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University of Michigan, Ph.D., 1961
Chemistry, biological

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TRYPTOPHAN METABOLISM IN THE KIDNEY

by
Clare Cornelius Johnston

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1961

Doctoral Committee:

Assistant Professor Merle Mason, Chairman
Professor Halvor N. Christensen
Professor Adam A. Christman
Professor Phillip Gerhardt
Professor Peter A. S. Smith

ACKNOWLEDGMENTS

It is a pleasure to credit a few of many generous persons whose help has made possible this thesis and the education of which it is a part.

The work was guided at all stages by the helpful, patient and thorough direction of Dr. Merle Mason.

The author also appreciates the support and interest shown in this work by Dr. H. N. Christensen.

Dr. A. A. Christman gave advice and help on many occasions.

The syntheses of L-kynurenine and 3-hydroxykynurenine were carried out with the help and advice of Dr. J. L. Warnell.

The fluorescent spectra were obtained with the aid of Dr. N. Ling.

The autoradiograms were obtained with the help of Dr. M. Schlesinger.

The work with Dowex-50 and the pK' determinations were carried out with the help of Mr. U. Al-Khalidi.

The xanthurenic acid was synthesized by Mr. W. Dempsey.

The 3-hydroxykynurenine standard was a gift of Dr. L. M. Henderson.

The 8-hydroxyquinaldic acid and 8-methoxyquinaldic acid were gifts of Dr. J. M. Price.

Constant encouragement and support were given by the author's parents and family.

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TRYPTOPHAN METABOLISM IN THE KIDNEY

FOREWORD

Rat kidney slices convert kynurenine to kynurenic acid and to a highly fluorescent unidentified metabolite (1). The purposes of this investigation were to isolate and characterize the unknown metabolite, to learn how it is formed from kynurenine *in vitro* and whether it is formed *in vivo*. Precursors of the unknown were synthesized for this purpose by procedures described in Appendix I. The metabolism of various related compounds was also studied.

Early in the investigation it was noted that the unknown was more intensely fluorescent than the substrate from which it was formed. The intense fluorescence made it appear to be much more abundant on paper chromatograms than it actually was and this led to special difficulties in purification. This difference in fluorescent intensities also led to an investigation of the fluorescent and phosphorescent properties of a number of compounds as related to their structures. Besides being of help in the identification of the fluorescent unknown, this study, described in Appendix II, provides information concerning the fundamental properties of these compounds which may be useful in the application of fluorescence measurements to the study of biochemical reaction mechanisms.

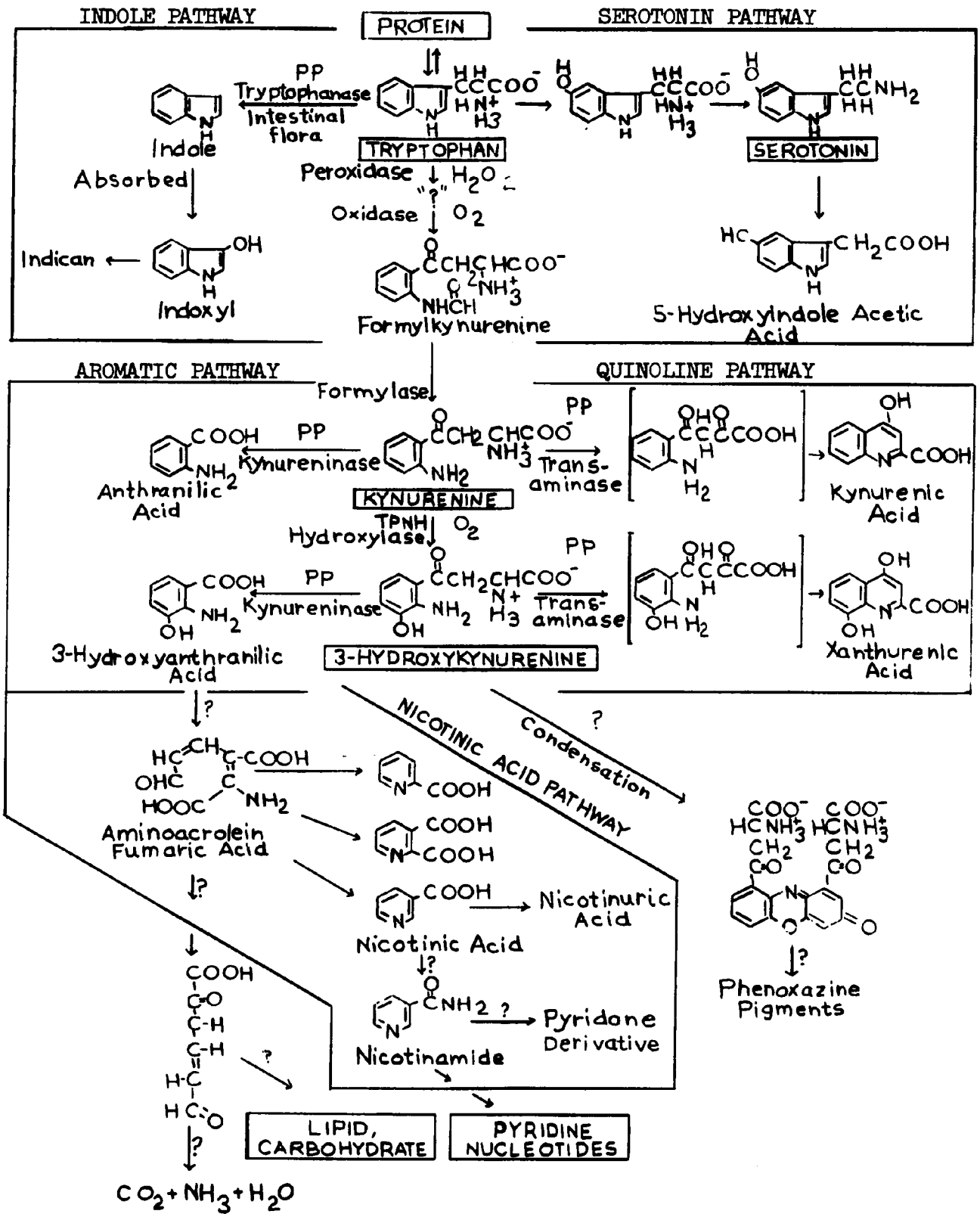
HISTORICAL BACKGROUND

A. General Aspects of Tryptophan Metabolism

Tryptophan is converted into a large number of metabolic products in animal tissues. Several recent and comprehensive reviews of tryptophan metabolism describe the reactions by which they are formed (2, 3). Although a detailed review of tryptophan metabolism is not necessary here, a diagram which summarizes many of the reactions is presented in Figure 1 for the sake of convenience in the discussion. A number of metabolites, especially those occurring in plants, microorganisms and insects, are not included in the diagram nor are the conjugated forms of some of the metabolites. Such conjugated tryptophan metabolites have been described. For example, in Table 1 conjugates are listed which were isolated from the urine of pyridoxine or riboflavin-deficient rats and tentatively identified (4).

A large portion of ingested tryptophan is rapidly metabolized in experimental animals to form carbon dioxide, water, urea and ammonia (5, 6). The details of the pathways of these conversions are still under investigation. 3-Hydroxyanthranilic acid, which is formed from tryptophan via kynurenine and 3-hydroxykynurenine, appears from isotopic studies to be a key intermediate (7). The rupture of the aromatic ring of 3-hydroxyanthranilic acid, known to occur in the formation of nicotinic acid derivatives, seems a logical step in the formation of aliphatic products of metabolism.

Figure 1. The intermediary metabolism of tryptophan



Note:

The abbreviations used are: pyridoxal phosphate (PP), reduced triphosphopyridine nucleotide (TPNH).

Table 1

Excretion of conjugated tryptophan metabolites by pyridoxine or riboflavin-deficient rats (4)

Metabolite Excreted	Pyridoxine Deficient Diet	Riboflavin Deficient Diet
N_{α}-Acetylkynurenine	Positive	Some
Anthranilyl glycine	Negative	Positive
Anthranilyl glucosiduronic acid	Negative	Positive
N_{α}-Acetyl 3-hydroxy-kynurenine	Positive	Some
O-(3-Hydroxykynureninyl) sulfate	Positive	Negative
O-(3-Hydroxykynureninyl) glucosiduronic acid	Positive	Negative

Note:
"Conjugated metabolite" denotes a product formed in a synthetic chemical reaction; especially one formed by chemical union (i. e., conjugation) of a reactant with one of the following moieties: acetate, cysteinyl, glucuronyl, glutamyl, glycyl, methyl, ornithinyl, or sulfate. The term "conjugation" is not used here in an electromeric sense.

B. Metabolism by the Quinoline Pathway

A much smaller amount of ingested tryptophan is metabolized via the Quinoline Pathway (Figure 1) (8). Quinoline derivatives such as kynurenic acid and xanthurenic acid have long been known to occur in urine. They are detected in small amounts in the urines of normal humans, even during periods when they are in negative nitrogen balance. For this reason, Price (8) has emphasized that these compounds should not be considered as abnormal metabolites as they have been frequently described.

Kynurenic acid was among the first intermediary metabolites to be isolated. It was isolated by Liebig (9) in 1853 from dog urine. It was shown to be a product of tryptophan metabolism by Ellinger (10) in 1904 and the structure was established by Homer (11) in 1914.

Xanthurenic acid was isolated by Musajo (12) from the urine of rabbits on a high-protein diet in 1934. He identified it as 4, 8-dihydroxyquinaldic acid (or 8-hydroxykynurenic acid) and synthesized it (13, 14). He demonstrated that the administration of either tryptophan or kynurenine or 3-hydroxykynurenine would cause the formation of xanthurenic acid (15) and that its excretion was responsible for the green color produced on the addition of iron salts to the urine of animals fed a high-protein diet (16). Lepkovsky et al (17) demonstrated that pyridoxine-deficient rats excreted large amounts of xanthurenic acid in their urine. More recent work indicates that this is primarily a result of depletion of kynureninase through the loss of its coenzyme, pyridoxal phosphate (18).

Reid et al (19) reported that when xanthurenic acid was fed to

rats along with pyridoxine none was excreted in the urine. This indicated the existence of enzymes capable of catalyzing its breakdown. Such breakdown is known to occur with bacteria of the Pseudomonas group (20). Kotake et al (21) performed experiments with rat and rabbit liver and kidney preparations which they interpreted as indicating this enzymatic breakdown. The enzyme was named "xanthurenicase". No extension or confirmation of their experiments has been reported.

Recently, two laboratories reported the formation of xanthurenic acid conjugates by rats. In 1954, Kotake and Nogami (22) reported the presence of two, fluorescent, xanthurenic acid metabolites in the urine of rats injected with xanthurenic acid. One was described as probably the 4-monoglucuronide of xanthurenic acid and the other as the disulfate ester (23).

While our work was in progress, Rothstein and Greenberg (24) reported the synthesis of xanthurenic acid-4 C^{14} and its metabolism by rats and rabbits. These authors were able to account for all of the administered xanthurenic acid (even when given concomitantly with pyridoxine) as free xanthurenic acid and two fluorescent derivatives in the urine. They recognized the similarity of the properties of the two derivatives with those described by Kotake and Nogami (22) and with the unidentified in vitro products of kynurenine and xanthurenic acid metabolism reported by Mason and Berg (1). They characterized the derivatives, however, as the serine conjugate of 8-xanthurenyl glucosiduronic acid and as the serine conjugate of 4, 8-xanthurenyl diglucosiduronic acid. This was the first reported instance of serine being utilized to conjugate a fed aromatic compound.

Although Rothstein and Greenberg (24) reported complete recovery of administered xanthurenic acid as unreacted xanthurenic acid and the two conjugated derivatives, Price et al (25, 26) have found small amounts of other quinoline derivatives in urine. They isolated the 8-methyl ether of xanthurenic acid from human urine. More recently they reported the presence of 8-hydroxyquinaldic acid in rat urine, arising presumably by reduction of the 4-hydroxyl group of xanthurenic acid (27). Rabbit urine has been found to contain a compound identified as quinaldylglycine. Price et al, have also found that kynurenic acid is converted by rats and humans to quinaldic acid, which has also been obtained from rabbit urine (28). Another quinoline derivative identified as 6-hydroxykynurenic acid was detected in pig urine (28). Other quinoline derivatives which have been reported are 4, 8-dihydroxyquinoline and kynurine (4-hydroxyquinoline). Kynurine was found in silkworm pupae (29) and 4, 8-dihydroxyquinoline in silkworm pupae urine (3). 4, 8-Dihydroxyquinoline was also formed from kynurenine by homogenates of mouse liver (31).

C. Tryptophan Metabolism in Various Diseases

The relationship of xanthurenic acid excretion to various diseases has been investigated in many laboratories, perhaps partly because it is easily measured by the distinctive green color formed with iron salts. The amount of xanthurenic acid excreted after a standard dose of tryptophan is often used as an index of pyridoxine deficiency (32). Excretion of xanthurenic acid is elevated less markedly when there is a deficiency of riboflavin, thiamin, or α-tocopherol (4, 33, 34).

Increased amounts of xanthurenic acid have been found in the

urines of patients with severe tuberculosis (35, 36), leukemia (37), and with certain neurological disorders (38). Increased excretion also occurs during pregnancy (39). Excretion was reported to be increased in rats after hypophysectomy and decreased after adrenalectomy (40).

The increased xanthurenic acid excretion occurring during various diseases is probably of little diagnostic importance. Dalgleish (41) has shown that the excretion of 3-hydroxykynurenine is elevated in patients with fever regardless of the disease causing the fever. Such excretion is apparently a result of the increased protein breakdown which accompanies fever. Since 3-hydroxykynurenine is the direct precursor of xanthurenic acid, increased excretion of xanthurenic acid probably has the same cause. Hence, xanthurenic acid excretion may be increased either by vitamin deficiency or by non-specific factors associated with fever of any origin.

Boyland et al (42, 43, 44) have found that 3-hydroxyanthranilic acid, 3-hydroxykynurenine and 8-methoxyxanthurenic acid are carcinogenic when impregnated into paraffin pellets and implanted into the bladders of mice. These compounds, structurally similar to the industrial carcinogen, β-naphthylamine, might be direct causitive agents of cancer of the bladder in humans.

There are a number of papers in which a possible relationship of xanthurenic acid to the etiology of diabetes mellitus is discussed. Okamoto (45) discovered that many zinc-binding reagents are diabetogenic. Presumably, these reagents exert their action by chelating zinc in the beta cells of the pancreas (which have a relatively high zinc content). Although Okamoto did not find xanthurenic acid to be diabet-

ogenic, Kotake et al (46 - 52) reported that rats fed on a high-fat diet which were injected with xanthurenic acid or fed tryptophan eventually developed the symptoms of diabetes. They did not observe a diabetogenic effect with the 8-methyl ether of xanthurenic acid (23). Moreover, they found xanthurenic acid in the urine of diabetics but not in the urines of healthy controls (53). In the urines of the controls, they found instead another fluorescent compound which they assumed to be the conjugate of xanthurenic acid described by Dalgleish et al (54). In an in vitro experiment, they found that xanthurenic acid inhibits the hexokinase reaction in 10^{-5} M concentration and thus may influence glucose metabolism at that stage (55).

