to duplicate the conditions used in the separation of the compounds on paper sheets. However, in contrast to paper, it was found necessary to decolorize the reaction mixture for use on the column. This operation removed higher soluble elements which interfered with the resolution of the separated compounds. It was also beneficial to homogenize intimately the dried reaction mixture with cellulose powder. This was a convenient and satisfactory way to introduce the reaction mixture onto the column since it could not be dissolved in the solvent.

In Fig. X the peak marked b reached a maximum intensity O.D. = 40 and c reached O.D. = 9. The ratio of respective areas of the two peaks was 2:1. The curve also shows the presence of peak e. The

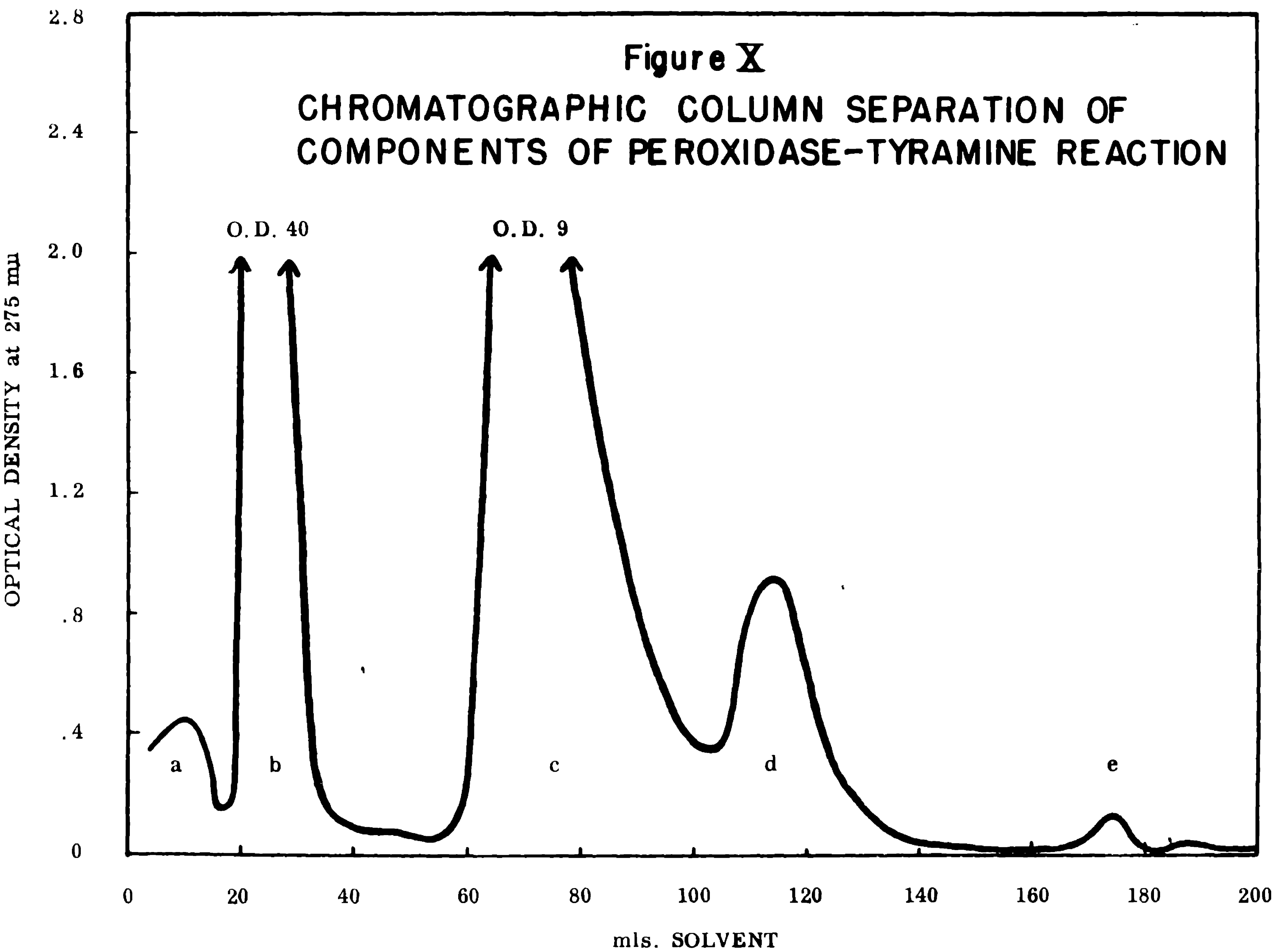


Figure X
CHROMATOGRAPHIC COLUMN SEPARATION OF COMPONENTS OF PEROXIDASE-TYRAMINE REACTION
O.D. 40
O.D. 9
a
b
c
d
e
OPTICAL DENSITY at 275 mμ
0
.4
.8
1.2
1.6
2.0
2.4
2.8
mls. SOLVENT
0
20
40
60
80
100
120
140
160
180
200

identity of each peak was established by paper chromatography; peak a was ninhydrin negative and neither absorbed nor fluoresced under ultra-violet light. It was considered to be inorganic salt (NaCl). Peak b migrated as tyramine, and peaks c, d and e, respectively, as Compounds I, II and III. It is probable that Compounds IV, V and any other components were removed by the charcoal adsorption step described above.

Compound I could thus be isolated by paper-column chromatographic technique. The yield was found to be about 50 mgm. per day from 200 mgm. of dried reaction mixture.

D. Addendum

In order to obtain I in a dried powder state, the following procedure was adopted.

To 100 ml. of solvent obtained from the column which contained Compound I were added 25 ml. distilled water and a few drops of 6N HCl. The mixture was shaken in a separatory funnel, the aqueous layer separated and the extraction repeated with another 25 ml. water. The organic layer was discarded.

To remove dissolved butanol, the aqueous extract was shaken twice with 25 ml. of di-ethyl ether. The extract was placed in a dessicator connected with a water suction pump, and the ether boiled off under reduced pressure. The extract now contained I, some formic acid and traces of HCl. It was lyophilized to a dry white powder which oxidized rapidly in air with the formation of a brown crust. The powder was extremely hygroscopic, Chromatography revealed it to be I of high purity.

IV. Characterization of Compound I

A. Properties of I

Although Compound I was obtained in a highly purified state by the procedure in Section IIID, it was brought to analytical purity by dissolving in absolute ethyl or methyl alcohol, filtering, and reprecipitating with absolute ethyl ether. The procedure was carried out a minimum of ten times to free I of contaminating salt. Charcoal decolorization and fractional precipitation were also employed. A white amorphous powder was obtained. I could not be crystallized with the use of ordinary laboratory solvents. Its melting point was ill-defined. Sintering appeared at 190° and melting with decomposition occurred over the range 210 - 235°. Sodium fusion qualitative analysis showed the presence of nitrogen and chlorine. Millon's test indicated a monophenolic grouping. Tests for amide, carbonyl and secondary amine groups were negative. The ninhydrin test for a primary amine was positive. Ultraviolet light absorption showed the compound to be aromatic. Resonance was indicated by fluorescence under ultraviolet light.

Fig. XI is a curve of the ultraviolet light absorption of pure Compound I at various pH's. There are two isosbestic points (as expected), one at 291 mμ and the other at 263 mμ. These are points where the extinction coefficient remains independent of pH. The pK of the phenol is approximately 7.3 (i.e., where it is half in the ionized and half in the unionized state). This compares with a value of approximately 10.0 for tyramine. For Compound I, λ_{max} (acid) = 284 mμ, * ϵ_{max} (acid) = 4610 liters per mole cm.; λ_{max} (basic) = 316 mμ, * ϵ_{max} (basic) = 5790 liters/mole cm. This represents an increase in extinction 2.5 times that

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* Calculated on the basis of molecular weight determined later.

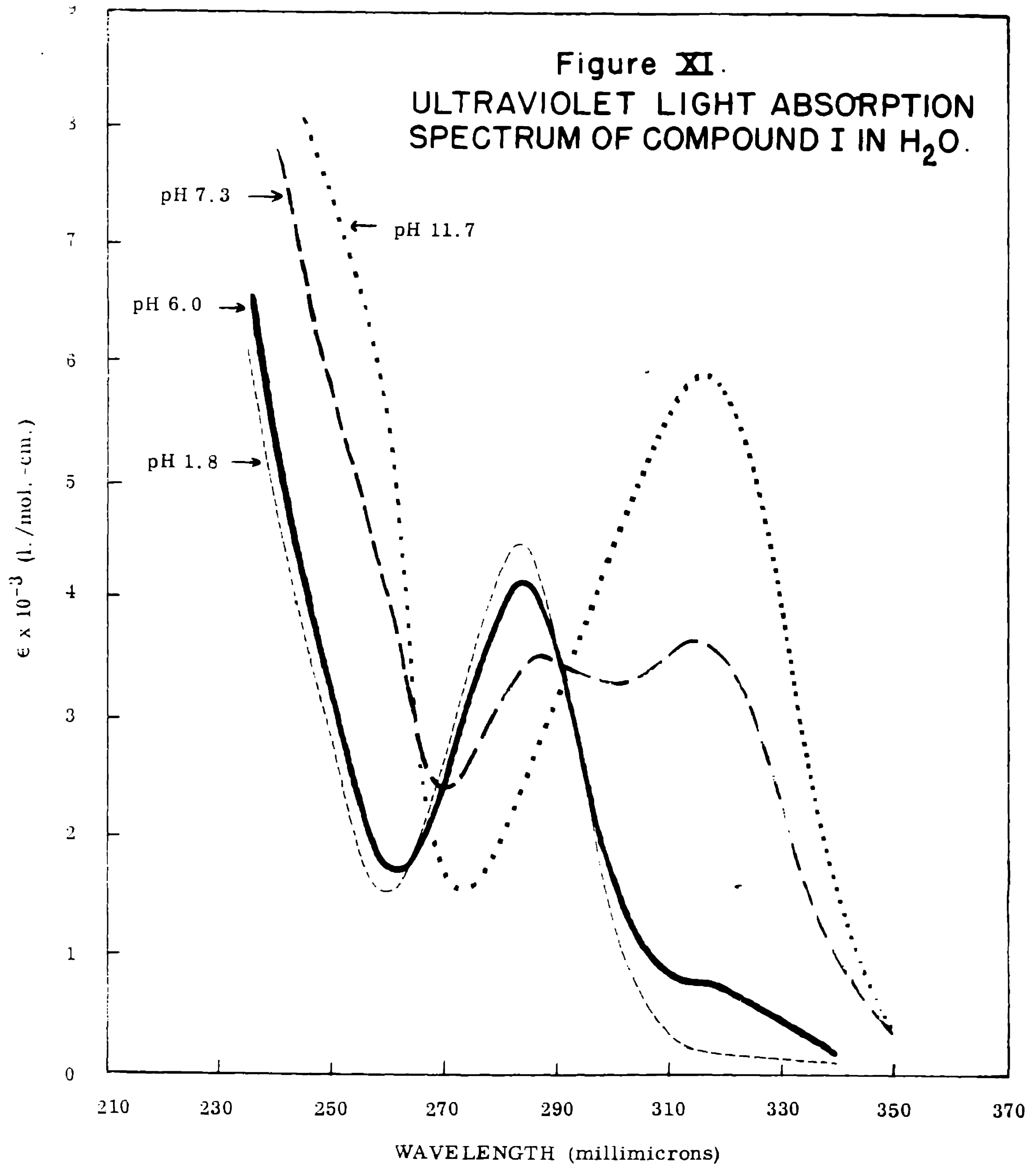


Figure XI.
ULTRAVIOLET LIGHT ABSORPTION SPECTRUM OF COMPOUND I IN H_2O.
pH 7.3
pH 11.7
pH 6.0
pH 1.8
$\epsilon \times 10^{-3}$ (l./mol.-cm.)
0
1
2
3
4
5
6
7
210
230
250
270
290
310
330
350
370
WAVELENGTH (millimicrons)

of tyramine.

Infra-red absorption spectra of tyramine and I are compared in Fig. XII. The KBr pellet technique was used. A calibration mark at 6.238 μ is indicated on each curve. There is a stronger OH-NH stretching band at 2.9 μ in I and also a stronger NH bending band at 6.1 μ. Both curves show the presence of C-H stretching at 3.3-3.4 as well as aromatic ring absorptions at 6.2 and 6.7 μ. Both show para ring-substitution at 12.0 - 12.2 μ. The tyramine spectrum is more sharply and distinctly defined than the curve for I.

On the basis of solubility, tyramine hydrochloride and I are very much alike. Both are extremely soluble in water, less soluble in methanol, and moderately soluble in ethanol. Both are insoluble in acetone, ethyl acetate, ether and chloroform.