Weitzel et al (56) found that a zinc-xanthurenic acid chelate (1:1) and a zinc-xanthurenic acid-insulin complex are formed. They concluded from the results they obtained with rabbits that xanthurenic acid is not a strong diabetogenic agent but that prolonged administration of tryptophan and fat can cause hyperglycemia and glycosuria. v. Holt et al (57) and Markees (58) could not show a diabetogenic effect of xanthurenic acid. Rosen et al (59) found more xanthurenic acid to be excreted by diabetics after test-loads of tryptophan than by non-diabetics.

In a study of quinoline derivatives, Kodata and Abe (60, 61) found that a free 8-hydroxyl group is essential for diabetogenesis. The presence of other hydroxyl or carboxyl groups abolished the effect and decreased the toxicity. Perhaps xanthurenic acid (which sometimes appears diabetogenic and at other times non-diabetogenic) is such a weak diabetogen that positive effects are not always to be expected, especially in short-term experiments.

Although the relationships of the intermediates of the Quinoline Pathway to the etiologies of diabetes, bladder cancer and other diseases are still unsettled, there is a good possibility that such compounds have specific effects on the economies of the organisms that produce them. The metabolite with which this investigation is concerned, a quinoline derivative, would be relevant in any such relationships.

D. Role of the Kidney in Tryptophan Metabolism

The metabolism of tryptophan differs in the various organs. Most of the reactions shown in Figure 1 can occur in rat liver. Rat kidney, however, appears to be unable to carry out several of these steps (1). The conversion of L-tryptophan to kynurenine apparently does not occur in the kidney, although kynurenine is converted to kynurenic acid and unidentified fluorescent metabolites. The kidney has a low kynureninase activity and a high transaminase activity and the production of anthranilic acid and 3-hydroxyanthranilic acid is small (62). Consequently, non-aromatic products which derive from 3-hydroxyanthranilic acid are not expected as major products of the metabolism of kynurenine in the kidney. However, 3-hydroxyanthranilic acid is rapidly metabolized by kidney slices (1), and is converted in large measure to quinolinic acid by kidney homogenates (63).

Because of the weak kynureninase activity and the relatively strong kynurenine transaminase activity, the major pathway of kynurenine metabolism in the kidney might be expected to occur by the Quinoline Pathway rather than by the Aromatic-Nicotinic Acid Pathway. For this and other reasons, it was suspected at the beginning of the work reported here that the fluorescent product formed from kynurenine by kidney

slices might be a quinoline derivative or that the Quinoline Pathway might be involved in its formation. Early experiments were, therefore, designed to test whether kynurenine and xanthurenic acid formed identical products and whether enzymes capable of converting kynurenine to xanthurenic acid were present in the kidney. Later efforts were directed toward the proof of the structure of the unknown metabolite.

PART I. THE ACTION OF RAT KIDNEY PREPARATIONS ON TRYPTOPHAN METABOLITES AND VARIOUS RELATED QUINOLINE DERIVATIVES

A. Introduction

Part I of this thesis describes preliminary attempts, based largely on chromatographic techniques, to characterize and compare the fluorescent compounds formed from kynurenine, 3-hydroxykynurenine and xanthurenic acid by kidney slices. Since the products from these three substrates had similar properties, the question arose as to whether kynurenine might be converted to the fluorescent derivative through the intermediate formation of 3-hydroxykynurenine and xanthurenic acid. As a test of the possibility of the occurrence of such a sequence of reactions, rat kidney mitochondria were assayed for kynurenine hydroxylase, the enzyme involved in the conversion of kynurenine to 3-hydroxykynurenine. Further information concerning the nature of the unknown(s) was sought through a study of the relationship of the structures of a variety of quinoline derivatives, some of these known to be tryptophan metabolites, to their abilities to form fluorescent metabolites when incubated with kidney slices.

B. Experimental

1. Incubation procedure

One milligram of the substrate was neutralized with an equivalent amount of 0.1 N NaOH and the volume made up to 3.0 ml. with Krebs-Ringer phosphate buffer, pH 7.4. Three or four slices of rat kidney, prepared by slicing one-half of a rat kidney with a Stadie-Riggs slicer, were added and the resulting incubation mixture incubated with shaking in a Dubnoff incubator at 37° in an atmosphere of oxygen for three hours.

2. Source of substrates

The preparation and properties of L-kynurenine, L-3-hydroxykynurenine, D-3-hydroxykynurenine, DL-3-methoxykynurenine, xanthurenic acid, 8-methoxyxanthurenic acid and 4, 8-dihydroxyquinoline are given in Appendix I. Kynurine (C Grade) was obtained from the California Foundation for Biochemical Research, Incorporated. Kynurenic acid was a product of Hoffman-La Roche, Incorporated. 8-Hydroxyquinoline (reagent grade) was a product of The G. Frederick Smith Chemical Company. Quinaldic acid was a product of Eastman Kodak Company. The 8-hydroxyquinaldic acid was a gift from Dr. J. M. Price, University of Wisconsin.

3. Chromatographic procedures

The incubation mixtures were spotted two inches from the margin of Whatman #1 chromatography paper. Chromatograms were then developed by the ascending method with the following solvents:

(a) methanol: n-butanol: benzene: water:: 2: 1: 1: 1, designated MBBW,

(b) n-butanol: glacial acetic acid: water:: 4: 1: 5, organic phase, designated BAW,

(c) 20% potassium chloride in water, w/v, designated KCl,

(d) isobutyric acid: water: ammonia water (a solution of 28-30% NH_3 in water):: 50: 30: 1, designated IBA.

When the solvent front had migrated approximately 30 cm., the paper was removed from the chromatography jar, dried and inspected under ultraviolet light. A Hanovia Inspectolite was used as a source of light rich in the longer wavelengths and a hand mineral lamp (Mineralite) as a source emitting the shorter wavelengths.

C. Results

1. A study of fluorescent artifacts produced on paper chromatograms

by interaction of kynurenine, 3-hydroxyanthranilic acid or xanthurenic acid with inorganic salts

When the incubation mixtures were chromatographed, fluorescent spots were observed which appeared to be related to the presence of the salts in the incubation media. Apparently some of the metabolites were bound to these salts during development of the chromatograms. Since something of the nature of this phenomenon had to be known to interpret the chromatograms, the following experiment was performed. Saturated solutions of a series of tryptophan metabolites were spotted on paper together with Krebs-Ringer phosphate solution and the chromatograms were developed with MBBW. The results are shown in Table 2. The formation of multiple spots due to the salts appears to be associated with the presence of the kynurenine, o-aminophenol or 8-hydroxyquinoline structure, indicating that the basis of the phenomenon may be chelation of the cations present in the medium. When the chromatograms were sprayed with silver nitrate (see Appendix III) and heated, a salt area with an R_f value of 0.49 was revealed. This latter spot also had initially a weak fluorescence.

2. Metabolism of L-kynurenine, L-3-hydroxykynurenine, D-3-hydroxykynurenine, 3-methoxykynurenine and xanthurenic acid by rat kidney slices

Figures 2-7 illustrate the appearance of chromatograms obtained when incubation mixtures containing L-kynurenine, L-3-hydroxykynurenine, D-3-hydroxykynurenine, DL-3-methoxykynurenine and xanthurenic acid were spotted before and after the incubation period. The R_f values are given in Table 3. In every case but 3-methoxykynurenine, a fluorescent product with a value, using MBBW, of about 0.25 was formed. All of these fluorescent products appear as especially brilliant, blue-white spots

Table 2

Production of fluorescent artifacts with certain tryptophan metabolites in the presence of Krebs-Ringer salts

Compound	R_f value obtained in MBBW	
	with salts	without salts
Anthranilic Acid	0.80	0.82
3-Hydroxyanthranilic Acid	0.74 0.48 0.38	0.71
L-Kynurenine	0.83 0.45	0.44
DL-3-Hydroxykynurenine	0.28 (D-) 0.34 (L-)	0.30 (D-) 0.36 (L-)
Kynurenic Acid	0.57	0.56
Xanthurenic Acid	0.57 0.39	0.56

Figures 2 and 3. Chromatograms obtained using MBBW solvent of media containing L-kynurenine, xanthurenic acid, L-3-hydroxykynurenine, or D-3-hydroxykynurenine before and after incubation with rat kidney slices

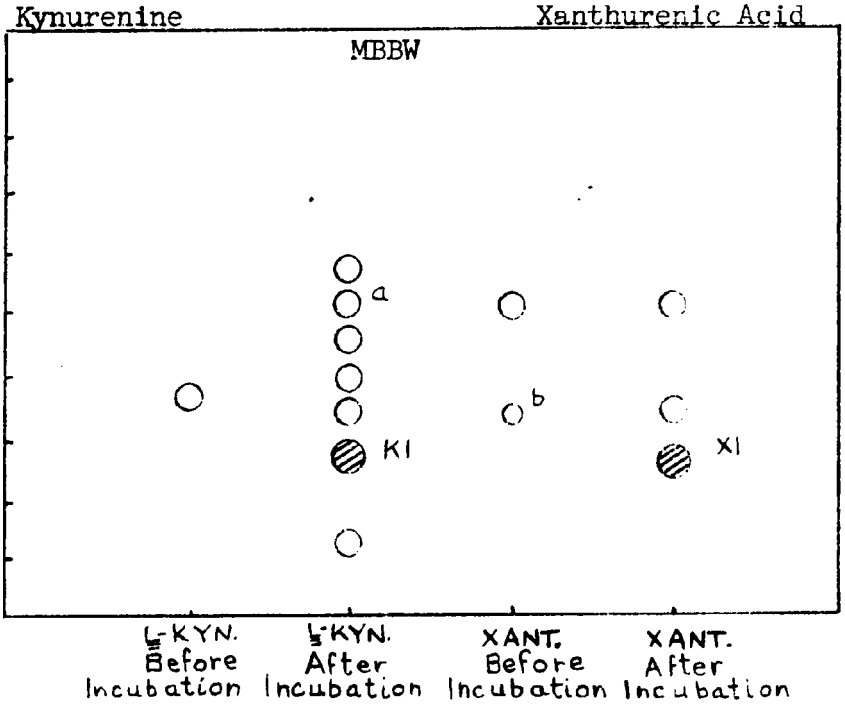


Figure 2.

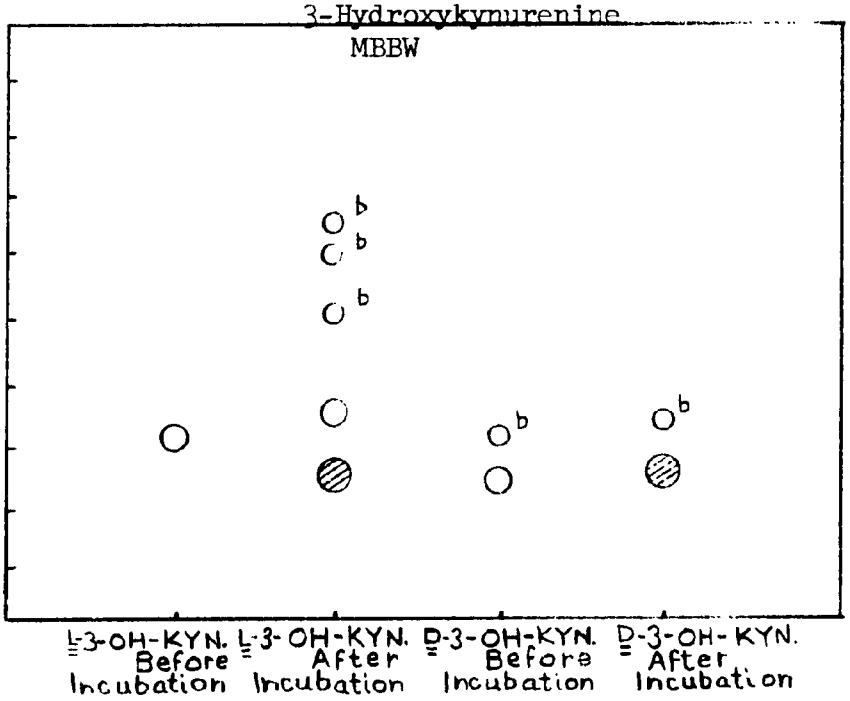


Figure 3.

[a]Kynurenic acid. [b]Weak fluorescence.

Note:
All chromatograms are depicted schematically. The hatched spots in Figures 2-7 represent similar derivative(s) formed during the incubation. See Table 4 for a comparison of R_f values.

Figures 4 and 5. Chromatograms obtained using KCl and BAW solvents of media containing xanthurenic acid or L-3-hydroxykynurenine before and after incubation with rat kidney slices[a]

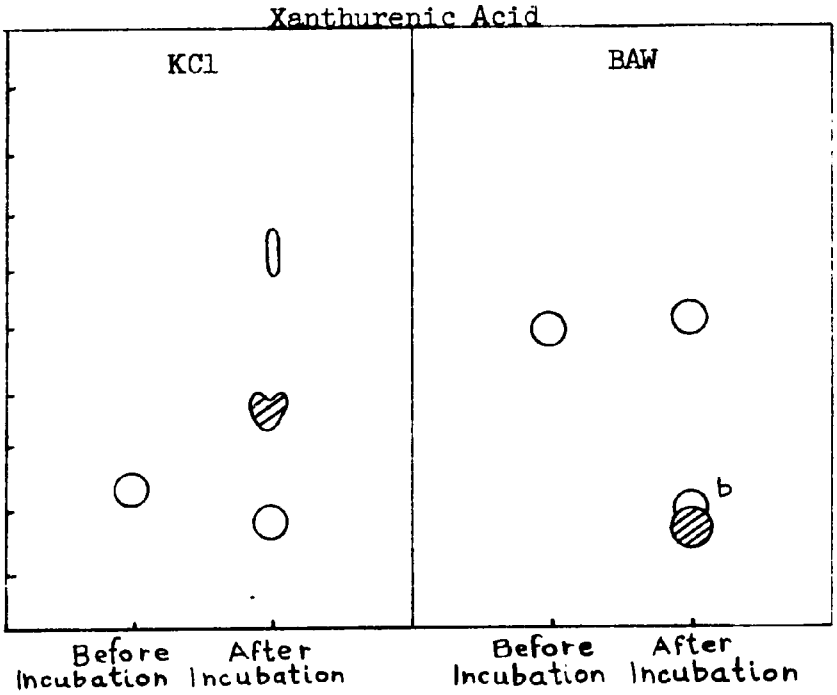


Figure 4.