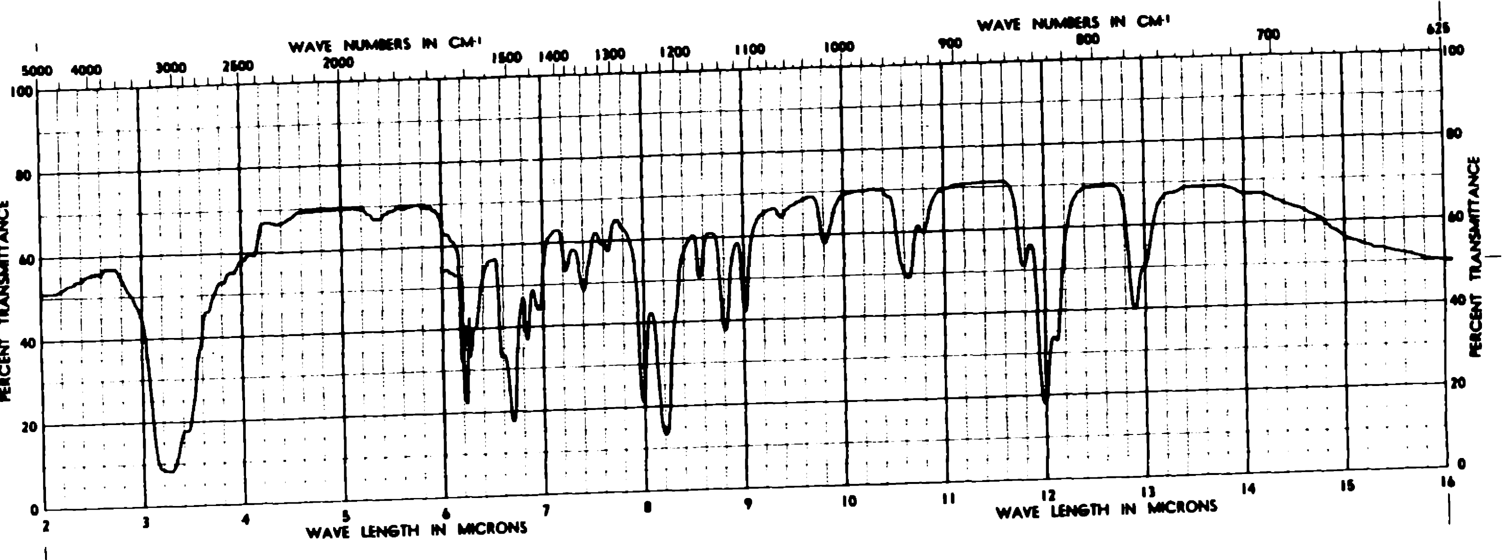
Compound I differs from tyramine in not being a substrate for tyrosinase. As previously stated, Compound I is extremely hygroscopic and unstable in air. It quickly oxidizes to a brown, vitreous pigment. In aqueous solution, I is stable in acid and neutral solutions even in the presence of heat. It is extremely difficult to hydrolyze to yield new unidentified chromatographic spots. In alkaline solution, I turns yellow, especially when heated.

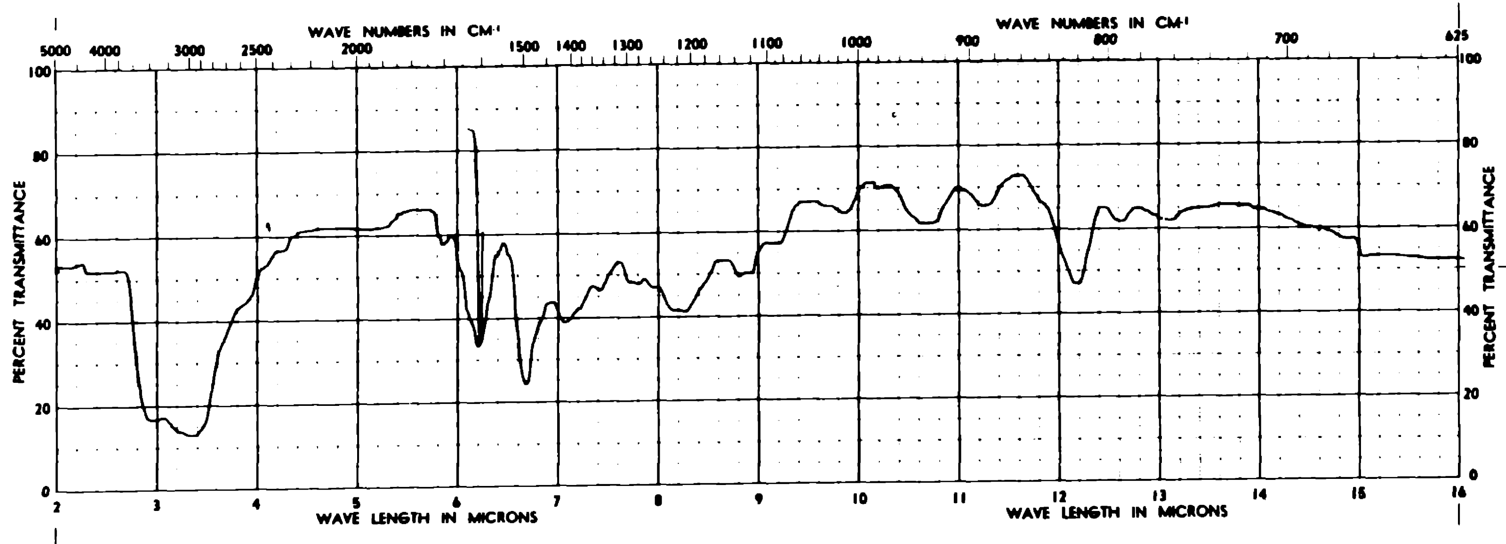
Compound I was sent to the Microchemical Laboratory, M.I.T. (Dr. S. M. Nagy, Director) for quantitative analysis. The figures obtained were:

C 55.42% H 6.69% N 7.85% Cl 20.26%
O (by difference) 9.78%

An empirical formula of $C_8H_{11}NOCl$ is required, but the correct one may be any multiple thereof. For $C_8H_{11}NOCl$ is calculated:

C 55.66% H 6.42% N 8.11% Cl 20.54% O 9.27%


I. R. SPECTROPHOTOMETER
NaCl PRISM
NO.
DATE
INDEX
SAMPLE
Fig. XIIa
FROM
SAMP. CELL: MM CMS.
REF. CELL MM CMS.
CHEM. MG. MG.
SOLV.
VOL. C.C. % C.C. %
F.S. SOLID C. GAS MM
BAIRD ASSOCIATES, INC.
CAMBRIDGE, MASS., U.S.A.
1029.9
WAVE NUMBERS IN CM⁻¹
WAVE LENGTH IN MICRONS
PERCENT TRANSMITTANCE


I. R. SPECTROPHOTOMETER
NaCl PRISM
NO. 7335
DATE
INDEX
SAMPLE
Fig. XIIb
COMPOUND I
FROM
SAMP. CELL: MM CMS.
REF. CELL MM CMS.
CHEM. MG. MG.
SOLV.
VOL. C.C. 1 % C.C. %
F.S. SOLID C. GAS MM
BAIRD ASSOCIATES, INC.
CAMBRIDGE, MASS., U.S.A.
1029.9
WAVE NUMBERS IN CM⁻¹
WAVE LENGTH IN MICRONS
PERCENT TRANSMITTANCE

These figures check well with the ones found. In order to determine the approximate molecular weight, so that the true empirical formula could be ascertained, it was necessary to make a camphor-soluble derivative for use by the Rast method (60). This derivative would also yield information about the molecular structure of Compound I.

B. Formation of Benzoyl Derivative of Compound I and a Study of its Properties

The Schotten-Baumann reaction (60, p. 88) was used to prepare this derivative. To a 125 ml. Erlenmeyer flask containing 50 ml. ice cold aqueous extract of I were added 21 ml. of 2M NaOH, then 0.5 ml. of benzoyl chloride. It was shaken vigorously for one minute, then at intervals for ten minutes. The flask was warmed under tap water and shaken gently until the odor of benzoyl chloride disappeared. The crude, sticky benzoyl derivative was filtered off and washed with water. It was dried at room temperature with a yield of about 100 mgms. of product.

Many attempts were made to purify and crystallize this compound by chemical means alone but not until chromatography on alumina was employed could this be attained.

A 65 x 1 cm. pyrex column was packed with 45 gms. neutral Al_2O_3 to a height of 45.5 cm. One hundred mgms. of crude benzoyl derivative of Compound I were dissolved in 4 ml. hot ethyl acetate and poured on the column. It was washed with a few ml. of fresh ethyl acetate, then eluted with the solvent. The results are presented in Fig. XIII. The inset shows the method of assaying the eluant. One drop was collected every 5 ml. on a sheet of Whatman No. 1 paper, dried, and examined under ultraviolet light for fluorescence and absorbency. Fluorescent material was eluted in early fractions followed by the appearance of an absorbent compound in the fractions between 50 and 110 ml. Fig. XIVa gives the ultraviolet absorption spectrum of the benzoyl derivative.

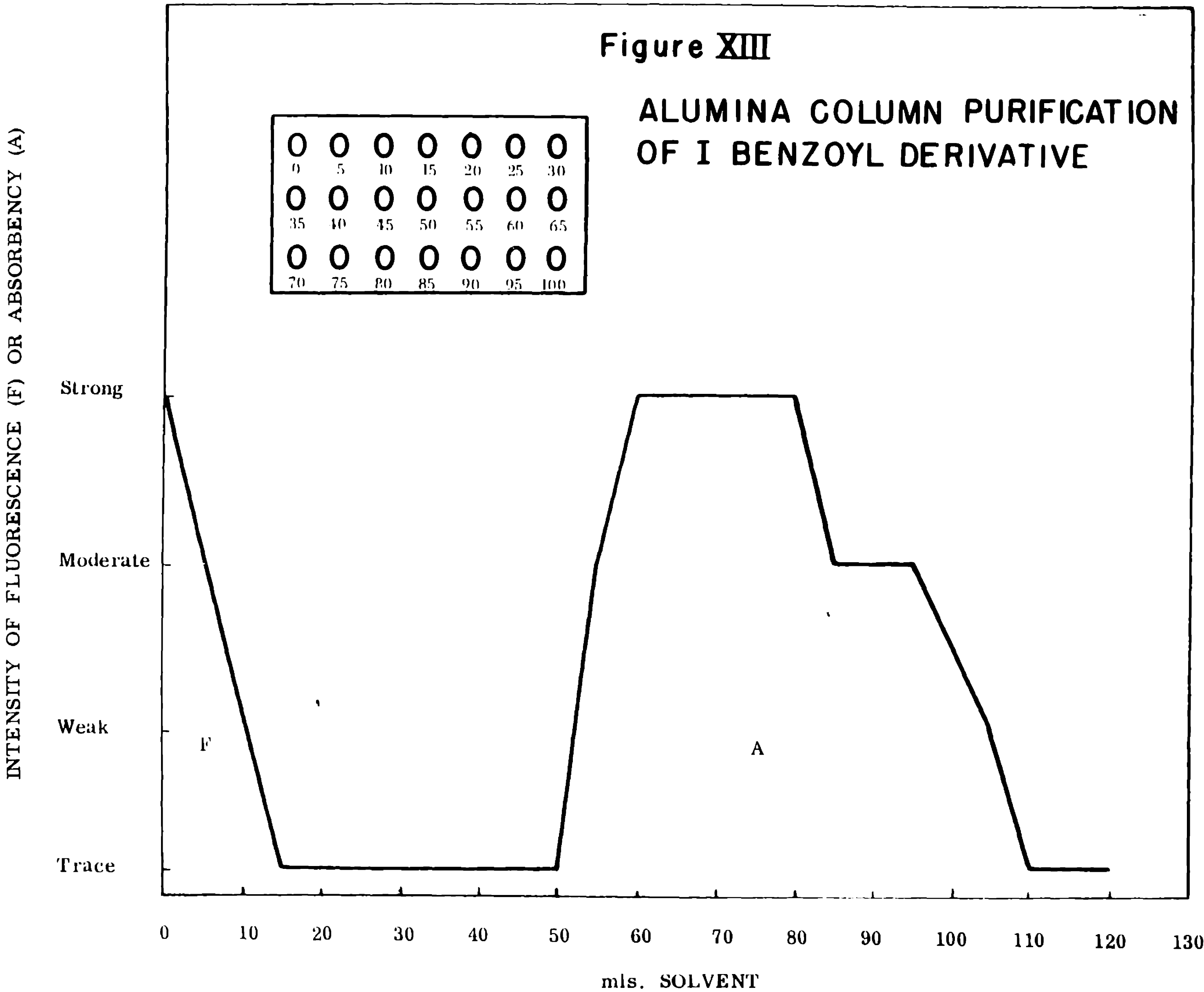
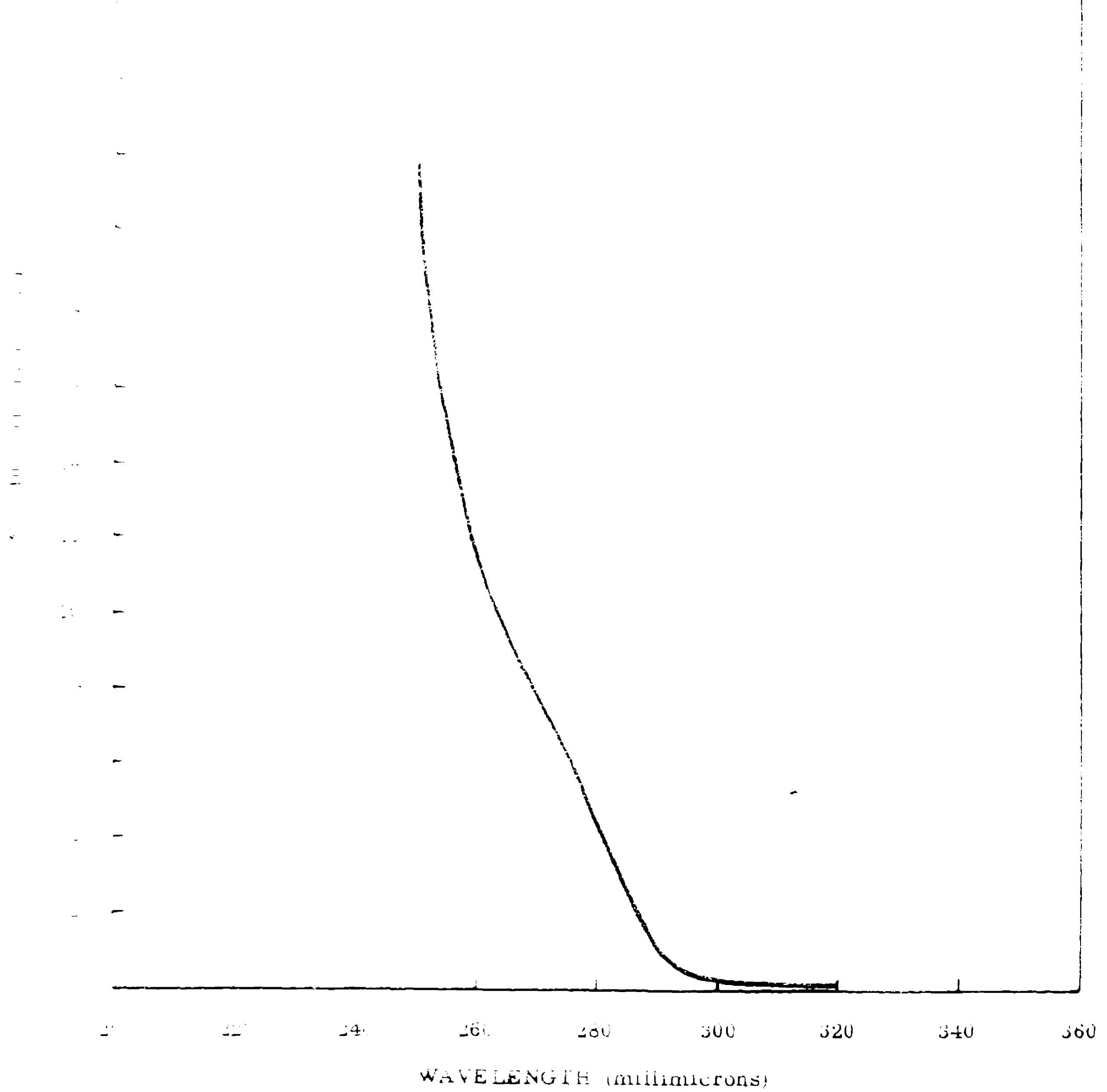