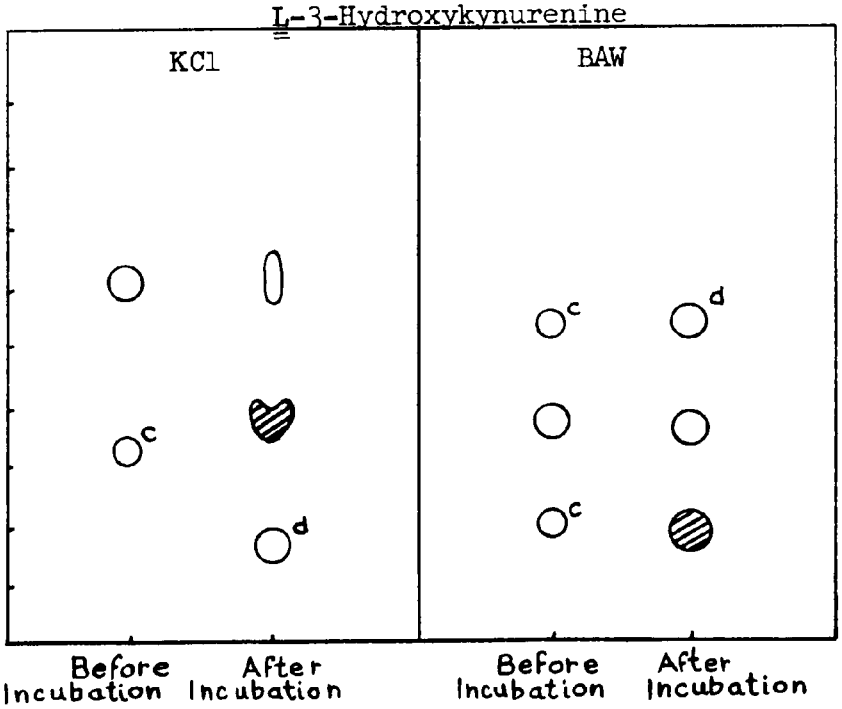


Figure 5.

[a]See note on page 16. [b]Overlapping spot assumed to be an artifact such as discussed on page 13. [c]Weak fluorescence. [d]Xanthurenic acid.

Figures 6 and 7. Chromatograms obtained using KCl and BAW solvents of D-3-hydroxykynurenine and of media containing D-3-hydroxykynurenine after incubation with rat kidney slices, and chromatograms obtained using KCl, BAW and MBBW solvents of media containing DL-3-methoxykynurenine before and after incubation with rat kidney slices[a]

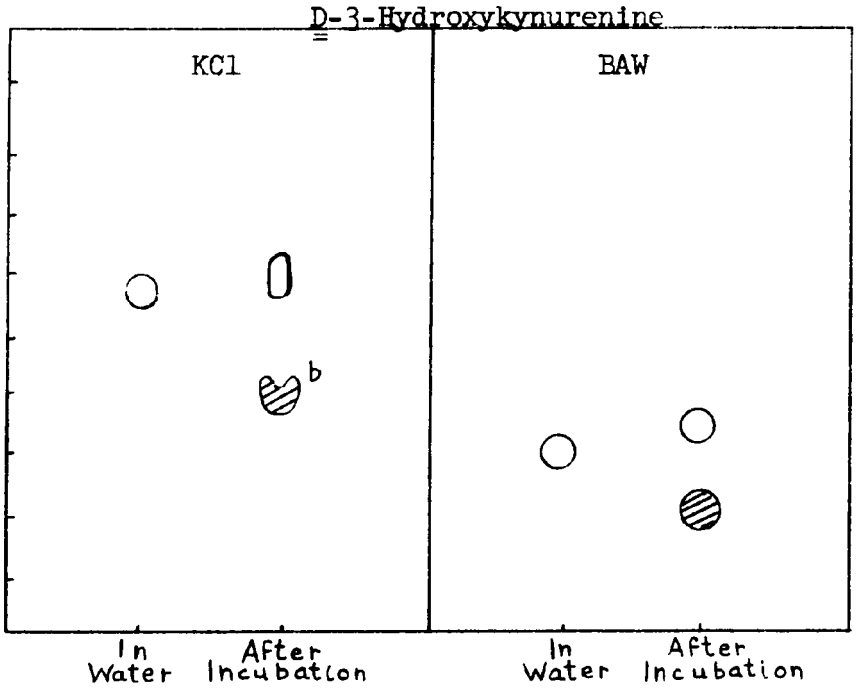


Figure 6.

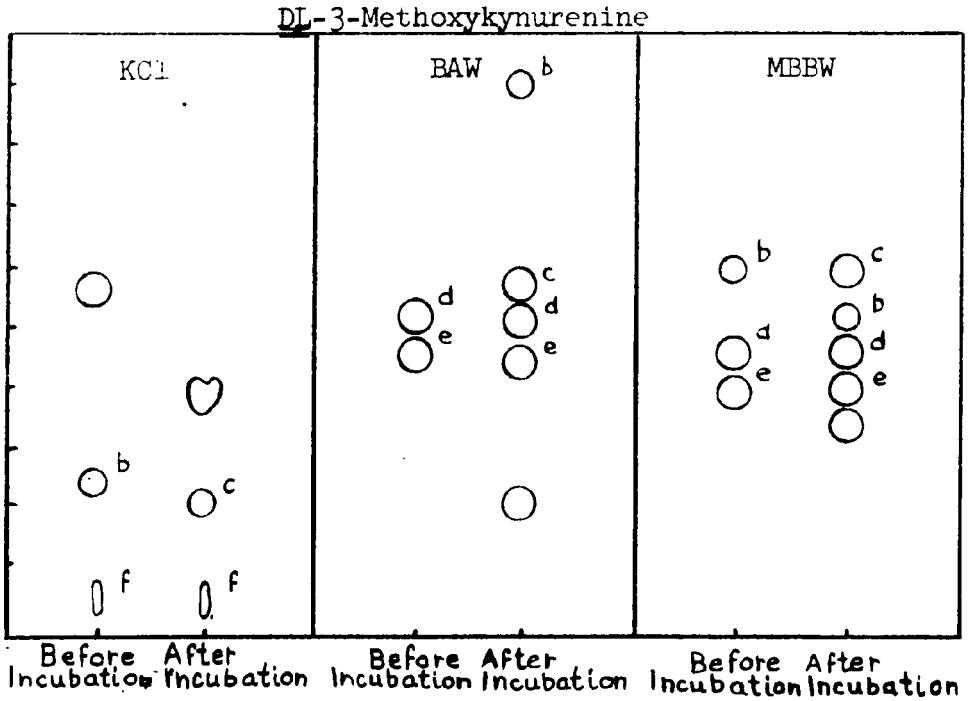


Figure 7.

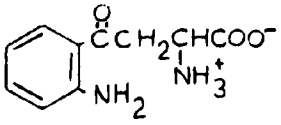
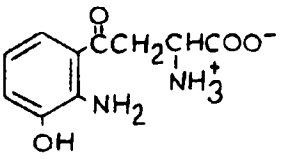
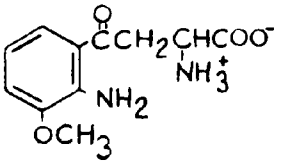
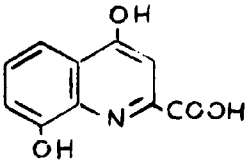
[a]See note on page 16. [b]Weak fluorescence.

[c]8-Methoxyxanthurenic acid. [d]L-isomer. [e]D-isomer.

[f]Fluoresces when illuminated with the Mineralite.

Table 3

Chromatographic data obtained after incubation of kynurenine, 3-hydroxykynurenine, 3-methoxykynurenine and xanthurenic acid with rat kidney slices[a]

Structure of substrate	Name of substrate	R_f values of major fluorescent spots using			Probable name of product
		KCl	BAW	MBBW	
	L-Kynurenine	0.48	0.18	0.28	KI
		0.33	0.44	0.53	Kynurenic Acid
		0.48	0.32	0.38	L-Kynurenine
	DL-3-Hydroxy-kynurenine	0.37	0.19	0.25	Unknown[c]
		0.16	0.54	0.51	Xanthurenic Acid
		0.49[b]	0.31	0.24	D-3-Hydroxy-kynurenine
		0.50[b]	0.32	0.32	L-3-Hydroxy-kynurenine
	DL-3-Methoxy-kynurenine	0.57	0.39	0.46	D-3-Methoxy-kynurenine
		0.57	0.44	0.51	L-3-Methoxy-kynurenine
		0.20	0.55	0.59	8-Methoxy-xanthurenic Acid[d]
	Xanthurenic Acid	0.35	0.17	0.25	XI
		0.16	0.51	0.51	Xanthurenic Acid

[a]These R_f values are only approximate. They were found to vary from one experiment to another.

[b]The isomers do not separate in this solvent; these two values obtained on different chromatograms.

[c]Compare with KI and XI.

[d]R_f values obtained for synthetic 8-methoxyxanthurenic acid in KCl, BAW and MBBW, respectively, are 0.26, 0.51 and 0.57.

under ultraviolet light and are apparently identical. However, to avoid assumption of identity on the basis of these chromatographic data alone, the products formed from kynurenine and from xanthurenic acid will be designated KI and XI to specify their origins (see Table 4).

3. Metabolism of other tryptophan metabolites and related quinoline derivatives by rat kidney slices

Figures 8-14 illustrate the appearance of chromatograms which were obtained when incubation mixtures containing 4, 8-dihydroxyquinoline, 8-hydroxyquinoline, 8-hydroxyquinaldic acid, quinaldic acid, kynurine, kynurenic acid and 8-methoxyxanthurenic acid were spotted before and after incubation. A chromatogram of the medium incubated without substrate gave a weakly fluorescent spot with an R_f value of 0.35 when MBBW was used, and both a faint spot with an R_f value of 0.27 and a weakly fluorescent streak with an R_f value of 0.55 when KCl was used.

Dihydroxyquinoline, kynurine and quinaldic acid do not fluoresce immediately upon exposure to ultraviolet light but require prolonged exposure. This was accomplished by leaving the developed chromatograms exposed to daylight for about one week.

It appears that fluorescent derivatives were formed only in those cases in which the substrate possessed an 8-hydroxyl group. Further investigation (of these apparent products) is required before definite conclusions can be drawn. The R_f values are shown in Table 5.

4. Hydroxylation of L-kynurenine by rat kidney mitochondria

The experiment described here is patterned after the work of de Castro, Price and Brown (64) which showed that rat liver mitochondria can convert kynurenine into 3-hydroxykynurenine. If the kynurenine

Table 4

R_f values of the blue-white, fluorescent products formed from kynurenine (KI), 3-hydroxykynurenine and xanthurenic acid (XI) by the rat kidney slice[a]

Substrate	R_f values using		
	MBBW	BAW	KCl
Kynurenine	0.26 0.27	0.08 0.18	0.48 --
3-Hydroxykynurenine	0.28 0.25*	0.15 0.17*	0.50 0.35*
Xanthurenic Acid	0.25*	0.19*	0.37*

[a] R_f values were found to vary in solutions from one experiment to another, probably due to variations in the amounts of contaminants present (see Part III). The values given in this table were not obtained simultaneously, except for the R_f values of 3-hydroxykynurenine and xanthurenic acid marked by an asterisk.

Figures 8 and 9. Chromatograms obtained using KCl, BAW and MBBW solvents of media containing 4, 8-dihydroxyquinoline or 8-hydroxyquinoline before and after incubation with rat kidney slices

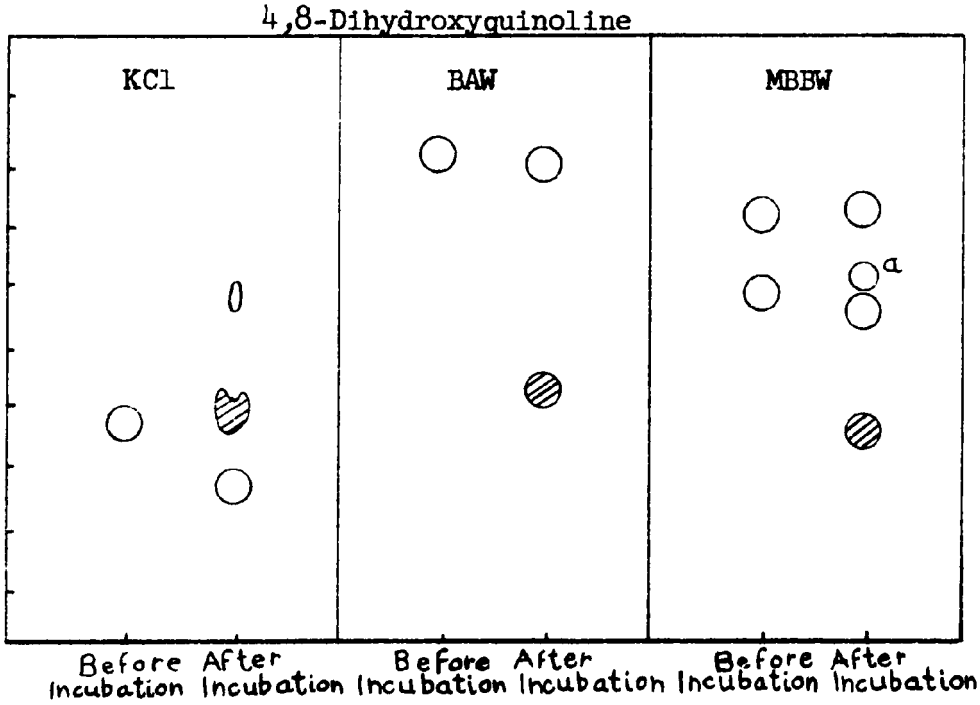


Figure 8.

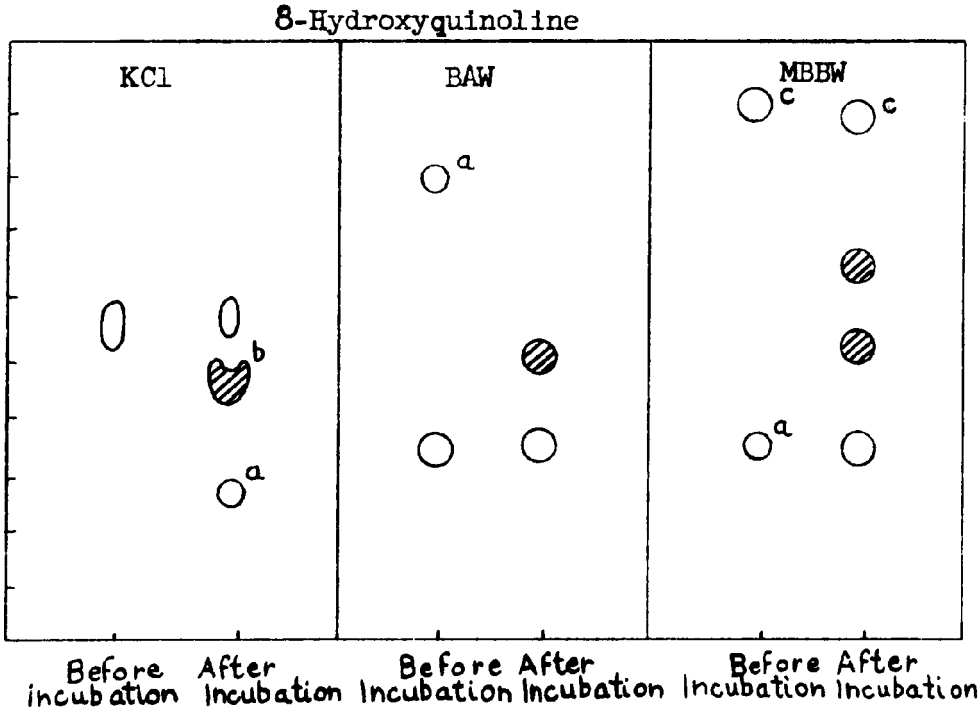


Figure 9.

[a]Weak fluorescence. [b]Fluoresces when illuminated with the Mineralite. [c]Absorbing region.

Note:

The hatched spots indicate the position of possible derivatives formed during the incubation. See footnote c, Table V.