Figure XIII
ALUMINA COLUMN PURIFICATION
OF I BENZOYL DERIVATIVE
0 5 10 15 20 25 30
35 40 45 50 55 60 65
70 75 80 85 90 95 100
INTENSITY OF FLUORESCENCE (F) OR ABSORBENCY (A)
Strong
Moderate
Weak
Trace
F
A
0 10 20 30 40 50 60 70 80 90 100 110 120 130
mls. SOLVENT

Figure XIV a.
ULTRAVIOLET LIGHT ABSORPTION SPECTRUM
OF I BENZOYL DERIVATIVE IN 95% ETHANOL



of eluant. This latter material represented the bulk of the chromatographed material. These fractions were combined and taken to dryness over a steam bath. The compound was easily crystallized from 75% ethanol into long white, translucent needles which melted sharply at 196.5° (corr.) and did not alter upon further recrystallization. This compound was insoluble in water, moderately soluble in methanol and ethanol, and easily soluble in acetone, ethyl acetate, chloroform and ether. It was also soluble in camphor.

The analytical figures for this compound were:

C 76.77% H 5.46% N 4.03% O (by difference) 13.74%

requiring a minimum empirical formula of $C_{22}H_{18}NO_3$ which upon calculation yields:

C 76.73% H 5.27% N 4.07% O 13.9%

The agreement is excellent. Molecular weight determinations by the Rast method gave an average figure of 785. There is an allowable error of 20% in this method.

$C_{22}H_{13}NO_3$	requires	M.W. 344
$C_{44}H_{36}N_2O_6$	requires	M.W. 689
$C_{66}H_{54}N_3O_9$	requires	M.W. 1033

$C_{44}H_{36}N_2O_6$ is evidently the molecular formula. We are able now to postulate the structural formula of Compound I by combining these results with the preceding data.

Assuming no nitrogen is lost in the formation of the compound, the presence of two tyramine residues is required. This accounts for sixteen carbon atoms and two oxygen atoms (see Table I). To bring it up to the total of six oxygen atoms present in the benzoyl derivative of Compound I, 4 benzoyl radicals are needed. This provides an additional 28 carbons and 20 hydrogens. It adds up to the correct total of $C_{44}H_{30}N_2O_6$

for the benzoyl derivative of Compound I (after subtracting 4 hydrogens lost in the reaction). In the Schotten-Baumann reaction, benzoylation takes place only for amines and phenols.

$$RNH_2 + C_6H_5COCl \dashrightarrow RNHCOC_6H_5 + HCl$$

$$ROH + C_6H_5COCl \dashrightarrow ROOCC_6H_5 + HCl$$

Table I

Empirical Formula of I Benzoyl Derivative

Two Tyramine Residues	Four Benzoyl Radicals	Total
C 16	C 28	C 44
H 20 (-4)	H 20	H 36
N 2	- -	N 2
O 2	O 4	O 6

The assumption that I was formed by the combination of two tyramine residues, allied with the fact that benzoylation yielded the tetra-substituted compound, indicated that phenol and amine sites were still present in I. This, with the negative test for a secondary amine, led to the belief that I was a diphenyl compound. In order to prove the assumption and arrive finally at the structure, it was necessary to degrade I, or some derivative, to a structure known and described in the literature. For this reason, it was mandatory to methylate the phenolic groups on I, so that I could be oxidized to a simpler known structure without destroying the aromatic groups present.

C. Formation of Methyl Sulphate Derivative of Compound I and a Study of Its Properties

To 50 ml. aqueous extract of I cooled to 0°, 6 gm. NaOH in 10 ml. water were added. Bubbling nitrogen gas into the extract beforehand prevented air oxidation of I. The solution was stirred magnetically

for 8-10 hours at room temperature while 12 ml. dimethyl sulphate were added dropwise. At the end of this time, ultraviolet absorption studies showed that there were no pH-variable phenolic groups left. The methylated derivative was separated from the solution by means of charcoal adsorption and alcohol elution. 650 mgm. Darco G-60 charcoal were added to the neutral or weakly acidic methylated solution of I and gently shaken and then allowed to stand 10 minutes. The charcoal, which adsorbed the methyl sulphate derivative of Compound I, was centrifuged down and transferred to a test tube where the rest of the operation was carried out. It was washed carefully three times with 10 ml. portions of distilled water. Overly vigorous agitation of the charcoal led to a colloidal suspension. The charcoal was centrifuged down between washings and the washings checked at 285 mμ to see whether any derivative had been eluted.

Absolute ethyl alcohol was found to be suitable for eluting the adsorbed material. Four 10 ml. aliquots were necessary as adjudged by spectrophotometric assay. The alcoholic solution was evaporated down to a few mls. on a hot plate and then to dryness over a steam bath. The residual material was redissolved in a small amount of absolute ethanol and precipitated by the addition of absolute ether. The precipitate was centrifuged down, taken up in hot absolute alcohol and filtered. Upon cooling, hygroscopic, translucent, cigar-shaped crystals formed. They were very fragile, birefringent, and melted sharply at 225° (corr.) The crystals were very soluble in water, less soluble in methanol and ethanol, and insoluble in ethyl acetate, acetone, ether and chloroform. They fluoresced under ultraviolet light. In an 80-10-10 (n-butanol, formic acid (88%), water) descending chromatogram, an R_F of 0.15 was obtained. Fig. XIV reveals the essential similarity of the ultraviolet

absorption curve of this compound with unionized Compound I (Fig. XI). λ_{max} is located at 283 mμ and has an ϵ of 6070 liters/mole cm. considerably higher than that of Compound I (ϵ_{max} (acid) of 4610). No ultraviolet absorption variability is shown with change in pH.

Fig. XVb presents a complicated spectral pattern, but there is no carbonyl band between 5-6 μ and the OH and NH stretching bands are reduced. (The KBr pellet used to suspend the specimen is hygroscopic and shows some intrinsic OH absorption.) The very strong band at 8.0 - 8.2 μ is probably due to absorption by methyl sulfate ion.

Chemical analysis of the methyl sulphate derivative of Compound I by sodium fusion revealed the presence of nitrogen and sulfur. Tests for inorganic sulfate were negative. The quantitative figures obtained from the microchemical laboratory were:

C 51.16% H 7.52% N 4.27% S 10.35% O(by difference) 26.70%

In addition, a quantitative Zeisel alkoxy determination gave:

$$CH_3O = 11.03\%$$

Based on the presence of 2 nitrogen atoms per molecule, (as shown previously), an empirical formula of $C_{26}H_{44}N_2S_2O_{10}$ is required which calculates to the following percentages:

C 51.30% H 7.29% N 4.60% S 10.53% O 26.28%

In addition, two CH_3O groups per molecule represent 10.20% of the weight, agreeing well with the figure found. One CH_3O group requires 5.10% and three groups, 15.30%. Therefore, it is concluded that I has two phenol groups expected on the basis of two tyramine residues. Furthermore, the presence of sulfur and the heightened amount of carbon and oxygen in the methyl sulfate derivative of Compound I may be accounted for by postulating that it is a double methylsulfate salt with quarternary methylammonium side-chains attached to a basic diphenyl

Fig. XVa gives the infrared absorption spectrum of the benzoyl derivative.