Figures 10 and 11. Chromatograms obtained using KCl, BAW and MBBW solvents of media containing 8-hydroxyquinaldic acid or quinaldic acid before and after incubation with rat kidney slices[a]

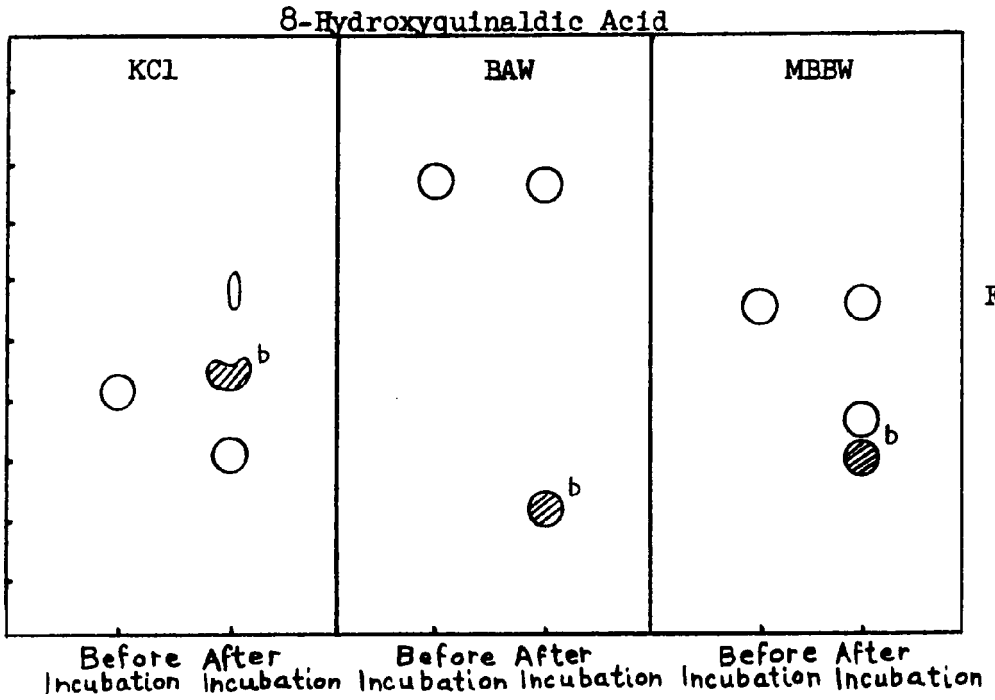


Figure 10.

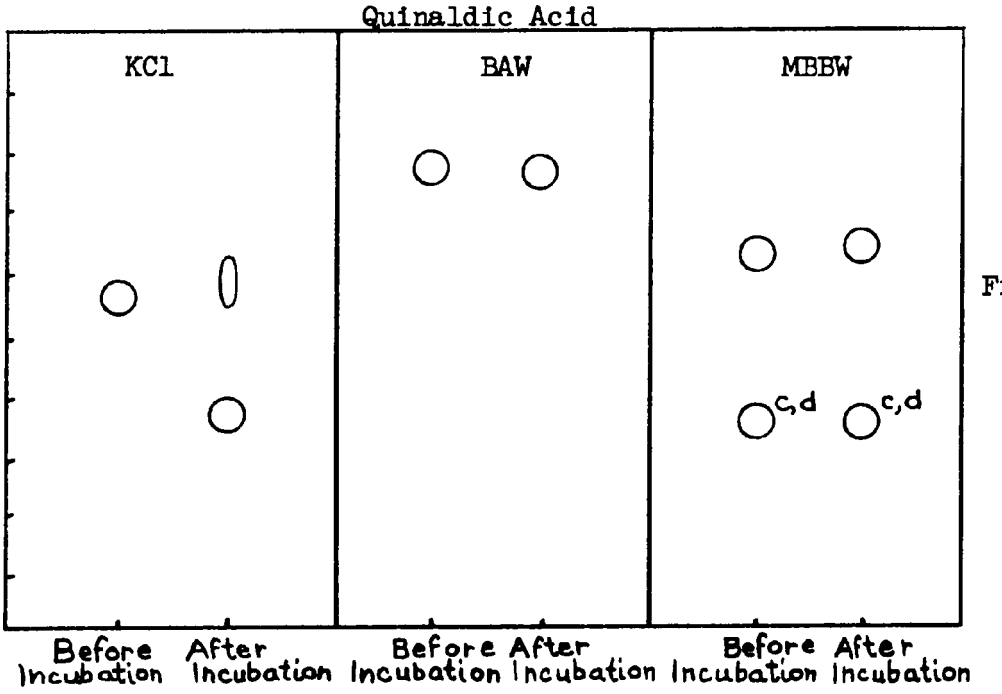


Figure 11.

[a]See note on page 22. [b]Fluoresces when illuminated with the Mineralite. [c]Weak fluorescence.

[d]Assumed to be artifacts such as are discussed on page 13.

Figures 12 and 13. Chromatograms obtained using KCl, BAW and MBBW solvents of kynurenic acid and of media containing kynurenic acid after incubation with rat kidney slices, and chromatograms obtained using KCl, BAW and MBBW solvents of 8-methoxyxanthurenic acid before and after incubation with rat kidney slices[a]

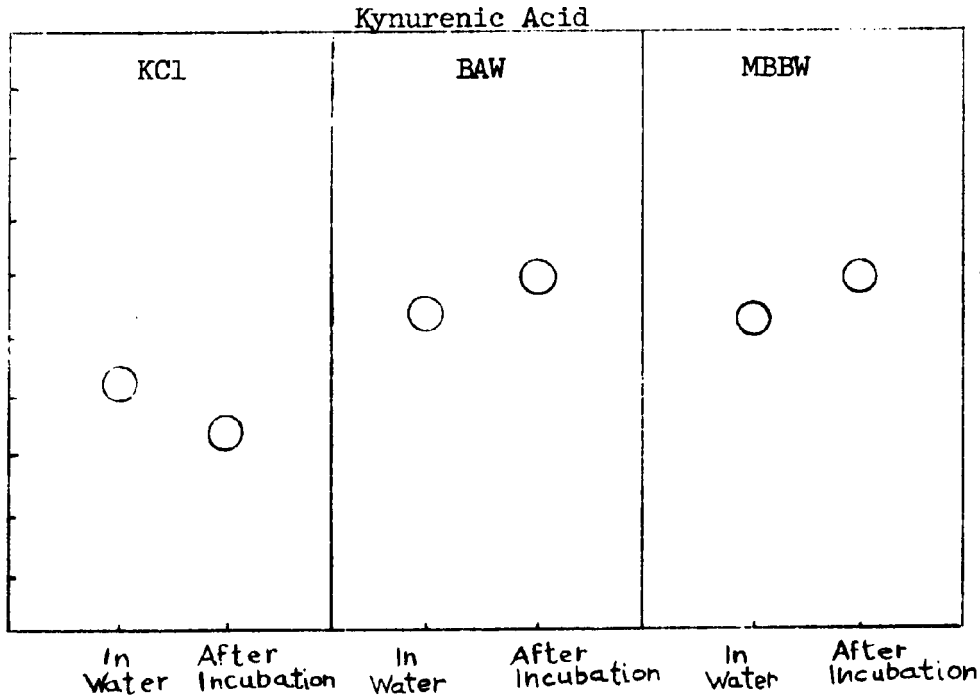


Figure 12.

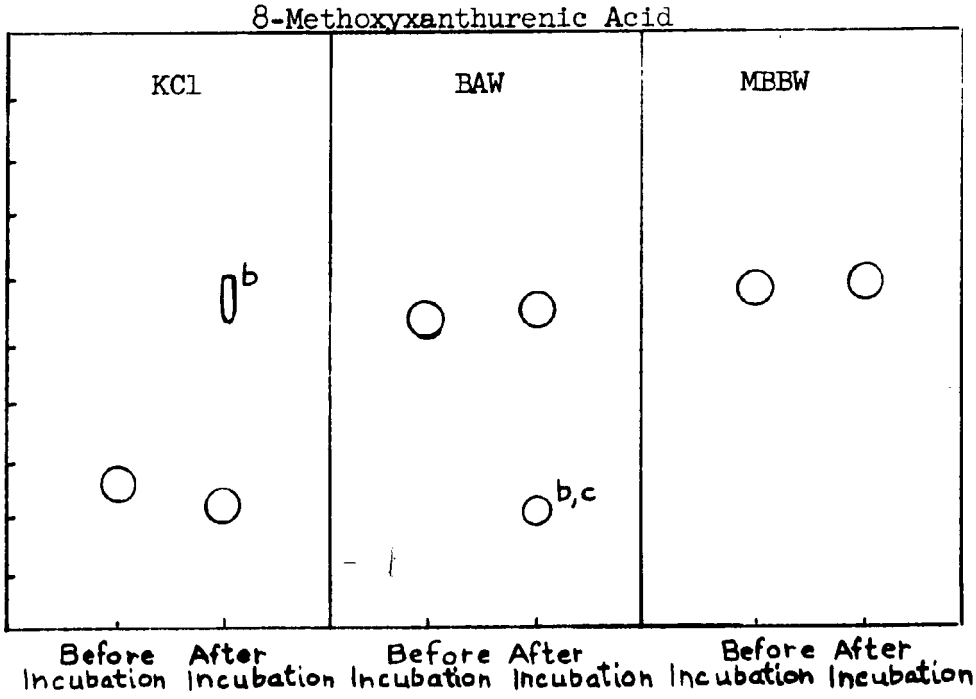


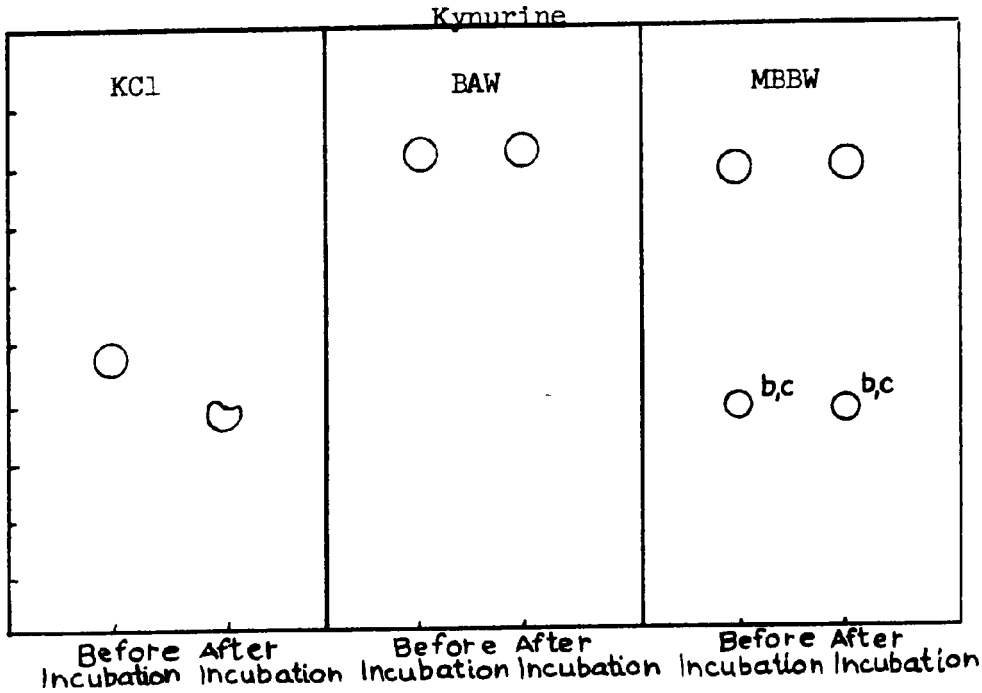
Figure 13.

[a]See Note on page 22.

[b]Assumed to be artifacts such as are discussed on page 13.

[c]Weak fluorescence.

Figure 14. Chromatograms obtained using KCl, BAW and MBBW solvents of media containing kynurine before and after incubation with rat kidney slices[a]



[a]See note on page 22.

[b]Assumed to be artifacts such as are discussed on page 13.

[c]Weak fluorescence.

Table 5

Chromatographic data obtained after incubation of quinoline compounds with rat kidney slices

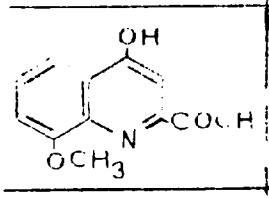
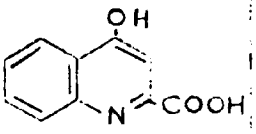
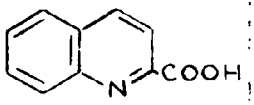
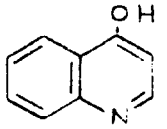
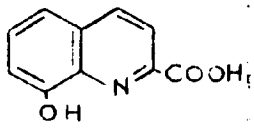
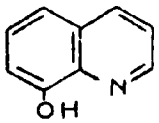
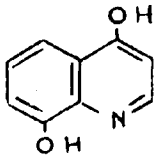
Structure of substrate	Name of substrate	Rf values of apparent, fluorescent derivatives obtained using		
		KCl	BAW	MBBW
	3-Methoxy-xanthurenic Acid	Negative	Negative	Negative
	Kynurenic Acid	Negative	Negative	Negative
	Quinaldic Acid	Negative	Negative	Negative
	Kynurine	Negative	Negative	Negative
	8-Hydroxy-quinaldic Acid	0.44[a]	0.21[a]	0.29[a]
	8-Hydroxy-quinoline	0.45[a,b,c]	0.50[b,c]	0.63[b,c] 0.51[b,c]
	4,8-Dihydroxy-quinoline	0.38[c]	0.41[c]	0.56[c]

Table 5- Continued

[a]Fluoresces when illuminated with the Mineralite.

[b]R_f values obtained for 8-hydroxyquinoline glucuronide (California Foundation, Purified Grade) were 0.67[a], 0.29 (faint) and 0.17 in KCl; 0.56 and 0.37 in BAW; and 0.76[a] and 0.54 in MBBW.

[c]These spots were weak and, in another experiment, no spots were obtained. Further experiments are required to establish the existence of these reactions in the kidney slice.

hydroxylase activity occurs in rat kidney, the conversion of kynurenine to 3-hydroxykynurenine and then to xanthurenic acid (by the kynurenine transaminase reaction) would be expected to occur. Xanthurenic acid might then be an immediate precursor of the fluorescent product formed from kynurenine although not necessarily an obligatory one.

Incubation of kynurenine with rat kidney slices did not cause an accumulation of 3-hydroxykynurenine, possibly because this product reacts further as soon as it is formed; hence, kidney mitochondria were examined for kynurenine hydroxylase activity. The mitochondria of the kidney were isolated by the method of Hogeboom (65). 0.3 Ml. of this preparation, representing 0.4 g. of kidney, were incubated aerobically for 2 hours at 37° with 2.5 micromoles of L-kynurenine, 0.65 micromoles of triphosphopyridine nucleotide, 33 micromoles of nicotinamide, and 5 micromoles of citric acid in 1.5 ml. of a 0.0125 M phosphate buffer, pH 7.4, containing 0.125 g. of sucrose. Chromatograms of the incubation mixture developed with MBBW showed a yellow, fluorescent spot at an R_f value of 0.32. Synthetic L-3-hydroxykynurenine has an R_f value of 0.34 with this solvent (see Appendix I). This spot gave a positive reaction with Folin's phenol spray, but not with Pauly's diazo spray (see Appendix III). Probably the compound appeared to react negatively with the latter spray because of its low concentration. It seems very likely that the product was L-3-hydroxykynurenine on the basis of its similarity on paper chromatograms to authentic L-3-hydroxykynurenine. (Subsequently to this work Price (66) has also reported the presence of kynurenine hydroxylase in the rat kidney.) This conclusion makes it reasonable to suppose that the blue-white, brightly fluorescent derivatives formed from kynurenine, 3-hydroxykynurenine and xanthurenic

acid are identical and that the product can be formed from kynurenine by the following reaction sequence:

L-kynurenine $\xrightarrow[\text{hydroxylase}]{\text{kynurenine}}$ L-3-hydroxykynurenine $\xrightarrow[\text{transaminase}]{\text{kynurenine}}$

xanthurenic acid $\xrightarrow[\text{enzyme}]{\text{unknown}}$ fluorescent product.

5. Preliminary isolation and characterization of an interfering contaminant

In order to obtain some idea of the nature of the brilliantly fluorescent material, an incubation with kynurenine was carried out by a procedure similar to the one described but on a larger scale. The pooled media were chromatographed using MBBW on Whatman #3 paper and the band containing KI was eluted with water. The spectrum of the eluate (Figure 15) had a maximum at 290 mμ which is not characteristic of xanthurenic acid or other quinoline derivatives. It soon became evident, however, that the eluate was impure. When it was rechromatographed using BAW, four separate bands were observed. One was the bright, blue-white fluorescent band (KI), two were fluorescent bands of lesser intensity, while the remaining band was an ultraviolet-absorbing region, especially visible under the Hanovia lamp. The maximum at 290 mμ in the spectrum mentioned above was due to this absorbing material. The spectra of KI and the contaminant are compared in Figure 16.

In a similar way, xanthurenic acid was incubated with kidney slices and the fluorescent product (XI) was isolated chromatographically using MBBW. The spectrum of the eluate of the chromatogram is shown in Figure 15. In this case, also, the presence of the contaminant could be detected by the absorption at 290 mμ. Compound XI, after the second chromatographic purification, also had a quinoline-type spectrum.

Figure 15. Spectra of KI and XI eluted from MBBW chromatograms

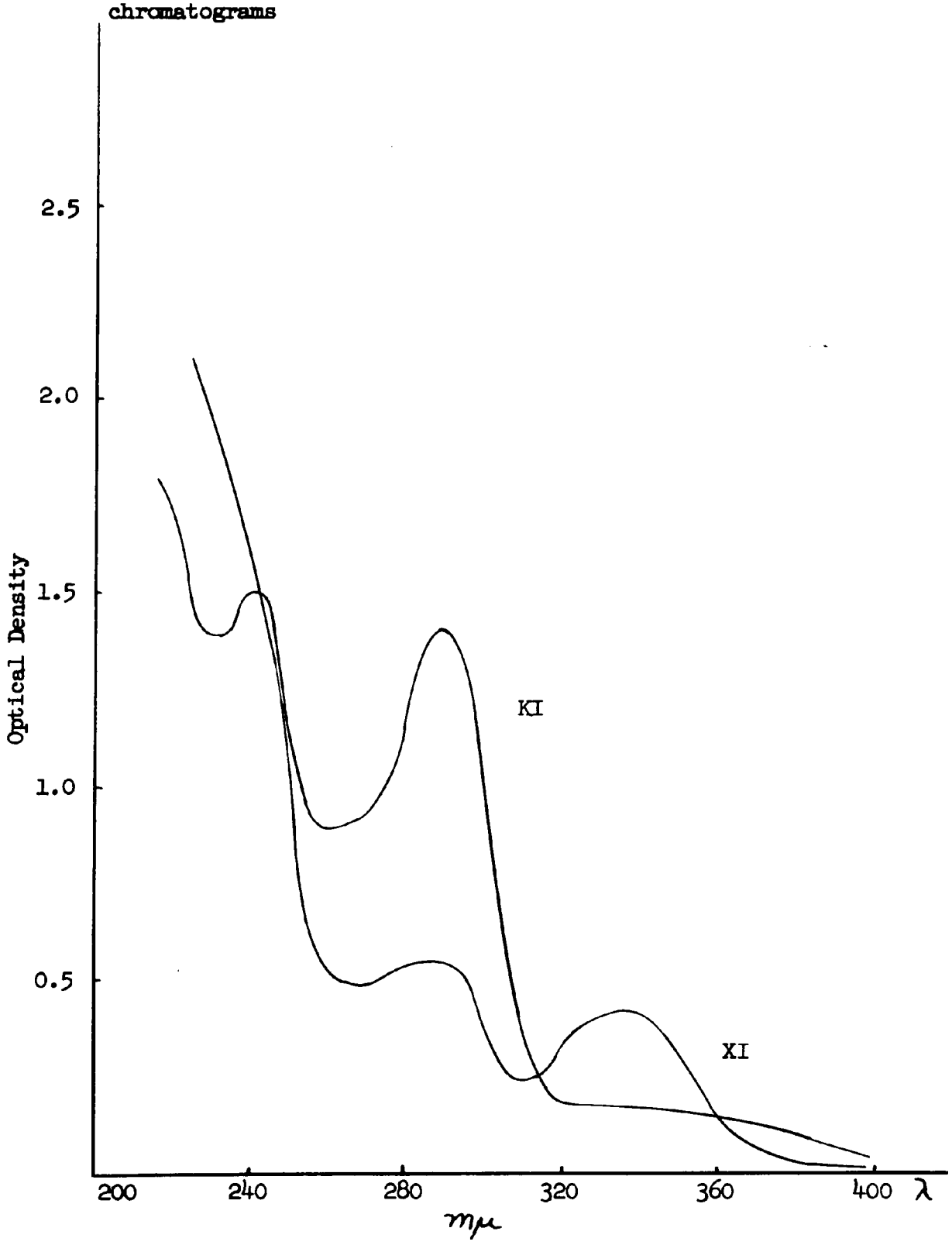


Figure 16. Spectra of KI and of interfering contaminant after separation on BAW chromatograms



Since the spectrum of the interfering material resembled that of uric acid, some of the eluate was chromatographed along with synthetic uric acid. The R_f values agreed (Table 6) and both reacted positively to ammoniacal silver nitrate spray (see Appendix III).

There was enough of the absorbing material remaining for one incubation with uricase. The results are shown in Table 7. The drop in optical density of the test solution relative to the blank indicates that the enzyme catalyzed some destruction of the absorbing substance, confirming that the material was uric acid.

D. Summary

1. The chromatographic development of kynurenine, 3-hydroxyanthranilic acid or xanthurenic acid in the presence of salts was found to produce artifactual spots on paper chromatograms.

2. L-kynurenine was hydroxylated by rat kidney mitochondria to form L-3-hydroxykynurenine.

3. In the incubation of a series of substituted quinoline compounds with kidney slices, only those compounds having an 8-hydroxyl group appear able to form fluorescent products, but further experiments are required before positive conclusions can be drawn.

4. Methoxykynurenine forms one fluorescent product, identified by chromatographic comparison as methoxyxanthurenic acid, upon incubation with kidney slices.

5. L-kynurenine forms kynurenic acid and L-3-hydroxykynurenine forms xanthurenic acid, respectively, upon incubation with kidney slices.

6. Kynurenine, 3-hydroxykynurenine and xanthurenic acid reacted with

Table 6

Comparison of the properties of uric acid with those of the interfering contaminant that absorbs at 290 mμ

Compound	Absorption maximum mμ	R_f value in		Reaction with $AgNO_3$
		MBBW	BAW	
Uric Acid	290	0.18	0.26	Positive
290 mμ Absorbing Material	290	0.18	0.24	Positive

Table 7

Action of uricase on the interfering contaminant that absorbs at 290 mμ

Sample	Optical Density at 290 mμ
1.0 ml. eluate containing the 290 mμ absorbing contaminant (test solution)	0.29
1.0 ml. eluate containing the 290 mμ absorbing contaminant plus cyanide (blank solution)	0.35

Conditions of the assay (67, 68):

Four milligrams of uricase were dissolved in 1.0 ml. of 0.1 M borate buffer, pH 9.2 and filtered. The blank solution contained 0.20 ml. of this enzyme solution, 0.1 ml. of potassium cyanide solution (4 mg./ml.), 0.20 ml. of water and 1.0 ml. of the eluate containing the contaminant. The test solution was the same except that 0.1 ml. of water was substituted for the cyanide solution. The samples were incubated for two hours at 37°.

kidney slices to form a product(s) which appeared on paper chromatograms as a blue-white, brightly fluorescent spot(s). The products had similar R_f values in three solvent systems and appeared by visual inspection to have the same color of fluorescence.

7. Endogenous uric acid distorted the spectra of eluates containing the fluorescent products, KI or XI.

8. The spectra of KI and XI obtained after removal of uric acid (by a second chromatographic development) were similar to spectra obtained for quinoline-type compounds.

PART II. COMPARISON OF THE FLUORESCENT PRODUCTS (KI AND XI) FORMED FROM KYNURENINE AND XANTHURENIC ACID BY RAT KIDNEY SLICES WITH SIMILAR ONES (TA AND AXA) FORMED IN VIVO FROM TRYPTOPHAN AND XANTHURENIC ACID

A. Introduction

In this section of the thesis, the preparation of KI and XI on a larger scale is described. Larger amounts of these compounds were prepared in order to study more completely their properties and to determine if they are identical.

There are compounds present in the urines of tryptophan or xanthurenic acid-loaded rats which also exhibit a bright, blue-white fluorescence on paper chromatograms and which have R_f values similar to KI and XI. Tryptophan and xanthurenic acid were administered to rats and these fluorescent compounds isolated from the urine in order to determine if they were the same as those formed in vitro by rat kidney slices.

Since there was a possibility that one or more of these products was a sulfate conjugate, the preparations were carried out in the presence of radioactive sulfate. In the in vivo preparations, radioactive sulfate was injected into the rats simultaneously with the administration of the tryptophan or xanthurenic acid; while for the in vitro preparations, radioactive sulfate was added to the incubation medium.

B. Experimental

1. Administration of tryptophan, xanthurenic acid and radioactive sulfate to rats

(a) A rat was placed on a pyridoxine-deficient diet for several weeks in order to ensure an ample production of xanthurenic acid from

ingested tryptophan (69). After fasting for 24 hours, 100 mg. of L-tryptophan in water solution were given by stomach-tube. At the same time, 25 microcuries of radioactive sulfate were injected intraperitoneally. The urine was collected over a period of 24 hours during which the rat continued to fast.

(b) Ten rats were fasted for 24 hours and then each was injected intraperitoneally with a neutralized solution containing 3.3 mg. of xanthurenic acid and 2.5 microcuries of radioactive sulfate. Their urines were collected over a 24 hour period during which their fasting was continued. The pooled urine was lyophilized and taken up in a small volume of water.

2. Incubation of kynurenine, xanthurenic acid and radioactive sulfate with rat kidney slices

Thirty milligrams of substrate (either L-kynurenine sulfate monohydrate or xanthurenic acid) and 25 microcuries of radioactive sulfate were neutralized and the solution made up to 60 ml. with Krebs-Ringer phosphate solution, pH 7.4. The kidneys from five, freshly-killed rats were sliced and three to four slices were incubated with 3.0-ml. aliquots of the above solution in twenty, 20.0-ml. beakers in the Dubnoff shaker for three hours at 37° under an atmosphere of oxygen. After incubation, the mixtures were centrifuged and supernatant layers were pooled.

3. Isolation of the fluorescent products from urine and from incubation mixtures

The isolation procedure described here follows somewhat that of Rothstein and Greenberg (24). The solution containing the material to be isolated, whether urine or incubation medium, was lyophilized and

then adjusted to a volume of approximately fifty ml. by the addition of water. The solution was then streaked along a line 2 inches from the margin of a sheet of Whatman #3 paper. By alternately streaking and drying the solution, a total of about 10 ml. was applied. Five sheets were prepared in this way. The sheets were then placed in a Chromatocab (a commercial glass and stainless-steel chromatographic cabinet for developing descending chromatograms) and developed with MBBW. After the chromatograms were developed and dried, they were examined under ultraviolet light. The fluorescent bands were marked with a pencil and labeled (see Figures 17, 20, 23 and 26). The sheets were also scanned with a radiation monitor (Nuclear Corporation Monitor, model 1516) by placing the thin-walled tube holder flat against the paper and recording the counts per minute registered by the monitor at various distances from the origin.

The band with the intense blue-white fluorescence (R_f=0.35), was cut out from each of the five sheets and eluted overnight with water in the Chromatocab. The eluates so obtained were again streaked on five sheets of Whatman #3 paper. The chromatograms were developed overnight in the Chromatocab with BAW. After the sheets were dried, they were inspected under ultraviolet light and the fluorescing bands were marked. Again the sheets were scanned as before with the radiation monitor. The band which fluoresced with the intense blue-white color and had an R_f value of about 0.1 was cut out of each of the five sheets and eluted.

The eluates were streaked on five sheets of Whatman #3 paper, developed as before with MBBW and then examined with the ultraviolet lamp and the radiation monitor. The blue-white, brightly fluorescent

band with an R_f value of about 0.35 was eluted. After the third chromatographic development, there was only one fluorescent band visible, except in the case of the KI preparation where another very faint band still remained (see Figures 19, 22, 25 and 28). The eluate was stored at approximately -15° until used in tests to be described later.

4. Chromatographic procedure

Solutions of isolated fractions (or, in some cases, tenfold concentrations of these solutions) were spotted on chromatography paper, dried and developed by ascending technique as described in Part I. Developed chromatograms were sprayed with various spray reagents (see Appendix III) and some were examined for radioactive areas.

5. Development of autoradiograms

The developed chromatograms were placed in X-ray cassettes and covered with a sheet of pliofilm to prevent chemicals on the paper from reacting with the film during exposure. Sheets of Kodak, medical, no-screen, X-ray film were placed upon the pliofilm sheet and the cassettes were closed. The films were exposed for one month and then developed.

C. Results

1. Isolation of KI, XI, TA and AXA

Using the procedure described on page 37, the blue-white, brightly fluorescent material (having an R_f value of about 0.25 on Whatman #1 paper) was isolated from (a) the urine of tryptophan-loaded rat, (b) the urine of xanthurenic acid-loaded rats, (c) incubation mixtures in which L-kynurenine had been the substrate and (d) incubation mixtures in which xanthurenic acid had been the subtrate. The fluorescent products from these four sources were designated respectively, TA, AXA,

KI and XI.

In Figures 17-28, the course of the purification of KI, XI, TA and AXA can be followed. These figures illustrate the appearance of the paper chromatograms obtained for each preparation at each of the three chromatographic steps. By this chromatographic technique, the fluorescent materials were separated from other fluorescing substances.

Although a radioactive area is closely associated with the fluorescent area on the chromatograms, the fluorescence and radioactivity can be separated as shown in Figures 19, 22, 25 and 28. This indicated that the fluorescent products were not sulfate conjugates. A radioactive material isolated from the urine of a rat fed tryptophan (number 8 in Figure 17), exhibited fluorescence throughout purification; the details of the further purification and identification of this material will be considered in Part II-C3.