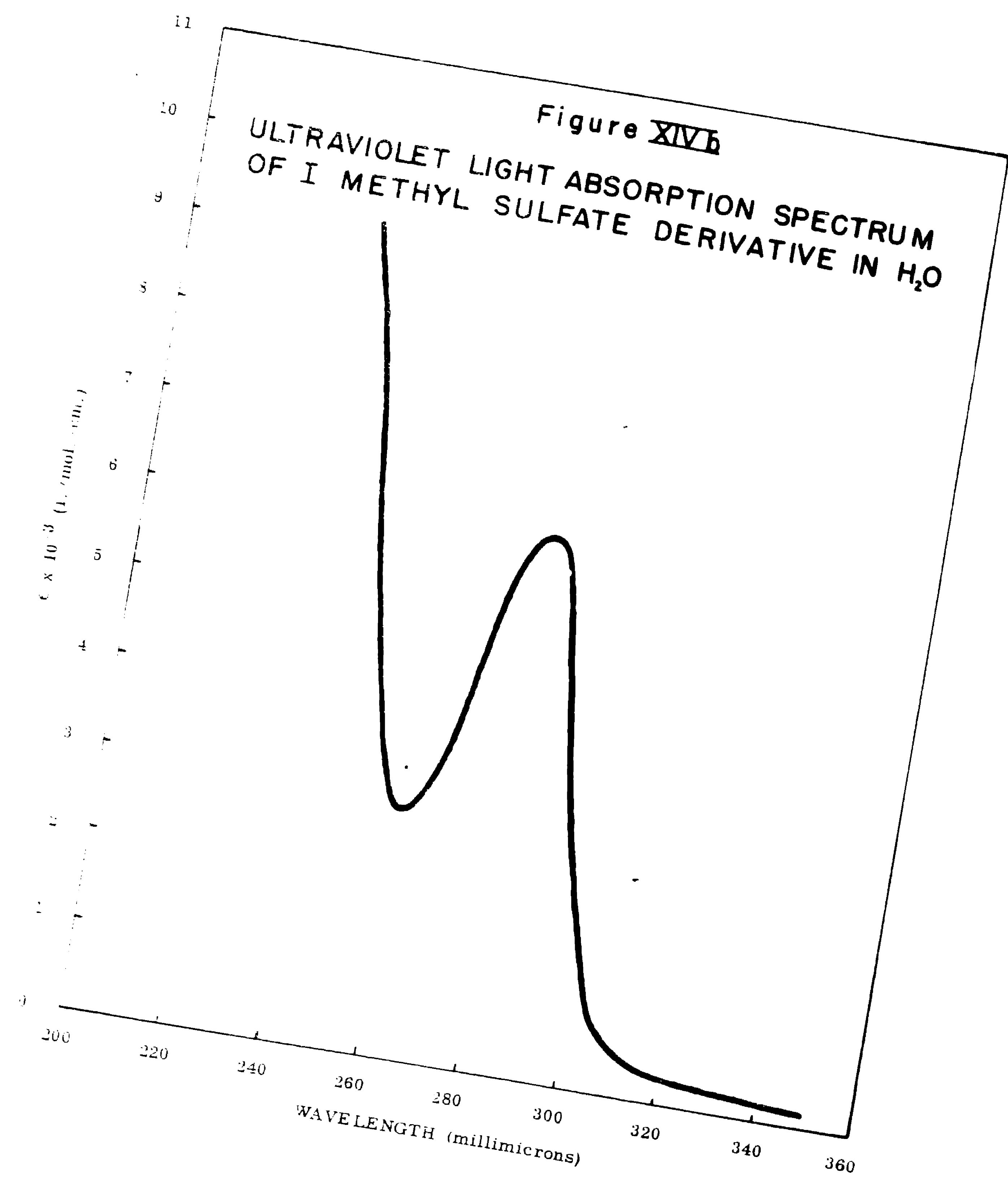
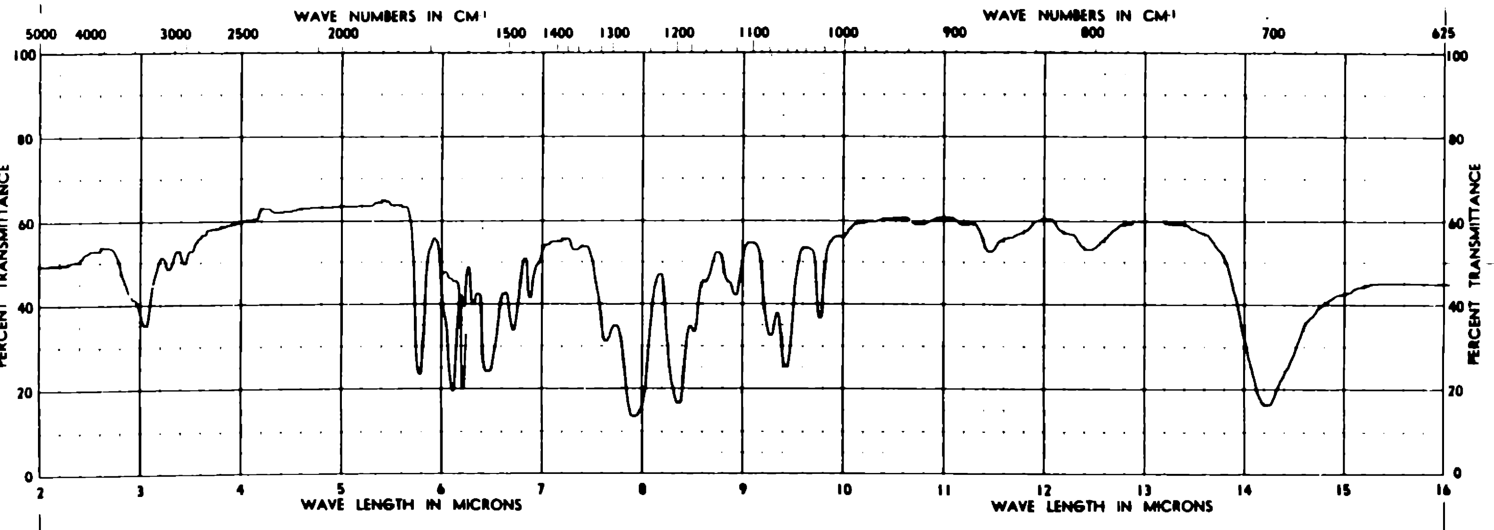


Figure XIV b
ULTRAVIOLET LIGHT ABSORPTION SPECTRUM
OF I METHYL SULFATE DERIVATIVE IN H_2O
WAVELENGTH (millimicrons)
200
220
240
260
280
300
320
340
360
11
10
9
8
7
6
5
4
3
2
1
0

I. R. SPECTROPHOTOMETER
NaCl PRISM

NO.			
DATE		INDEX	
SAMPLE Fig. XVa I BENZOYL DERIVATIVE			
FROM			
SAMP. CELL:	MM	CMS.	
REF. CELL	MM	CMS.	
CHEM.	MG.	MG.	
SOLV.			
VOL.	C.C.	%	C.C. %
F.S.	SOLID	C.	GAS MM

BAIRD ASSOCIATES, INC.
CAMBRIDGE, MASS., U.S.A.
1079-9



I. R. SPECTROPHOTOMETER
NaCl PRISM

NO. 7336			
DATE		INDEX	
SAMPLE Fig. XVb I METHYL SULFATE DERIVATIVE			
FROM			
SAMP. CELL:	MM	CMS.	
REF. CELL	MM	CMS.	
CHEM.	MG.	MG.	
SOLV.			
VOL.	C.C.	%	C.C. %
F.S.	SOLID	C.	GAS MM

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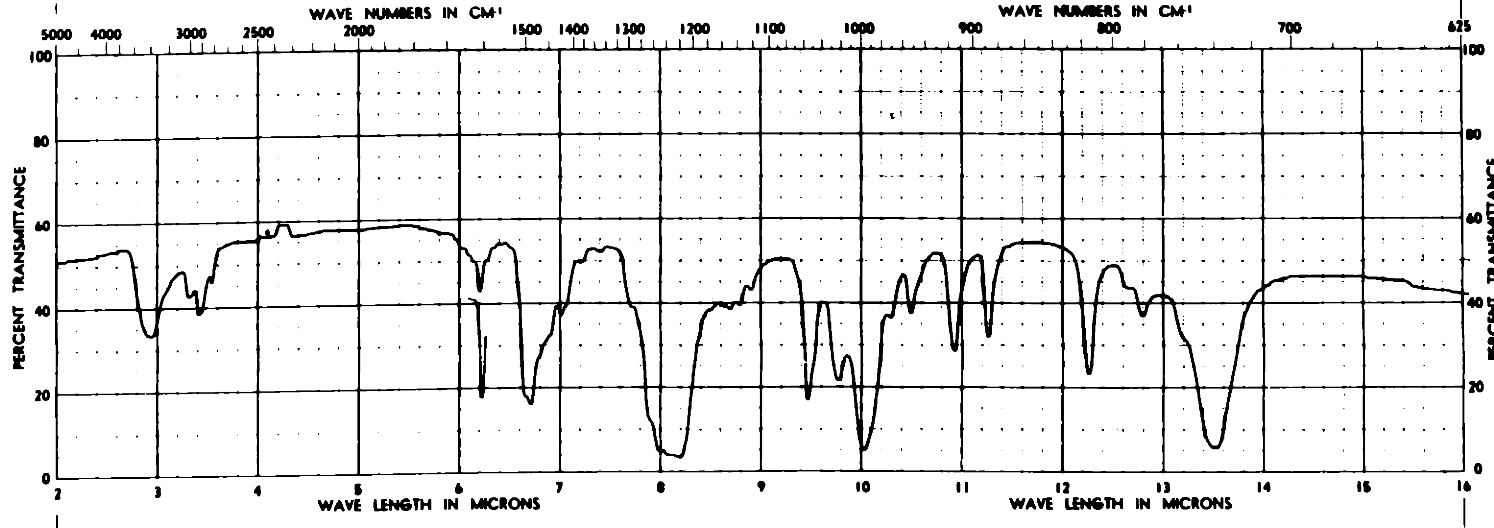


structure. The diphenyl structure has not yet been proven. Section D, following, describes how this proof was obtained.

Fig. XVI is introduced merely to provide additional basis for identifying the tetrabenzoyl derivative and the methyl sulphate derivative of Compound I. The photographs are x-ray diffraction powder patterns taken on flat film at 5 cm. with the nickel filtered copper Kα line (1.54Å). By means of the Bragg law, the rings are converted into the crystal lattice spacings of Table II. (S = strong, m, medium and w, weak diffraction rings.)

Table II

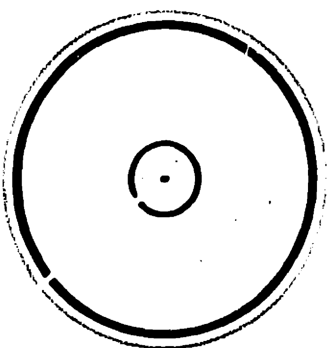
X-Ray Diffraction Spacings

Compound;	I benzoyl derivative	I methyl sulphate derivative	
Solvent:	ethanol - H_2O	absolute ethanol	
Ring diameter cm.	Lattice Spacing Å	Ring diameter cm.	Lattice Spacing Å
5.6 w	3.0	6.3 w	2.8
4.3 m	3.8	5.0 w	3.3
3.9 s	4.2	4.4 s	3.7
3.5 m	4.6	4.2 s	3.9
2.8 m	5.7	3.7 w	4.4
2.5 w	6.3	3.4 s	4.7
2.1 m	7.5	3.2 m	5.0
1.8 w	8.7	2.8 m	5.7
0.9 s	17.2	2.2 w	7.1

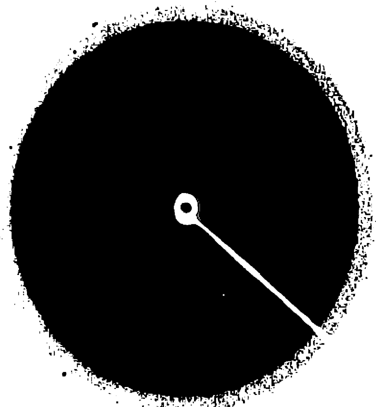
D. Degradation of methyl sulphate derivative of Compound I

If the potassium permanganate oxidation procedure of Shriner and Fuson (60, p. 198) is followed, all side-chains on the methyl sulphate derivative of Compound I should be converted into carboxyl groups. The following adaptation of this procedure was carried out:

Sixty mgms. of the methyl sulphate derivative of Compound I



(a)



(b)

Fig. XVI X-Ray Diffraction Powder Patterns of Benzoyl (a) and Methyl Sulfate (b) Derivatives of Compound I

were dissolved in 4.8 ml. water, whereupon 240 mgms. $KMnO_4$ and .06 ml. 10% NaOH were added. The solution was refluxed for 30 minutes, during which time the permanganate solution turned from purple to brown. After addition of 0.6 ml. 6N H_2SO_4, the solution was refluxed an additional 30 minutes. Upon cooling, sodium bisulfite was added carefully until the solution cleared. A large, fluffy, white precipitate formed (evidently largely $Mn(CH_3SO_4)_4$). This was purified by dissolving it in a minimum of dilute NaOH, filtering, and reprecipitating with a few drops of 6N H_2SO_4. This procedure was repeated four to five times until only a trace of manganese salt remained. Upon drying, a white amorphous compound was obtained, which decomposed above 300°. No further attempt was made to crystallize this substance since it had very poor melting point properties. It was evidently a carboxylic acid as demonstrated by its ease of solubility in base and precipitation with acid. The methyl ester was made by suspending the carboxylic acid in a few drops of methanol, (which acts as a catalyst), then adding 4-5 ml. diazomethane in ether (10 mgms. per ml.), stirring until evolution of nitrogen gas ceased and evaporating to dryness over the steam bath. The compound was dissolved in absolute ethanol and filtered. It was recrystallized six to seven times from ethanol-water, until flat, diamond-shaped crystals were obtained which melted sharply at 173° (corr.). This compound was insoluble in water, and easily soluble in alcohol, acetone and ether. It fluoresced under ultraviolet light. A sample sent to the microchemical laboratory for analysis yielded the following percentages of C, H and O.

C 65.89% H 5.49% O (by difference) 28.62%

This compound is known and has been synthesized by Sugii (61) and Gilman, Swiss and Cheney (62). Its physical characteristics are

in agreement with those found here. Sugii, who crystallized it out of methanol, describes flat, rhomboid crystals which melt sharply at 173°. In addition, the melting point of the dicarboxylic acid precursor was found by Sugii to be greater than 302°, again in agreement with the findings here. Gilman, Swiss and Cheney confirm Sugii's data.