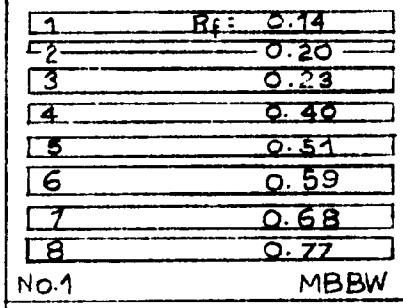
2. Comparison of properties of KI, XI, TA and AXA

Having prepared KI, XI, TA and AXA by the procedure just described, their properties were next investigated in order to establish whether or not they are identical. In order to facilitate comparison, the concentrations of the four eluates were first adjusted so that all had the same intensity of fluorescence as measured in the Lumetron fluorimeter (B-1 filter).

(a) Spectral and chromatographic data obtained on eluates of KI, XI, TA and AXA: The R_f values obtained upon spotting and developing approximately 20-lambda aliquots of the eluates were substantially identical in four solvent systems and the compounds did not show any separation when cochromatographed. All gave negative tests on chromatograms when sprayed with Pauly's, Ehrlich's, ammoniacal silver

Figures 17, 18 and 19. Fluorescent and radioactive products in the urine of tryptophan-loaded rat

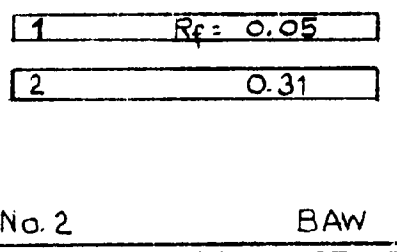
Figure 17.



Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
-	Yellow-gray	Very weak
-	Red	-
9,000	Blue-white	Strong
10,000	Blue-white	Strong
1,000	Yellow-gray	Weak
300	Gray	Very weak
1,000	Gray-blue	Strong
8,000	Blue	Strong

Urine of a tryptophan-loaded rat after descending paper chromatography using MBBW

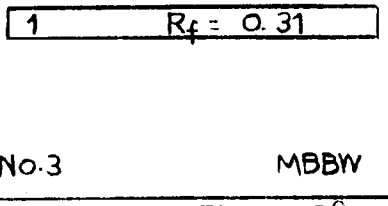
Figure 18.



Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
7,000	Blue-White	Strong
100	Blue	Weak
60		

Number 3 in Figure 17 after descending paper chromatography using BAW

Figure 19.



Radioactivity C.P.M.[a,b]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
-	Blue-White	Strong

Number 1 in Figure 18 after descending paper chromatography using MBBW

[a]Describes chromatogram at left at a corresponding distance from the origin.

[b]There was still radioactivity near this band so that chromatographic development was continued until the band was near the bottom of the sheet. By this time it had separated from most of the radioactivity.

Figures 20, 21 and 22. Fluorescent and radioactive products in the urine of xanthurenic acid-loaded rats

Figure 20.

CHROMATOGRAM Origin	Radioactivity C.P.M[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	15,000		
1 R_f = 0.26	5,000	Blue-white	Strong
2 0.37	3,000		
	1,000		
3 0.74[b]	700		
	1,500		
	400		
No. 1 MBBW	40		

Urine of xanthurenic acid-loaded rats chromatographed with MBBW

Figure 21.

CHROMATOGRAM Origin	Radioactivity C.P.M[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
1 R_f = 0.05	-	Blue-white	Strong
2 0.15[b]	-		
3 0.44	-		Weak
No. 2 BAW			

Number 1 in Figure 20 after descending paper chromatography with BAW

Figure 22.

CHROMATOGRAM Origin	Radioactivity C.P.M[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	20		
	50		
1 R_f = 0.33	50	Blue-White	Strong
	50		
	20		
No. 3 MBBW	20		

Number 1 in Figure 21 after descending paper chromatography with MBBW

[a]Describes chromatogram at left at a corresponding distance from the origin.

[b]May be an overlap of two fluorescent bands.

Figures 23, 24 and 25. Fluorescent and radioactive products in media in which L-kynurenine was incubated with rat kidney slices

Figure 23.

CHROMATOGRAM Origin	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	1,500		
	1,000		
	6,000		
1 R_f = 0.40 [b]	35,000	Blue-white	Strong
2 0.57	15,000		
	4,000		
	300		
	70		
No.1 MBBW			

Incubation mixture in which kynurenine was substrate chromatographed with MBBW

Figure 24.

CHROMATOGRAM Origin	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	4,000		
1 R_f = 0.20	50	Blue-white	Strong
	20		
2 0.42	30		
	30		
	30		
	30		
No.2 BAW			

Number 1 in Figure 23 after descending paper chromatography with BAW

Figure 25.

CHROMATOGRAM Origin	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	40		
1 R_f = 0.35	50	Blue-white	Strong
	50		
2 0.47	50		Very weak
	30		
No. 3 MBBW			

Number 1 in Figure 24 after descending paper chromatography with MBBW

[a] Describes chromatogram at left at a corresponding distance from the origin.

[b] May be an overlap of more than one fluorescent band. (An aliquot of the incubation mixture was developed in an ascending manner using MBBW. Four fluorescent spots were visible on the paper. The R_f values of these spots were 0.31, 0.41, 0.49 and 0.54.)

Figures 26, 27 and 28. Fluorescent and radioactive products in media in which xanthurenic acid was incubated with rat kidney slices

Figure 26.

Origin CHROMATOGRAM	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	15,000		
1 R_f = 0.30	25,000	Blue-white	Strong
2	1,000		
3 0.48 [b]	2,000		
	600		
4 0.77 [b]	50		
No.1 MBBW	40		

Incubation mixture in which xanthurenic acid was the substrate chromatographed with MBBW

Figure 27.

Origin CHROMATOGRAM	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	20,000		
1 R_f = 0.20	40	Blue-white	Strong
	40		
	0		
	0		
	0		
No.2 BAW	0		

Number 1 in Figure 26 after descending paper chromatography with BAW

Figure 28.

Origin CHROMATOGRAM	Radioactivity C.P.M.[a]	Color of Fluorescence[a]	Intensity of Fluorescence[a]
	70		
	50		
1 R_f = 0.40	40	Blue-white	Strong
	50		
	60		
	90		
No.3 MBBW			

Number 1 in Figure 27 after descending paper chromatography with MBBW

[a]Describes chromatogram at left at a corresponding distance from the origin.

[b]May be an overlap of more than one fluorescent band.

nitrate or ninhydrin reagents (see Appendix III). The R_f values are presented in Table 8.

Ultraviolet absorption spectra were obtained on the eluates and are shown in Figure 29. All had maxima at 245 and 340 mμ. Their fluorescence activation and emission spectra were obtained with the Aminco-Bowman spectrophotofluorimeter and are shown in Figure 30. All had activation maxima at 350 mμ and emission maxima at 450 mμ.

(b) Autoradiographic and chromatographic data obtained on one-milliliter samples of eluates of KI, XI, TA and AXA: One milliliter of each eluate was evaporated to dryness in a vacuum desiccator and then the residue taken up in 0.1 ml. of water. This solution was applied as a single spot on Whatman #1 paper by alternately spotting and drying. After ascending development, the chromatograms were exposed to photographic film for one month in X-ray cassettes. They were then sprayed with various color reagents and the films were developed.

In all cases, the fluorescent spots were found to be nonradioactive. The eluates KI, XI and AXA were free of radioactivity. There was a faint radioactive spot obtained with the TA eluate, but it did not coincide with TA. The R_f values of the fluorescent spots from the four eluates are shown in Table 9.

KI, XI, TA and AXA showed no reaction with Pauly's, Ehrlich's, ammoniacal silver nitrate or ninhydrin solutions. However, all preparations contained another material which reacted positively with ninhydrin spray and which had an R_f value in BAW of about 0.15. (This R_f value compares quite closely with serine which has a value of 0.06 in BAW and 0.34 in MBBW.) Also, the eluate of TA contained salts which were revealed with ammoniacal silver nitrate spray and which had an R_f

Table 8

R_f values obtained using twenty-lambda samples of eluates of KI, XI, TA and AXA

Solvent used	R_f values			
	KI	XI	TA	AXA
MBBW	0.32	0.32	0.31	0.30
BAW	0.16	0.14	0.13	0.11
KCl	0.46	0.47	0.46	0.48
IBA	0.21	0.17	0.17	0.19

Table 9

R_f values obtained using one-milliliter samples of eluates of KI, XI, TA and AXA

Solvent used	R_f values			
	KI	XI	TA	AXA
MBBW	0.25 0.14[a]	0.28	0.24 0.12[a]	0.19 0.12[a] 0.42[a]
BAW	0.11 0.17[a]	0.11 0.18[a]	0.16	0.11
KCl	0.46	0.50	0.48 0.22[a]	0.48 0.24[a]
IBA	0.16	0.19	0.23 0.15[a]	0.22

[a]This spot had a weak fluorescence.

Figure 29. Ultraviolet absorption spectra of KI, XI, TA and AXA

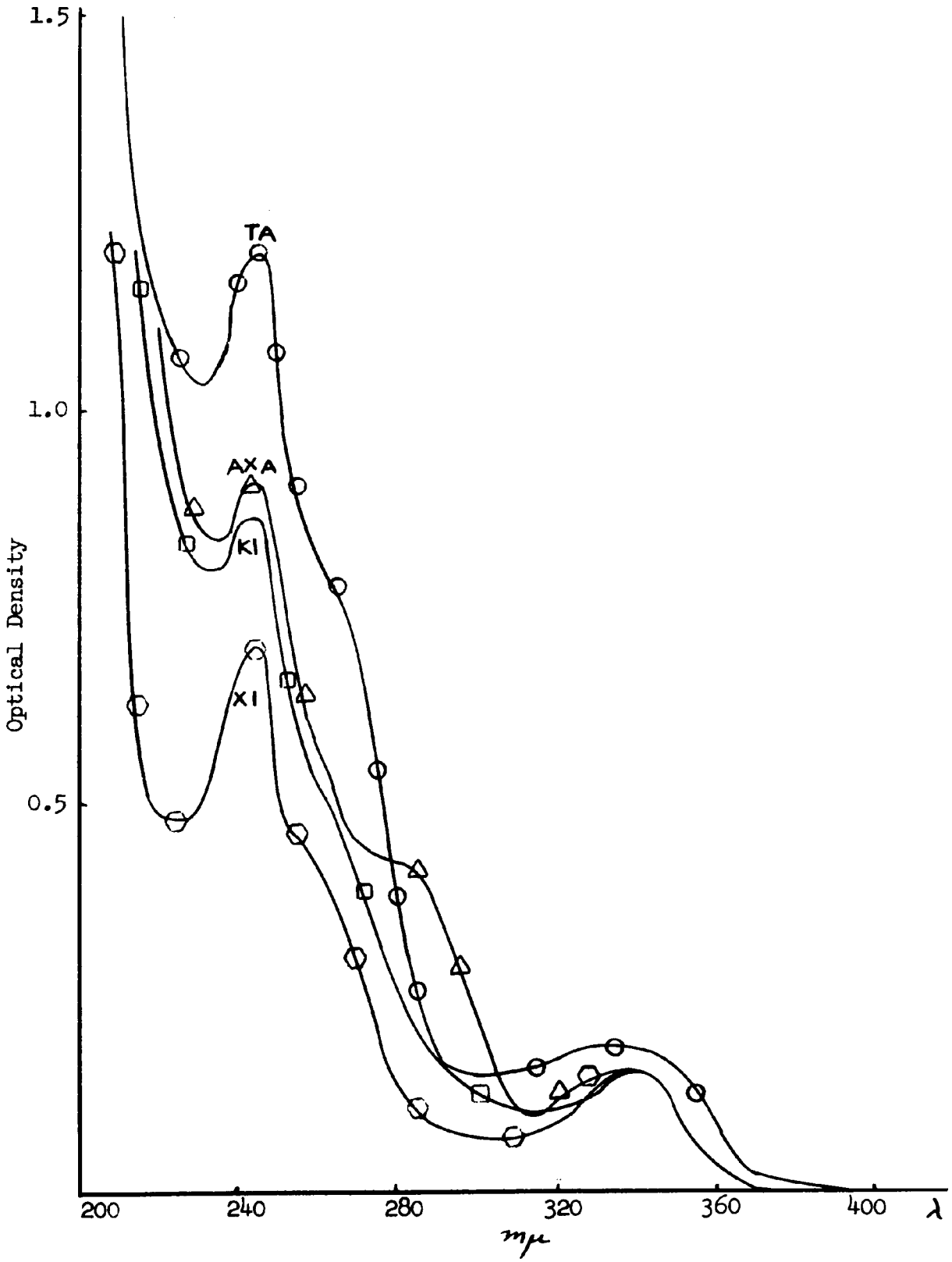
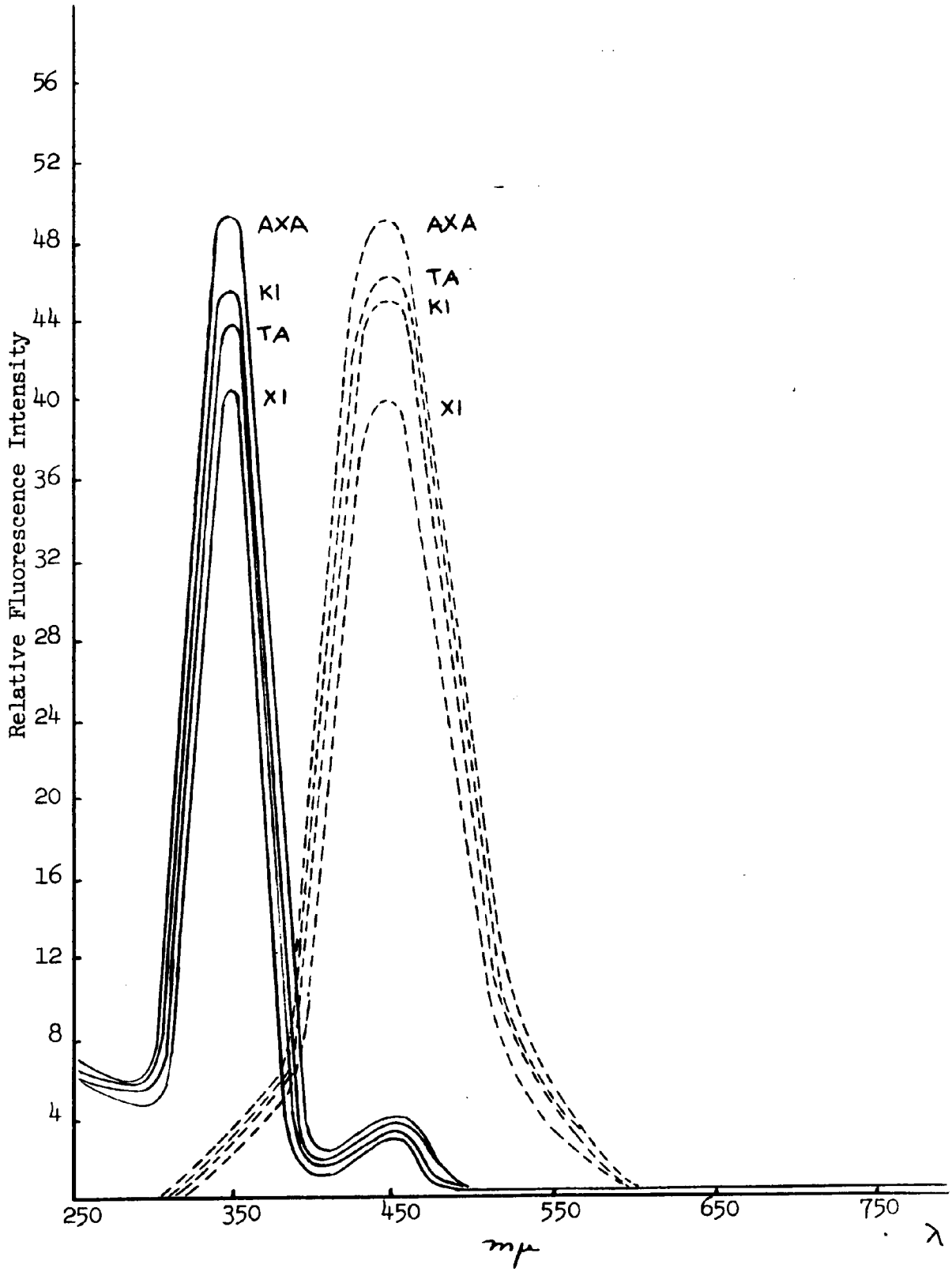


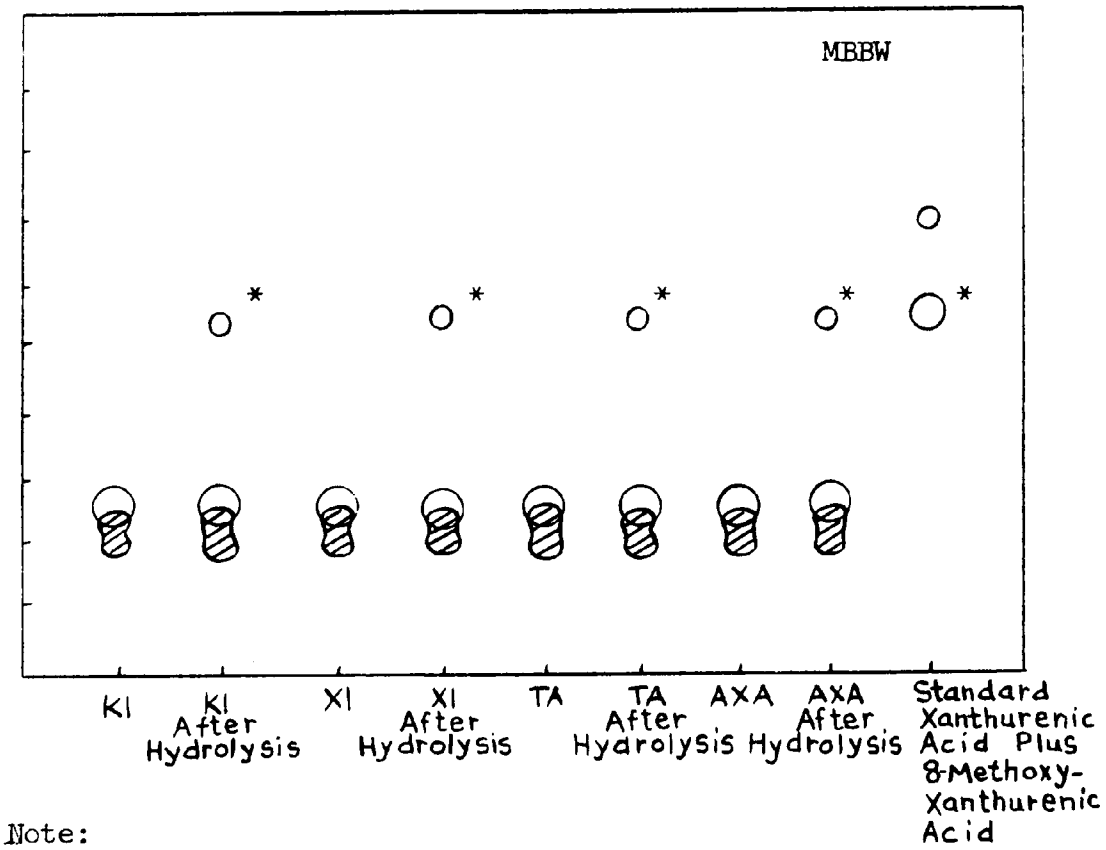
Figure 30. Fluorescent spectra of KI, XI, TA and AXA



value of 0.11 in IBA.

(c) Hydrolysis data obtained on one-milliliter samples of eluates of KI, XI, TA and AXA: Experiments were carried out to determine what products might be formed by hydrolysis of KI, XI, TA and AXA. One-milliliter samples of the eluates were mixed with equal volumes of 0.2 N HCl in thick-walled test tubes. A second and third series of one-ml. samples were mixed with equal volumes of 4.0 N HCl and 12 N HCl. The tubes were sealed, wrapped in cloth for safety, and placed in an oven at 100° for one hour for the 0.1 N solution, for 2 hours for the 2.0 N solution and for 6 hours for the 6 N solution. After the heating period, the tubes were cooled and the seals broken. They were then placed in a vacuum desiccator and evaporated to dryness. This procedure obviates the necessity of neutralizing the solution with alkali before chromatography, thereby avoiding the distortion and artifacts caused by salts. The dried hydrolyzates were taken up in 0.1 ml. of water and chromatographed as before. The fluorescent spots on the developed chromatograms were marked under ultraviolet light and then the sheets were sprayed with ninhydrin reagent to discover if any amino acid had been split off during hydrolysis. The results of this experiment can be seen in Figures 31, 32 and 33. They are made somewhat difficult to interpret owing to the presence in all the eluates of a non-fluorescent, ninhydrin-reacting contaminant. However, it is seen from the chromatograms that, regardless of origin, the fluorescent compound upon hydrolysis yields xanthurenic acid. The trace of an additional fluorescent material showing in some of the chromotograms may be 4, 8-dihydroxyquinoline derived from the liberated xanthurenic

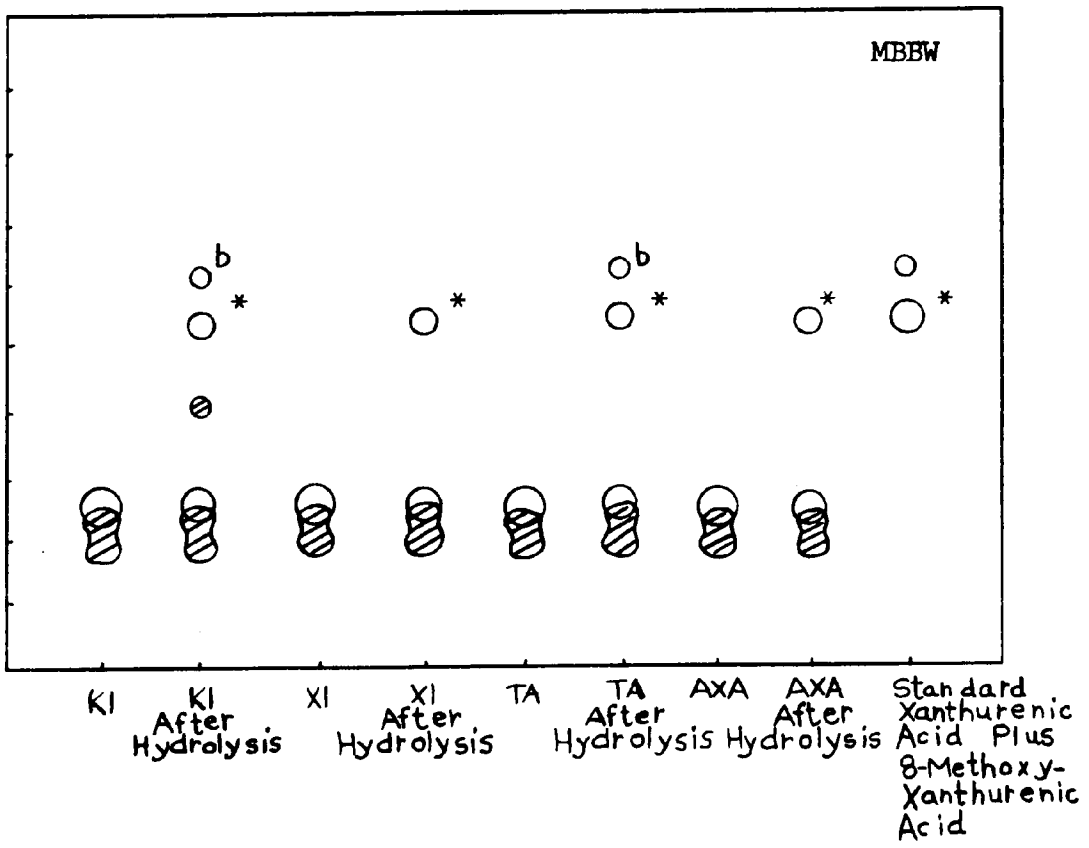
Figure 31. Chromatographic comparisons of unhydrolyzed KI, XI, TA and AXA with samples heated in 0.1 N acid for one hour



Note:

The open circles represent fluorescent spots found on the chromatogram. Those spots marked by an asterisk were positive to Pauly's spray reagent. The hatched areas represent ninhydrin-positive spots.

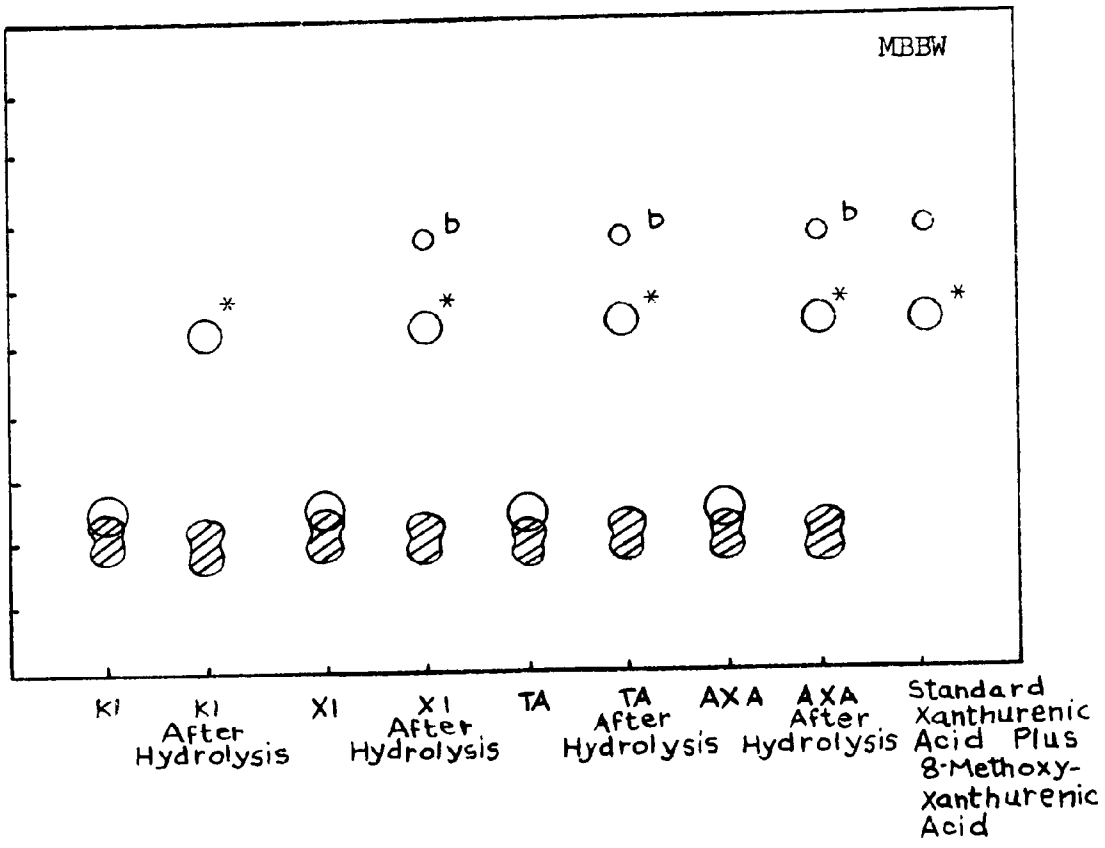
Figure 32. Chromatographic comparisons of unhydrolyzed KI, XI, TA and AXA with samples heated in 2 N acid for two hours[a]



[a]See note on page 50.

[b]Although the trace of material had an R_f value comparable to that of 8-methoxyxanthurenic acid (which was an impurity in the xanthurenic acid), it was probably not 8-methoxyxanthurenic acid. Instead, the material may have derived from a contaminant of the conjugates (KI, XI, TA and AXA), from the conjugates, themselves, or, most probably, from minor destruction of the liberated xanthurenic acid in the hot, acid solution. A possible derivative would be 4, 8-dihydroxyquinoline. No attempts were made to produce larger amounts of this material or to establish its identity.

Figure 33. Chromatographic comparisons of unhydrolyzed KI, XI, TA and AXA with samples heated in 6 N acid for six hours[a]



[a]See the note on page 50.

[b]See footnote b on page 51.

acid by decarboxylation during the heating period. The hydrolysis does not appear to liberate appreciable additional amounts of amino acid; the spot showing in the case of KI hydrolysis with 2.0 N HCl was too faint to be considered significant.

On the basis of the tests just described, KI, XI, TA and AXA are considered to be identical conjugates of xanthurenic acid. They apparently are not sulfate conjugates.

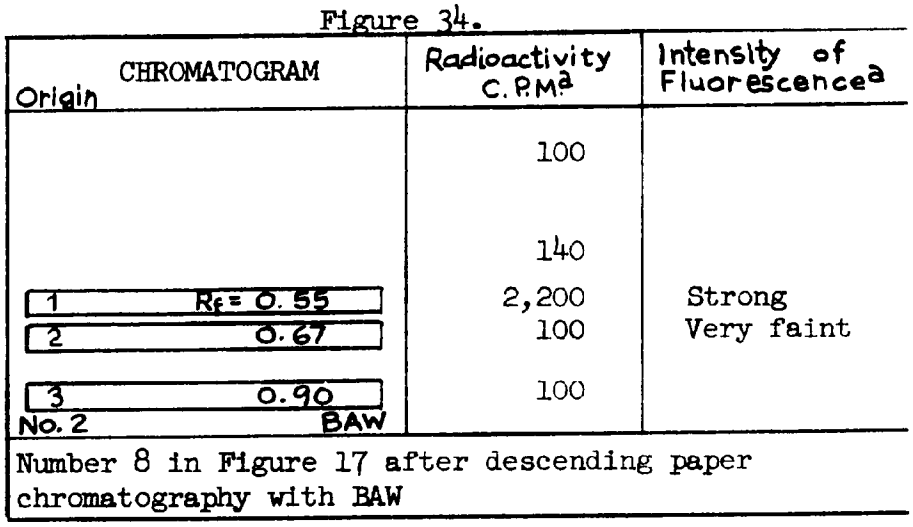
3. Isolation and identification of a radioactive, fluorescent compound found in the urine of a tryptophan-loaded rat

The radioactive, fluorescent band (Number 8 in Figure 17) which was observed on chromatograms of the urine of a tryptophan-loaded rat was cut out and eluted. The radioactive material was then purified by the same procedure used to obtain TA. Figures 34 and 35 show the appearance of the chromatograms obtained during such purification. The radioactivity was clearly associated with the fluorescent material. Eluates containing the purified material were used for the determination of the ultraviolet absorption spectrum (Figure 36) and the fluorescence excitation and emission spectra (Figure 37). The compound had an ultraviolet absorption maximum at 255 mμ, a fluorescence activation maximum at 290 mμ and a fluorescence emission maximum at 400 mμ.