E. Resumé

A series of compounds and their transformations have been discussed in the preceding sections. They are presented in graphic form in Fig. XVII. All names and symbols used in previous references to them are included, together with their correct proper and common chemical names, and their melting points. For every compound submitted, calculated and experimental elementary analyses were in agreement within the limits of error. In the two cases where the compounds were known beforehand, the melting points and physical characteristics concurred as well with the figures previously reported. Fig. XVIII provides formulae and reported melting points of related diphenyl compounds with the empirical formula $C_{18}H_{18}O_6$ and their demethylated derivatives. It is apparent that the compounds derived from dityramine fall exclusively into the 2, 2'-dihydroxydiphenyl series.

The formula and structure of I have been proven in this manner through the formation of derivatives, and the subsequent degradation of one of these derivatives to compounds of known structure and properties. I has been shown to be dityramine hydrochloride.

F. Discussion

The formation by peroxidase of dityramine from tyramine and dityrosine from tyrosine is not improbable. Examples have been given of diphenyl compounds produced by the action of peroxidase. They were: divanillin, di-p-cresol and tetramethylbenzidine. Perhaps

Figure XVII - REACTION SCHEME RESULTING IN CHARACTERIZATION OF COMPOUND I

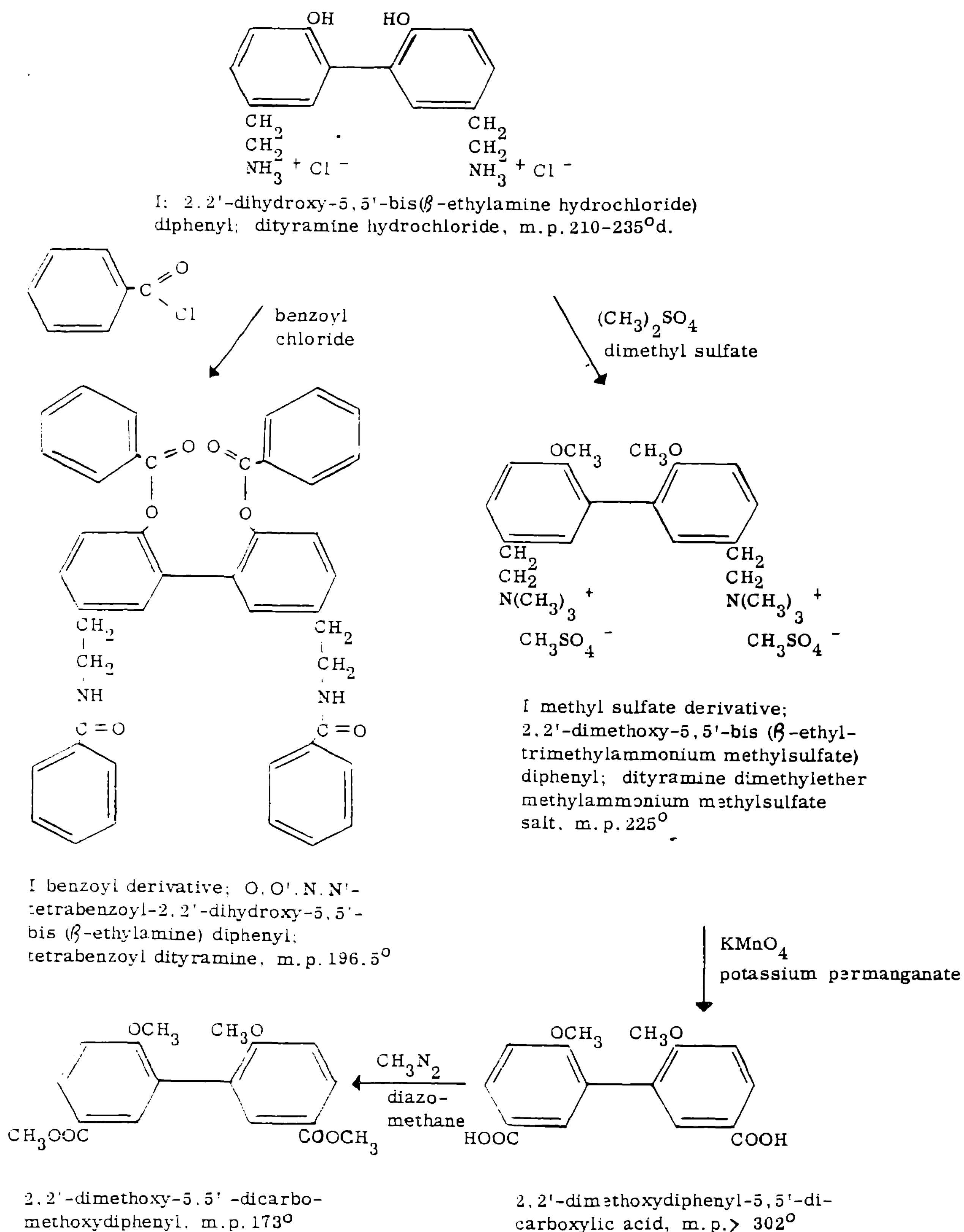
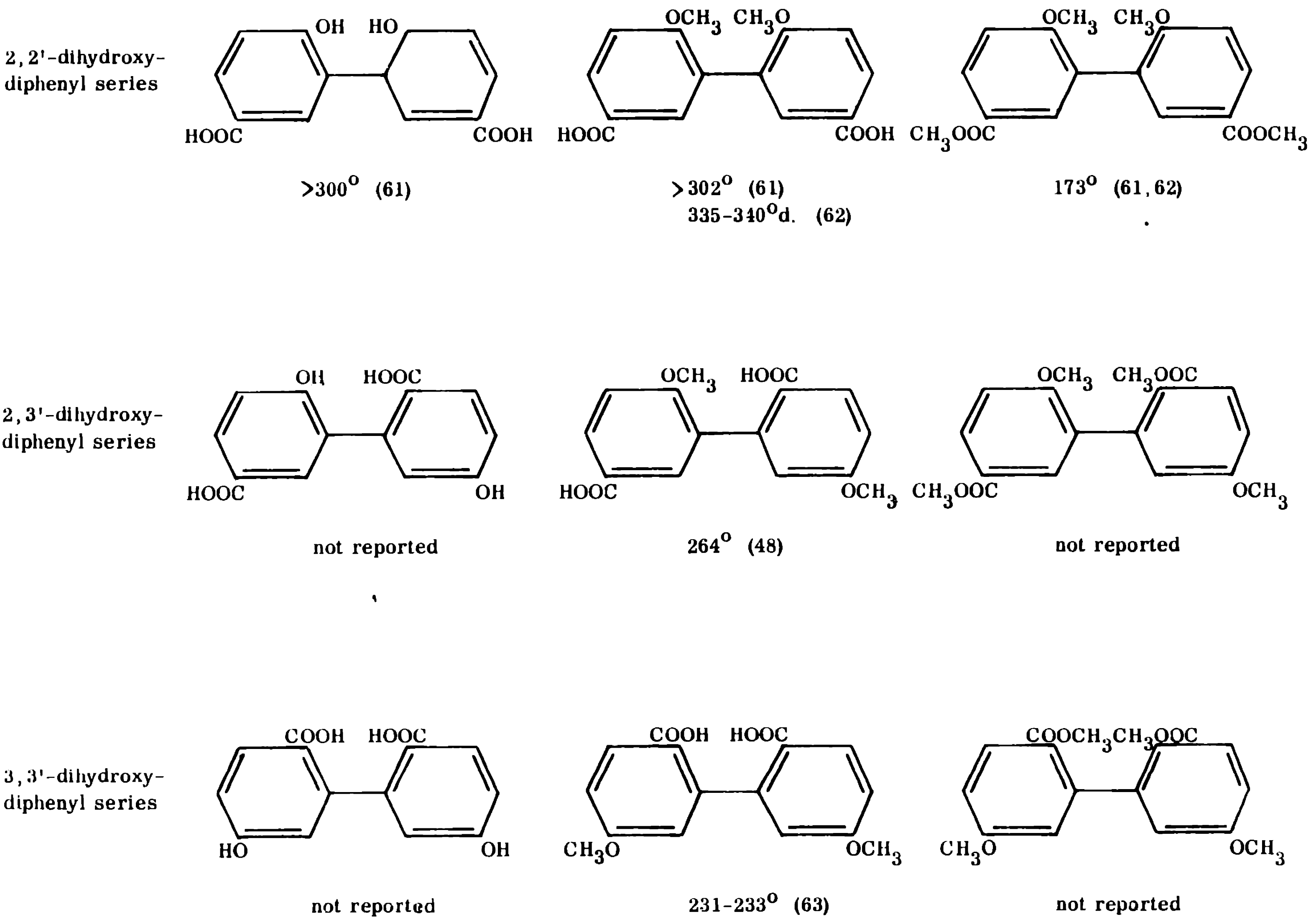
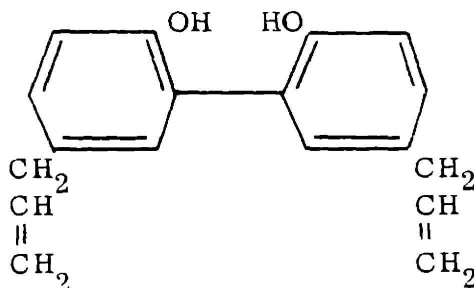


Figure XVIII - DIPHENYL COMPOUNDS AND METHYLATED DERIVATIVES RELATED TO DITYRAMINE



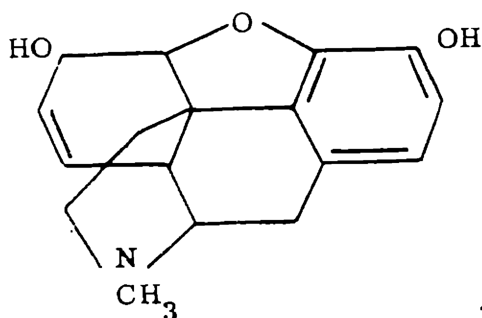
divanillin is the most interesting since, at present, there is speculation whether diphenyl elements are normally present in the plant substance lignin (64). Since tyrosine and peroxidase are found together in blood eosinophils (65), it is conceivable that dityrosine is formed as a physiological entity. Even in the presence of catalase, Knox (66) has shown that myeloperoxidase will oxidize tyrosine with enzyme-generated peroxide, an event that might occur within the cell. If dityrosine is a physiological entity, it might possibly play a role in the phagocytic function of leucocytes, and in the anti-hypersensitivity function of eosinophils in particular.

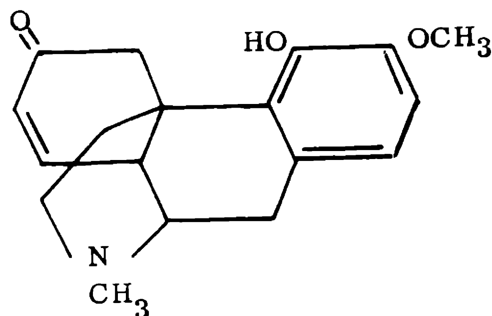
It is interesting to speculate whether or not peroxidase is important in the synthesis of plant substances. Sugii described the compound magnolol, (2, 2'-dihydroxy-5, 5'-diallyldiphenyl), in the bark of the magnolia tree (61). This substance conceivably may have been formed by the action of peroxidase on the monomeric elements.



MAGNOLOL

Peroxidase may be the principal enzyme in the synthesis of alkaloids of the morphine group. These compounds all show a diphenyl-type linkage; frequently, a related dibenzofuran group:





MORPHINE SINOMENINE

A new theory for the biogenesis of morphine alkaloids requires the presence of an enzyme capable of producing coupling reactions of this nature (67).

From the structure of dityramine hydrochloride, it is expected that it would show the same solubility and general chemical characteristics as the parent material. This is largely found to be true. Millon's test is positive signifying a monohydric phenol. Tests for secondary amine and ketone groups are negative. However, whereas tyramine forms an excellent yellow picrate in the form of needles (m.p. 206°), dityramine produces a yellow oil which cannot be crystallized by ordinary procedures. I shows an enhanced aromatic character when compared with tyramine. There is a shift of λ_{max} to longer wave-lengths, and the extinction coefficient is higher than the value obtained by adding together two tyramine residues; the phenolic groups are more acid, and fluorescence appears. Fluorescence is expected when two aromatic rings are joined, especially when phenolic groups are present (68).

The difficulty in crystallizing dityramine hydrochloride adequately for study may be mentioned. The compound can, in effect, be crystallized in the form of interlacing translucent needles by drying down an aqueous solution of the salt. The needles obtained are sticky

and impure whereas the reprecipitated amorphous salt used here was shown to be pure.

V. Synthesis of Compound I

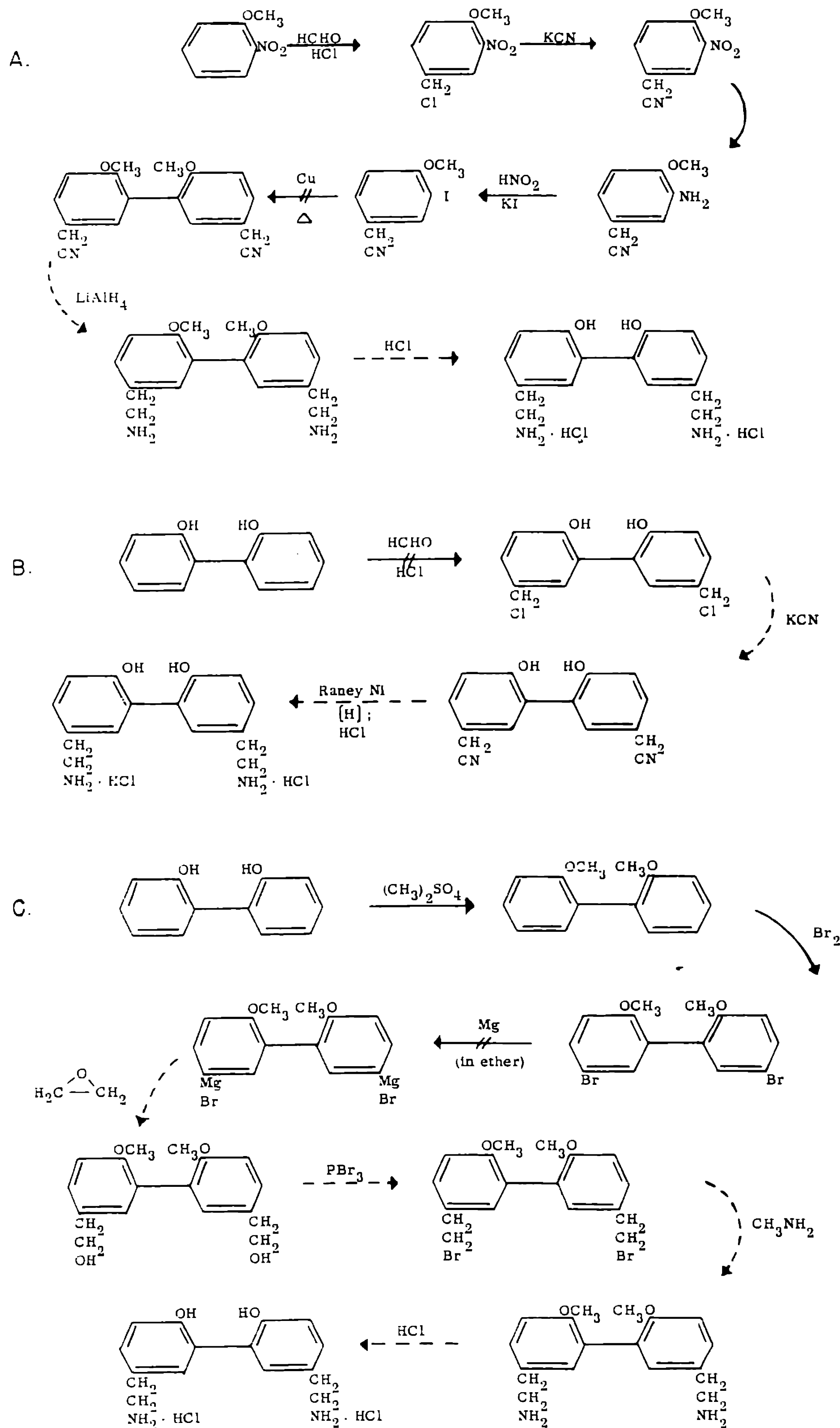
Although several attempts were made to synthesize I rigorously, with the assistance of Dr. N. A. Nelson, Department of Chemistry, M.I.T., they were not successful. Fig. XIX shows three attempted routes. The arrows with crossbars indicate points where the syntheses failed. In the case of A, the Ullman step (69) did not succeed. Evidently the nitrile group was unstable under the conditions necessary for the condensation. In B, the chloromethylation (70) of 2, 2'-dihydroxy-diphenyl resulted in practically none of the desired compound. Route C failed at its most sensitive point, the formation of the di-Grignard compound, even though Snyder, Weaver and Marshall had success (71) with the very closely related 3, 3'-dibromodiphenyl. The problem arising here was the very low solubility of the dimethylether-dibromo compound in absolute di-ethyl ether.

Despite the fact that these more rigorous synthetic methods failed, I was effectively synthesized by metal ion oxidation in one instance, and by the use of CuO and high temperature in another. The presence of I was determined by chromatography. Because of low yield and the questionable significance of these syntheses, no attempt was made to isolate the compound. The procedures were as follows:

a) Two hundred milligrams of free tyramine were refluxed for 6 hours with an equivalent amount of ferric chloride in 20 ml. water. The iron was removed with hydrogen sulfide and the solution evaporated to near dryness and chromatographed. A fluorescent ninhydrin positive spot having the same R_F as I was found using different solvent systems.

b) One hundred milligrams of tyramine hydrochloride, intimately mixed with an equivalent amount of copper oxide, was heated to 200° for five minutes in a Wood's metal bath. The mixture was cooled

Figure XIX - ROUTES ATTEMPTED IN DITYRAMINE HYDROCHLORIDE SYNTHESIS



and chromatographed directly. The appearance of a spot with the same properties as authentic I was observed.

There is a considerable amount of precedent for the formation of coupled ring systems using these methods. Divanillin has been synthesized from vanillin and iron salts (45). Pummerer et al (49, 92) used potassium ferricyanide as an electron-abstracting agent to form di-p-cresol from its monomer. Di β-naphthol has been synthesized using $FeCl_3$ (73), $CuAc_2$ (74) and CuO (73). A free radical mechanism is postulated for all of these reactions (75).

It is interesting to note that when tyrosine, or tyramine, reacts with ferrous sulphate in the presence of H_2O_2, coupling does not take place. Instead, the dihydric phenols, 3, 4-dihydroxyphenylalamine and 3, 4-dihydroxyphenylethylamine, are formed (76). This Fenton reaction (75, p. 1157; 81) also catalyzes the hydroxylation of phenylalanine to tyrosine (76).

VI. Separation and Identification of Compound II and Tyramine Pigment

II was isolated in the same manner described for I. However, it was present in very much smaller quantity and could not be purified to the same extent without endangering loss. II was a water soluble, hygroscopic compound with solubility characteristics much the same as I. It was unstable in air and basic solution. Its melting point was indeterminate, with decomposition. II gave a yellow color with Millon's reagent but formed no precipitate. Tests for secondary amine and ketone functions were negative. II was ninhydrin positive. It fluoresced under ultraviolet light. Its ultraviolet absorption spectrum, Fig. XX, is interesting to compare with I, (Fig. XI), II shows the same λ_{max} in acid and base, with, however, a slight shift of λ_{min} to shorter wavelengths. The compounds are presumably very similar, but two distinctions may be noted: II has a considerably higher extinction coefficient (not measured quantitatively due to lack of material); and ϵ_{max} (base) shows no increase in extinction over ϵ_{max} (acid). This leads to the conclusion that II has more resonance capacity than I, and also, that the phenoxide ion of II does not have increased resonance structure.

II did not form a crystalline benzoyl derivative, nor could any satisfactory derivative be made. However, II was methylated with methyl sulphate according to the procedure given in Section IVC and a few mgms. of impure product were obtained. This derivative could not be crystallized, but was partially purified by dissolving in absolute ethanol, then precipitating with absolute ether. In solubility, the compound was similar to the methyl sulphate derivative of I except for increased solubility in the alcohol series. The ultraviolet absorption

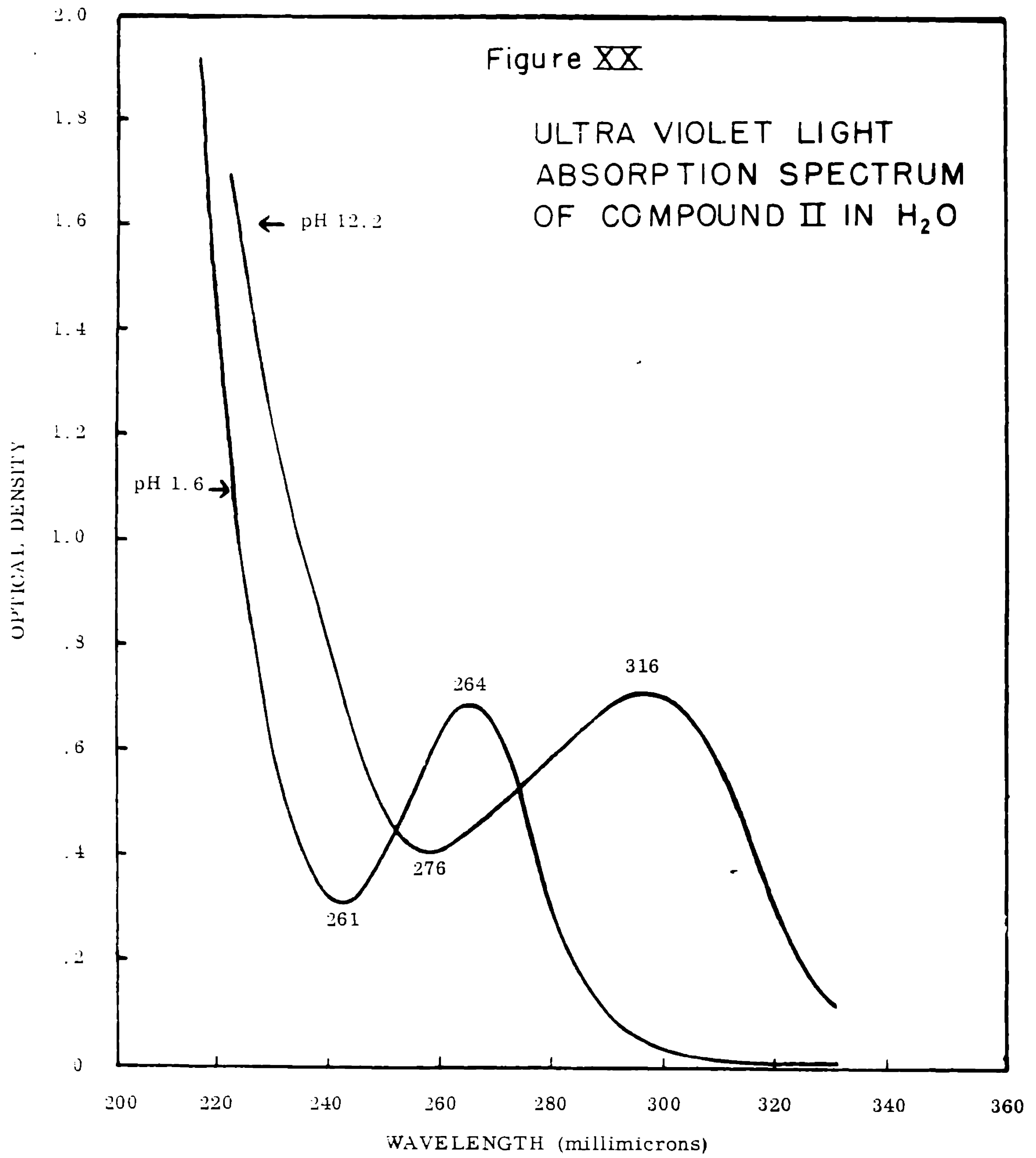
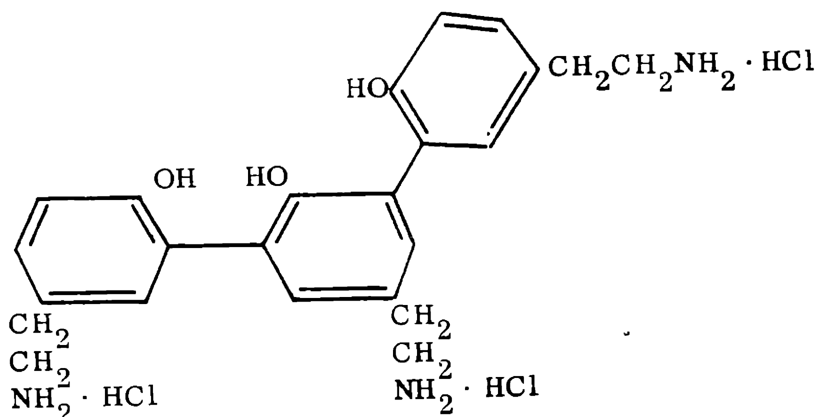


Figure XX
ULTRA VIOLET LIGHT ABSORPTION SPECTRUM OF COMPOUND II IN H_2O
pH 12.2
pH 1.6
OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
264
316
276
261
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

spectra were practically identical for both (Figs. XXI and XIV).

Microchemical elementary analyses were unsatisfactory because the samples contained 5.5% salt. On the basis of the evidence at hand, however, a terphenyl structure is suggested for II.



This compound may be termed trityramine. It is analogous to tri-p-cresol suggested by Westerfeld and Lowe in their work on the peroxidase oxidation of p-cresol (48).

Tyramine pigment is obtained as a brown precipitate by maintaining the solution at proper pH while the reaction is underway. Precipitation of pigment is not brought about by peroxidase action. This may be proved by the following experiment:

A reaction mixture is brought to pH 7.5 then incubated at 37° for one hour. The reaction is stopped by the addition of catalase. Aliquots of reaction mixture are pipetted into individual test tubes. By addition of very dilute HCl or NaOH, the pH is varied over the range from pH 1.3 to pH 12.3. Upon standing for 6 hours at 4°, pigment precipitates in the tubes with pH lying between 8.5 and 10.5. If the tubes not in this range are brought within it by appropriate additions of acid or base, pigment again precipitates out. However, once a precipitate forms, it cannot be redissolved in either strong acid or base.

Pigment molecules thus aggregate irreversibly when in a

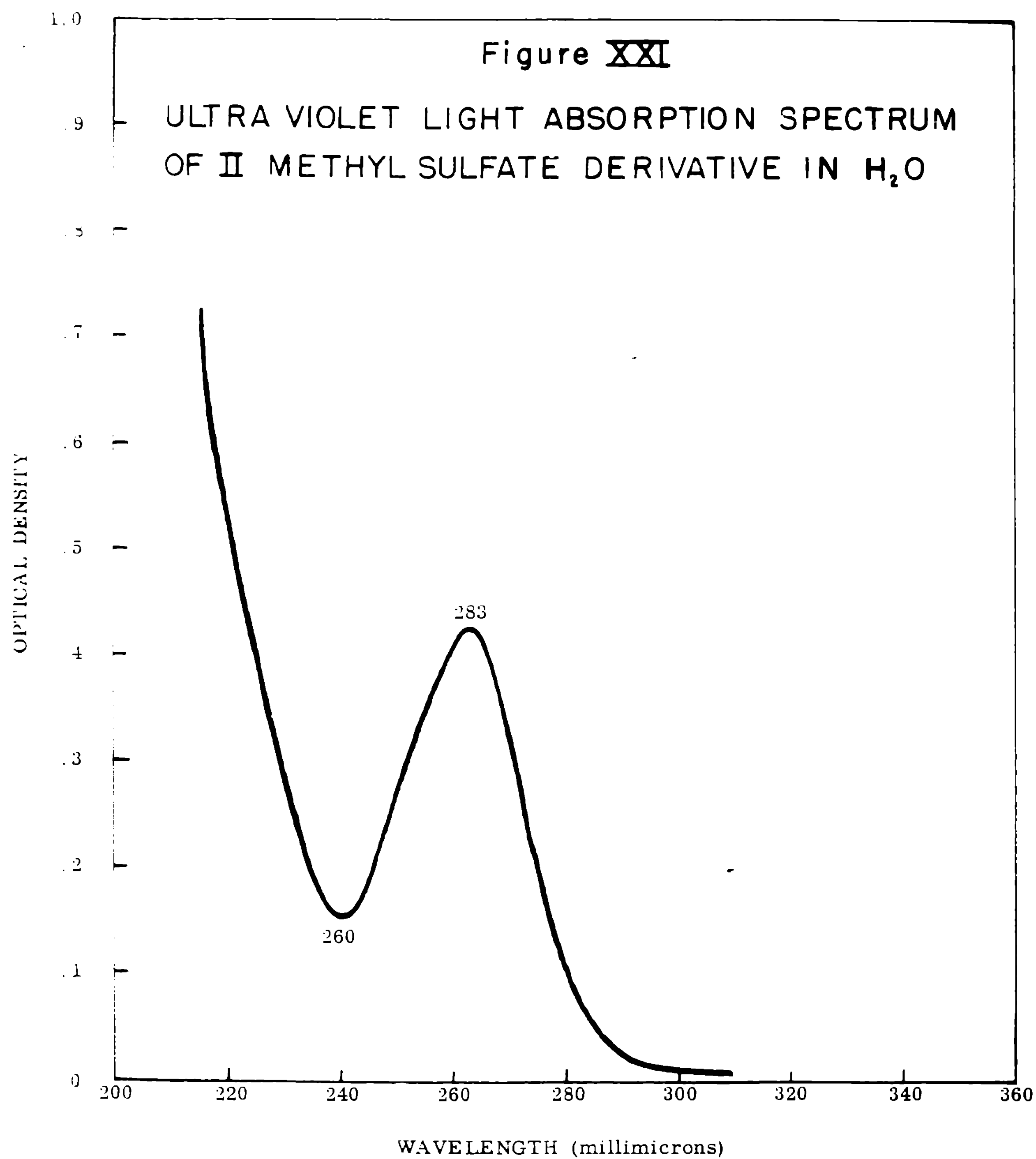


Figure XXI
ULTRA VIOLET LIGHT ABSORPTION SPECTRUM
OF II METHYL SULFATE DERIVATIVE IN H_2O
OPTICAL DENSITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
283
260
200
220
240
260
280
300
320
340
360
WAVELENGTH (millimicrons)

suitable pH range. This may be partially due to hydrogen bonding between ionized amino and phenolic groups. The pK values of these groups are respectively 10.0 and 10.5.

The precipitated pigment was insoluble in all laboratory solvents. When heated above 300° it decomposed slowly. Upon prolonged hydrolysis in 6N HCl it yielded the series of fluorescent compounds I, II, III, etc., which were observed chromatographically.

The pigment is not homogeneous. Elementary analyses of two samples provided the following figures:

a) C 59.17% H 6.32% N 7.32% O (by difference) 26.65%

b) C 65.52% H 5.95% N 8.68% O (by difference) 19.85%

If we calculate the empirical formulae derived from these figures on the basis of the eight carbon atoms present in one tyramine residue, the following data are obtained:

Table III

Empirical Formula of Tyramine Pigment

	a)			b)		
	%	Divide by Atomic Weights	Empirical Formula based on 8 C Atoms	%	Divide by Atomic Weights	Empirical Formula based on 8 C Atoms
C	59.71	4.98	8	65.52	5.46	8
H	6.32	6.28	10.0	5.95	5.91	8.7
N	7.32	.523	8.4	8.68	.620	9.1
O	26.65	1.67	2.7	19.85	1.24	1.8

The last columns show (assuming no carbon is lost) that the pigment has lost a small amount of nitrogen and gained a considerable amount of oxygen, on the order of 2-3 times. This information, together with the pigment hydrolysis data, signifies that after the pigment forms in solution, there is a great deal of non-enzymatic surface oxidation before, or during, precipitation. Phenolic compounds are well known to

be air unstable and easily oxidizable in basic solution. In the case of the pigment, the gain in oxygen is greater than can be accounted for by all the H_2O_2 present at the beginning of the reaction. Air oxidation is therefore taking place.

The loss in nitrogen was not detectable as free NH_3. It could not be ascertained to what place this nitrogen disappeared.

It is assumed, on the basis of evidence accumulated, that the pigment is built up of monomeric groups similar in structure to I and II. These groups polymerize to form a polytyramine structure. Hydrogen bonding takes place between neighboring phenolic and amino groups. Finally, at the proper pH, base catalyzed air oxidation occurs, yielding the brown, insoluble tyramine pigment which precipitates from solution. It is likely that during the air oxidation many of the surface aromatic rings form colored, active quinonoid groups which are capable of coupling with neighboring rings. The orange color formed during the peroxidase catalyzed reaction of tyramine or tyrosine may be due to the presence of these groups.

Tyrosine does not form an insoluble pigment due to the solubilizing effect of the carboxyl group.

VII. Action of Peroxidase on Compounds Related to Tyramine

In order to determine whether or not peroxidase could catalyze other compounds of the tyramine-tyrosine series according to the now established tyramine pathway, reactions were carried out using the standard reaction mixture, however, with ten times the usual amount of peroxidase. The results are given in Table IV. The chromatographic solvent used was a 55-15-30 mixture of n-butanol, formic acid (88%) and water.

Table IV

Products of Peroxidase Action on Compounds Related to Tyramine

Substrate	Color Formed	R_F of Substrate	R_F of Major Product	Fluorescence of Product In UVL
Phenylalanine	None	.48	-	-
p-methoxy phenylalanine	None	.58	-	-
Glycyl tyrosine	Yellow	.41	.12	+
Tyrosine ethyl ester	Yellow	.60	.19	+
N-formyl tyrosine	Pink	.75	.68	+
N-formyl tyrosine ethyl ester	Orange	.91	.79	+
Diiodotyrosine	Brown	.65	.37	Absorbs UVL
Methyl salicylate	None	.91	-	-
Tyrosine	Yellow	.38	.15	+
Tyramine	Yellow-brown	.49	.21	+

Referring to the table, one notes that all compounds acting as substrates for peroxidase, except one, formed fluorescent major products. The products migrate more slowly than the original substrates. It is postulated that, excepting diiodotyrosine, diphenyl compounds analogous to dityramine and dityrosine are formed.

Dihydric phenols follow a different pathway of oxidation with peroxidase. O-quinones are formed and undergo polymerization to melanins (37, 77). Peroxidase acts in this way like tyrosinase.

VIII. Action of Peroxidase System on Proteins

Bovine fibrinogen, zinc-free insulin, gelatin and pepsin were incubated at 37° for 6 hours in the following systems with peroxidase. The pH was kept at 7.0 except in the case of pepsin when it was maintained at 5.0.

A.	Protein	20 mgms.
	Phosphate Buffer	3 ml. .07 M
	Hydrogen Peroxide	.5 ml. .1%
	Peroxidase	2 mgms. in 2 ml. distilled water
B.	Same as A, but 5 mgms. tyrosine present	
C.	Same as A, but 5 mgms. tyramine hydrochloride present	
D.	Phosphate Buffer	3 ml. .07 M
	Hydrogen Peroxide	.5 ml. .1%
	Peroxidase	2 mgms. in 2 ml. distilled water
	Tyramine Hydrochloride	5 mgms.

The reaction was incubated 30 minutes, then stopped by the addition of .05 ml. catalase solution (250 units per ml.). After one hour 20 mgms. protein were added and the reaction incubated further for 2 hours.

E. Same as D, but 5 mgms. tyrosine present, instead of tyramine hydrochloride.

Controls:

F. Same as D, without addition of protein

G. Same as E, without addition of protein

H, I, J, K Same as A, B, C, D, but without hydrogen peroxide.

After incubation, these reactions were dialyzed against running tap water (15-20°) for 72 hours. Their volume was then doubled

by the addition of concentrated HCl, and they were hydrolyzed by evaporation to dryness over a boiling water bath. The solutions were filtered and chromatographed. The aromatic amino acids were concentrated by adsorption on charcoal and elution with acid alcohol. The final solutions were chromatographed as well.

Table V

Peroxidase System Effect on Protein

Experimental Group	Fluorescent Spot Corresponding to Dityramine or Dityrosine
A. (no tyrosine or tyramine)	0
B. (tyrosine present)	+
C. (tyramine present)	+
D. Catalase added, then protein	+
E.	+
F. Catalase added, no protein	±
G.	±
H. I. J. K.	0
Hydrolyzed tyramine pigment	+++
Hydrolyzed tyrosine pigment	+++

This experiment was performed in an attempt to determine whether peroxidase would bring about in situ diphenyl compound formation on proteins by condensation of external tyramine or tyrosine elements in the solution with protein tyrosine. Since the diphenyl linkage is comparatively stable to acid hydrolysis, it was expected that fluorescent spots corresponding to dityramine or dityrosine would be observed upon chromatography after hydrolysis, if diphenyls were actually formed on the protein itself. In Table V, the results of this experiment are given. The proteins showed no individual characteristics. The charcoal elution chromatograms were technically superior and qualitatively identical with the chromatograms of the whole hydrolysates.

By spraying with NaOH solution fluorescence was greatly enhanced.

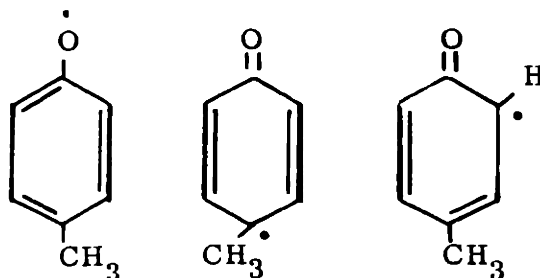
The results indicated that diphenyl formation in situ on the surface of the protein tested probably did not take place. The products of B and C reactions did not fluoresce more strongly than those of D and E which is necessary if peroxidase were condensing external tyrosine or tyramine elements onto protein tyrosine. It is likely that all the observed fluorescence was due to protein adsorption of diphenyl compounds resisting dialysis. Since F and G showed a trace of fluorescence, it is also likely that some trapped pigment was present. The pH was kept low purposely to inhibit pigment formation.

Studies were carried out in which the proteins were separated from the reaction mixtures by a dialysis sac. After hydrolysis, the proteins showed no fluorescent elements.

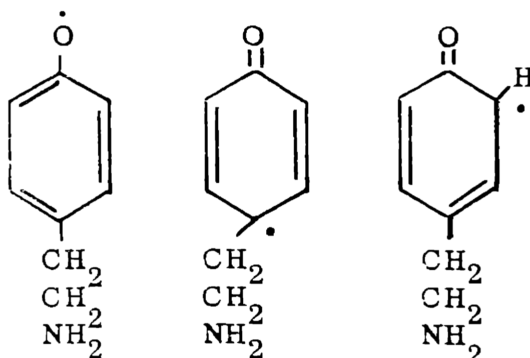
In conclusion, it may be stated that these experiments prove that there is no considerable in situ formation of diphenyl linkages on protein in the presence of peroxidase-H_2O_2-tyramine, (or-tyrosine), systems. There was no evidence of any kind of peroxidase action on proteins that could be detected by the methods employed in this study.

IX. Mechanism of Peroxidase-Tyramine Reaction

Pummerer's studies of the oxidation of phenols with various inorganic electron-abstracting agents (72) showed clearly that intermediate free radical forms were involved. Goldschmidt later confirmed this work (78). Free radical formation has long been associated with the formation of diphenyls (79). Usually this mechanism leads to very complicated mixtures of products unless one free radical form is favored by resonance stability. Waters has reviewed many of these oxidation processes (75), and believes that when p-cresol is oxidized by potassium ferricyanide in the presence of sodium carbonate, the following free radicals are involved:



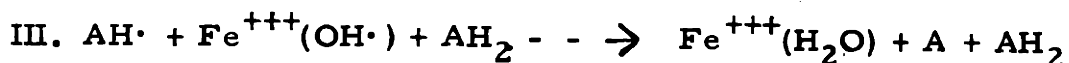
Transcribing this for the case of tyramine:



Whereas, in the dimerization of α- or β-naphthol, the free radical forms are stabilized by the double ring system, the peroxidase enzyme may serve this function here. Peroxidase may also favor one of the three isomeric forms written for the free radical of tyramine.

In accordance with Pummerer's reaction sequence for the formation of di-β-naphthol from β-naphthoxy radical (80), the presumed mode of peroxidase dityramine production is schematized in Fig. XXII. Note that p-methoxy phenylalanine cannot form a free radical. From Section I, it will be recalled that Chance's series of oxidation equations require the existence of a free substrate radical. These important reactions are reviewed here:

II. $Fe^{+++}(H_2O_2) + AH_2 \dashrightarrow Fe^{+++}(OH\cdot) + AH\cdot + H_2O$



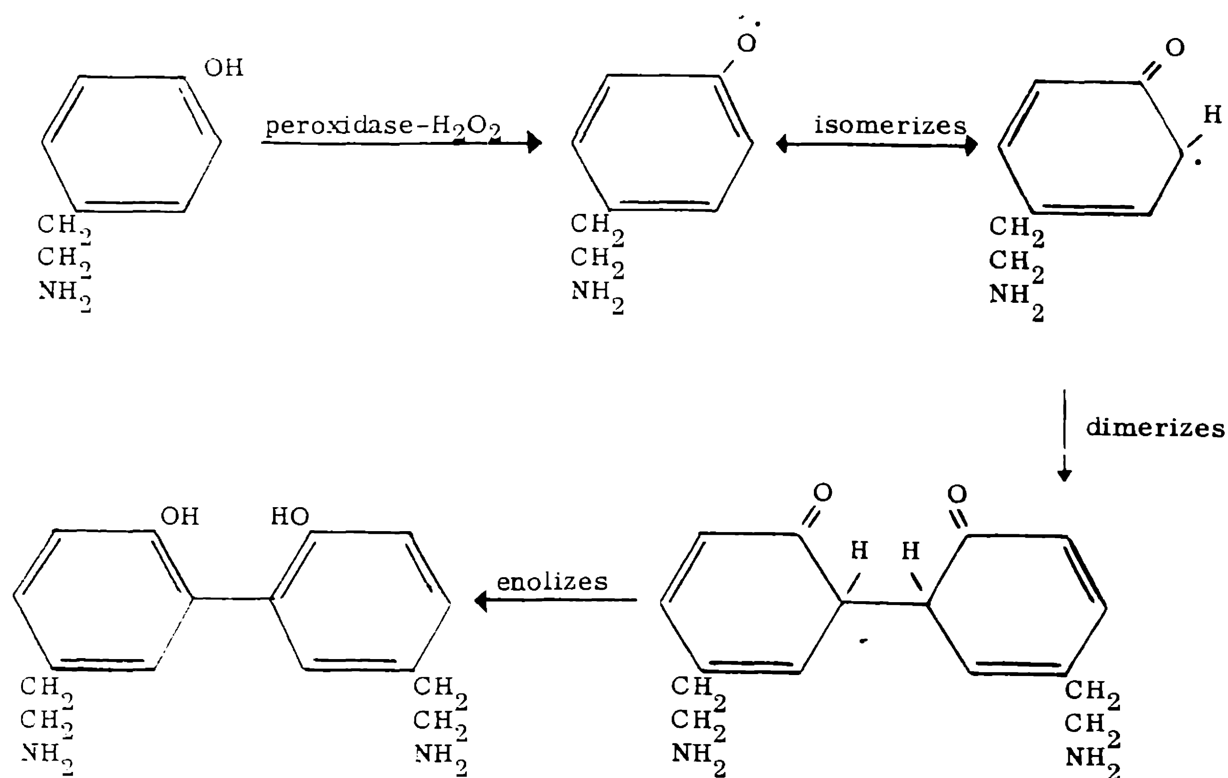
III. $AH\cdot + Fe^{+++}(OH\cdot) + AH_2 \dashrightarrow Fe^{+++}(H_2O) + A + AH_2$

Equation III may be rewritten to agree with the reactions studied here:

III. $AH\cdot + Fe^{+++}(OH\cdot) + AH_2 \dashrightarrow Fe^{+++}(H_2O) + 2AH\cdot \dashrightarrow HA\text{-}AH$

This reaction mechanism is not only probably true for tyramine, but also probably holds for tyrosine and all analogues which form diphenyl compounds as a result of the action of peroxidase. Polyphenyl compounds may also be oxidized in this manner.

Figure XXII - MECHANISM OF PEROXIDASE-TYRAMINE OXIDATION



X. General Summary and Discussion

1. A method is given for the isolation of the chief products of the reaction between horseradish peroxidase and tyramine(β-p-hydroxyphenylethylamine).

2. The product occurring in highest yield is shown to be dityramine (2, 2'-dihydroxy-5, 5'-bis (β-ethylamine) diphenyl). The dihydrochloride salt melts over the range 210 - 235° with decomposition. It shows blue fluorescence under ultraviolet light. Ultraviolet light absorption values are: λ_{max}(acid) = 284 mμ; ϵ_{max}(acid) = 4610 liters/mole cm. λ_{max}(base) = 316 mμ; ϵ_{max}(base) = 5790 liters/mole cm. The infra-red absorption spectrum is given. Dityramine is not a substrate for tyrosinase.

3. The O, O', N, N'-tetrabenzoyl derivative of dityramine, m.p. 196.5° (corr.) was prepared by the Schotten-Baumann method. ϵ_{275}(95% ethanol) = 6450 liters /mole cm. Infra-red absorption and x-ray diffraction data are given.

4. The methyl sulfate derivative of dityramine, 2, 2'-dimethoxy-5, 5'-bis (β-ethyltrimethylammonium methylsulfate) diphenyl, was prepared with dimethyl sulfate in 10% NaOH solution. It melted at 225° (corr.). λ_{max} = 283 mμ; ϵ_{283} (water) = 6070 liters/mole cm. Infra-red absorption and x-ray diffraction data are given.

5. The methyl sulfate derivative was degraded to 2, 2'-dimethoxy-5, 5'-dicarboxylic acid diphenyl, melting above 302°, with potassium permanganate. This was further derivatized to 2, 2'-dimethoxy-5, 5'-dicarbomethoxydiphenyl, m.p. 173°, using diazomethane.

6. Dityramine was synthesized catalytically from tyramine by heating with ferric chloride in aqueous solution, and by heaing with copper oxide at 200° for five minutes, as determined by chromatographic and ultraviolet absorption data.

7. Evidence is presented suggesting that trityramine is formed in the peroxidase-tyramine reaction along with dityramine. It is suggested also that the insoluble pigment formed is composed of aggregated di-, tri-, and polytyramine elements together with more highly oxidized, related substances.

8. Horseradish peroxidase catalyzes the oxidation of the following compounds to diphenyl derivatives structurally analogous to dityramine, as shown by chromatographic evidence: tyrosine, glycyltyrosine, tyrosine ethyl ester, N-formyltyrosine, N-formyltyrosine ethyl ester. Evidence for dityrosine formation is strongly afforded by ultraviolet light absorption data. Complete analogy between the peroxidase-tyramine and peroxidase-tyrosine reactions is also shown chromatographically. Phenylalanine and p-methoxyphenylalanine are not oxidized by horse-radish peroxidase.

9. The coupling of tyrosine residues of bovine fibrinogen, insulin, and pepsin with tyramine or tyrosine in the presence of peroxidase to form in situ diphenyl linkages probably does not take place.

10. The formation of diphenyl compounds by peroxidase is explained by the formation of phenoxide free radicals which undergo isomerization and dimerization. The inability of p-methoxyphenylalanine to act as a substrate for peroxidase is understood on this basis.

A great deal of work has been carried out in recent years on peroxidase. There have been many studies of peroxidase-substrate complexes by Chance (31-33) and George (34,35). But the physiological significance of this enzyme , present in dry weight concentrations up to 1 - 2% in certain tissues (26), has not been explained. The work undertaken in this paper may be a step toward solving this problem. In Section IV the suggestion is made that peroxidase is involved in the synthesis of plant alkaloids containing diphenyl-like or dibenzofuran structures.

Examples are thebaine and morphine. Magnolol, found in the bark of the magnolia tree is presented as an example of a non-alkaloid of the diphenyl type. From the work of Knox (66), it is apparent that peroxidase can function in the presence of catalase, if peroxide is supplied by a flavoprotein system.

The presence and significance of diphenyl compounds in animal tissues, and their physiological activities, have never been studied. Dityramine, a phenylethylamine, is expected to show pressor activity similar to epinephrine. Whether it exercises a specific, unique function in addition is an open question.

Opportunity for the production of dityrosine in the body is high: tyrosine is present in concentrations up to 1.1 mgm.% in the blood stream (65), and blood and tissue granulocytes are replete with myeloperoxidase. Whether dityrosine is decarboxylated before it exhibits physiological activity is another unanswered question.

One point seems to be clear. This is the fact that peroxidase produces organic free radicals. Whether these reactive species leave the enzyme surface to react with cellular constituents invites speculation. It is believed (81) that carcinogens will form reactive, free radical species highly stabilized by their resonance systems which disrupt cellular processes. It is not implied that peroxidase is involved in this fashion, but that it can form free radicals which might be important in cellular reactions.

Protein inactivation by peroxidase cannot be attributed to tyramine or tyrosine free radical attack with resulting diphenyl formation. From the work of Agner (39) and others, it is believed that the inactivating, oxidative effect is brought about by the oxidative coupling of small carrier molecules such as ascorbic acid, uric acid, catechols,

iodides, and various metal ions. At least one of these dialyzable species is present in every living cell. When sufficient peroxidase concentration is reached, they may bring about inactivation of susceptible protein substances. Although carriers were not added by various investigators who obtained positive results studying protein-peroxidase inactivation systems (42-44), the presence of impurities cannot be ruled out.

The work remaining in this area will include an investigation of the physiological activity of dityramine and dityrosine, and an examination of the products of the peroxidase-tryptamine and peroxidase-tryptophane reactions. With these studies completed, the problem of peroxidase function may be unfolded.

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Biographical Note

The author was born in New York City, New York in 1926 and attended the city public elementary and high schools. He entered Antioch College in 1942, but transferred to Bates College and finally to the University of Rochester under the Navy V-12 College Training Program during World War II. He received the B.A. degree in General Science from the University of Rochester in 1946. In 1950, the M.D. degree was awarded him by the same university. After completing a Naval internship at the U. S. Naval Hospital, Chelsea, in 1951, he entered the Mfassachusetts Institute of Technology. He was a fellow of the National Foundation for Infantile Paralysis, 1952-1954, and is a member of Sigma Xi.