Enough of the eluate containing the radioactive material was spotted on papers to give a distinct fluorescence and then the papers were developed using four different solvents. After development, the papers were exposed to photographic film for one month in an X-ray cassette. Finally, each of the four sheets was sprayed with a different spray reagent. The results of this experiment are shown in Tables 10, 11 and 12. The fluorescent, radioactive material is seen to con-

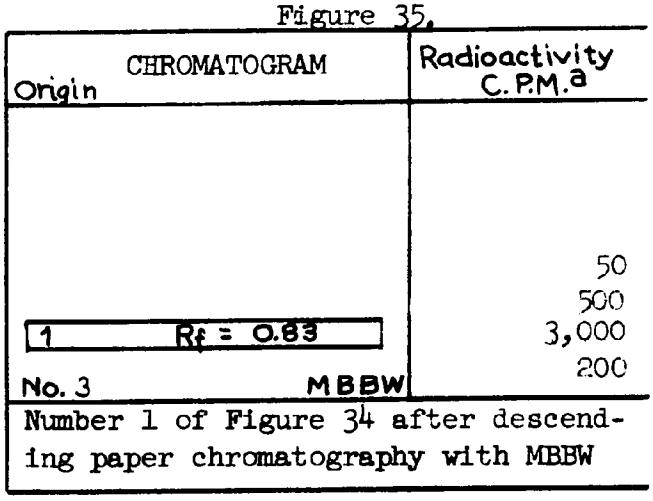
Figures 34 and 35. Fluorescent and radioactive products in the urine of tryptophan-loaded rat

Figure 34.



Number 8 in Figure 17 after descending paper chromatography with BAW

Figure 35.



Number 1 of Figure 34 after descending paper chromatography with MBBW

[a]Describes chromatogram at left at a corresponding distance from the origin.

Figure 36. Ultraviolet absorption spectrum of the purified, fluorescent, radioactive material obtained from the urine of tryptophan-loaded rat

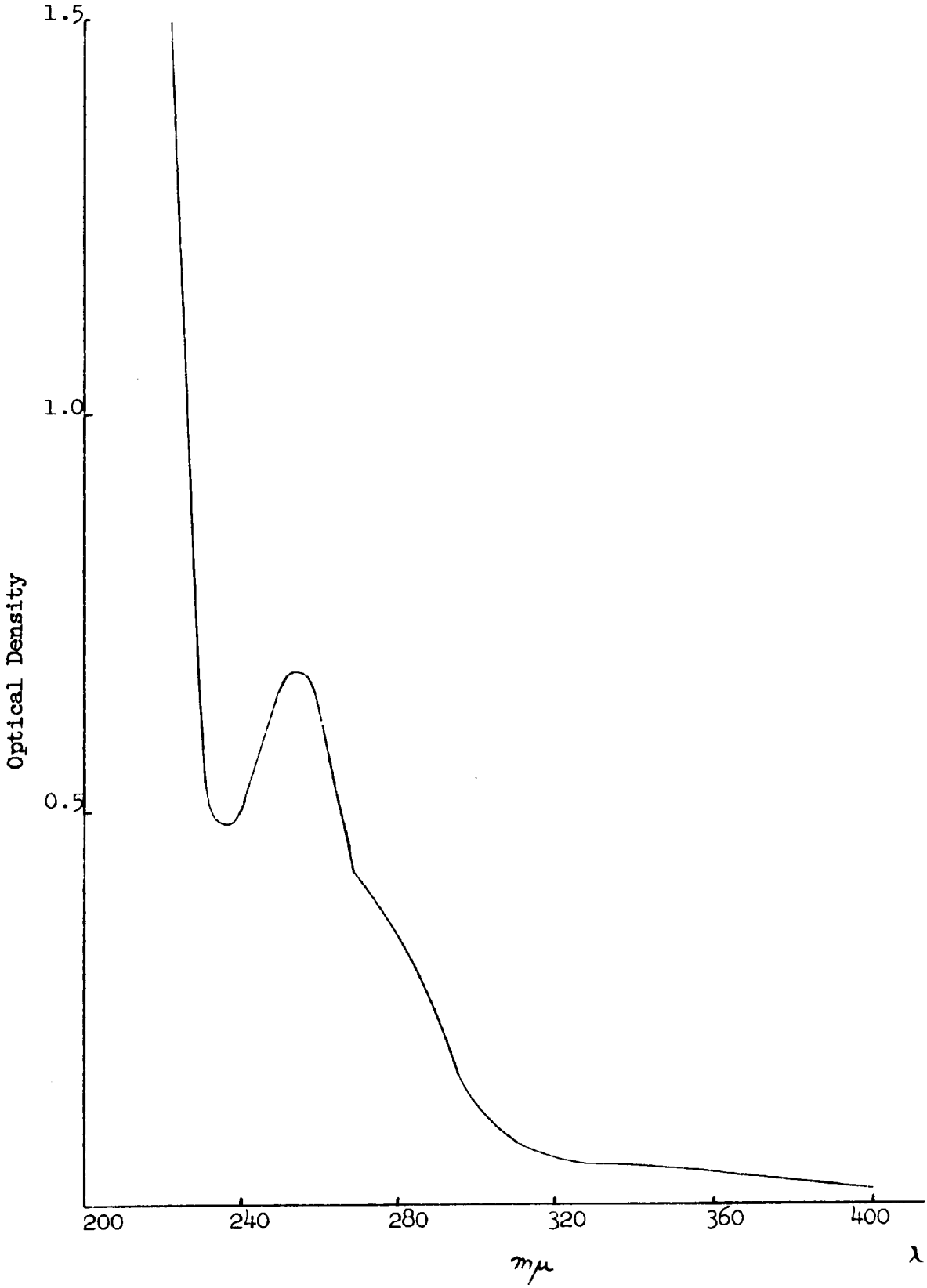


Figure 37. Fluorescence spectra of the purified, fluorescent, radioactive material obtained from the urine of tryptophan-loaded rat

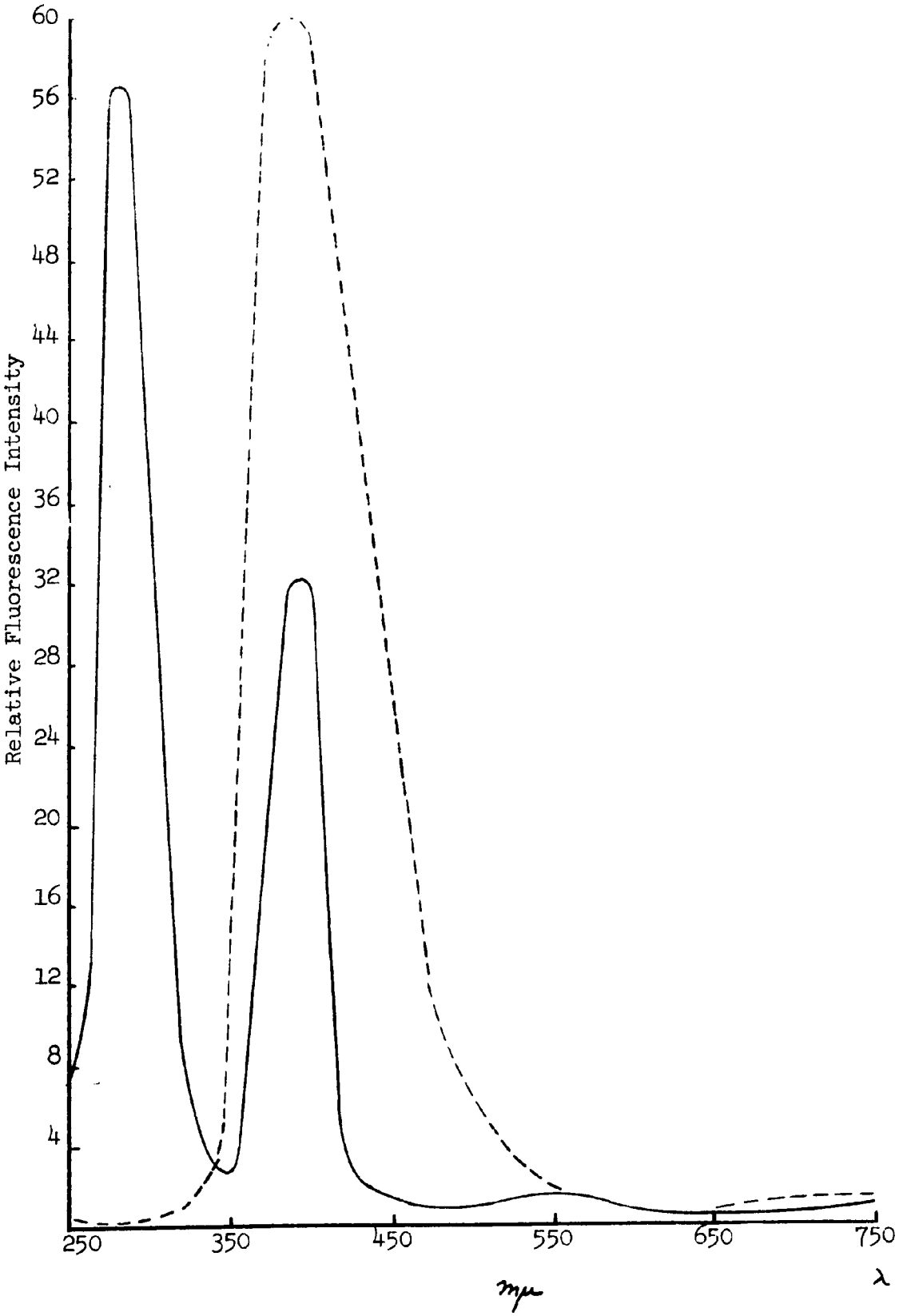


Table 10

R_f values of the purified, fluorescent, radioactive material, obtained by inspection of chromatograms with ultraviolet light

R_f values of the fluorescent, radioactive material using			
MBBW	BAW	KCl	IBA
0.60	0.41	0.60	0.36
0.36[a]	0.80[a]	0.50[a]	0.52
0.54	0.89		0.81
0.76[a]			

[a]This spot had a weak fluorescence.

Table 11

R_f values of the purified, fluorescent, radioactive material, obtained by inspection of films exposed to chromatograms

R_f values of the fluorescent, radioactive material using		
MBBW	KCl	IBA
0.60	0.60	0.36

Table 12

Color reactions obtained with the purified, fluorescent, radioactive material

Spray reagent	Solvent used to develop chromatogram	R_f values obtained with spray reagent	Color of spot obtained with spray reagent
Ninhydrin	BAW	Negative	Negative
Pauly's	KCl	0.60	Yellow
Ehrlich's	MBBW	0.60 0.54	Pink Yellow
$AgNO_3$	IBA	0.36	Brown-black[a]

[a]Color developed on standing overnight.

Table 13

Comparison of R_f values of the fluorescent, radioactive material with those published for indican

Solvent system used	Unknown	Indican
BAW (70)	0.41	0.40
KCl (70)	0.60	0.65
n-Butanol: Acetic Acid: Water:: 12: 3: 5 (71)	0.41	0.45
Isopropyl Alcohol: Ammonia Water: Water:: 8: 1: 1 (71)	0.56	0.53

tain radioactive sulfate, to react with Ehrlich's and Pauly's sprays, and to give a delayed reaction with ammoniacal silver nitrate.

These properties suggested that the compound in question might be indican, the sulfate conjugate of indoxyl. Published R_f values of indican for four solvents systems (70, 71) agreed closely with those of the radioactive tryptophan derivative (Table 13).

D. Summary

1. The blue-white, brightly fluorescent product(s) formed in vitro from kynurenine (KI) and xanthurenic acid (XI) were isolated (in eluates) by means of preparative paper chromatography using three successive chromatographic developments.
2. The blue-white, brightly fluorescent product(s) found in rat urine following administration of tryptophan (TA) or xanthurenic acid (AXA) and which have R_f values similar to KI and XI were isolated by the procedure used for KI and XI.
3. KI and XI were prepared using a medium containing radioactive sulfate; TA and AXA were obtained from rats injected with radioactive sulfate.
4. KI, XI, TA and AXA have substantially identical R_f values in four solvent systems and do not separate when chromatographed together.
5. KI, XI, TA and AXA do not react with Pauly's, Ehrlich's, ninhydrin or ammoniacal silver nitrate sprays.
6. The eluates of KI, XI, TA and AXA contained a non-fluorescent, ninhydrin-reacting contaminant(s) which had an R_f value similar to that of serine.
7. None of the four products were radioactive.

8. All of the four products yield xanthurenic acid upon acid hydrolysis.

9. KI, XI, TA and AXA have substantially identical ultraviolet absorption spectra and fluorescence activation and emission spectra.

10. Radioactive indican was isolated from the urine of a tryptophan-loaded rat which had been injected with radioactive sulfate.

PART III. ISOLATION AND CHARACTERIZATION OF THE PRODUCT (XI) FORMED FROM XANTHURENIC ACID BY RAT KIDNEY SLICES

A. Introduction

KI, XI, TA and AXA were isolated and shown to be identical. The problem remained to determine the structure. For this purpose, work was confined to XI because of the advantages in using an *in vitro* preparation in which only one fluorescent compound was formed. The fact that XI yielded xanthurenic acid upon hydrolysis suggested that it is a conjugate. Conjugation with sulfate seemed unlikely because of the failure to incorporate radioactive sulfate; hence, conjugation with glucuronic acid was considered. The possibility of conjugation with an amino acid, as proposed by Rothstein and Greenberg (24) for the fluorescent conjugate in rat urine, was also examined.

Quantitative methods were employed with the purpose of determining the molar ratios of glucuronic acid, xanthurenic acid and amino acid present in eluates or hydrolyzates of XI. The methods were found to be inapplicable to the eluates isolated as described in Part II, because of the presence of interfering contaminants. However, once a method of preparing XI in a form free of the contaminants was found, quantitative methods were adapted for the required analyses and the molar ratios of the constituents of the molecule were determined, and, thereby, the structure of XI was determined. The purified XI preparations were also used for the determination of various characteristics of XI, such as R_f values in several solvent systems, behavior toward color reagents, spectral properties, behavior on hydrolysis and the pK' values of the dissociating groups.

B. Experimental

1. Preparation and use of chromatographic media

(a) Dowex-50 (100-200 mesh, 5.2 meq./gm., 65 - 71% water by weight): About 100 ml. of the resin are suspended in 500 ml. of 2 N HCl and heated to boiling with mechanical stirring. Care is required to avoid serious bumping. The suspension is allowed to settle and the acid is decanted. Then 500 ml. of 2 N NaOH are added and the suspension is again brought to boiling, allowed to settle and drained. The treatment with acid and alkali are repeated as before, followed by a third treatment with 2 N HCl. Finally, the resin is boiled with 500 ml. of distilled water.

After the resin has settled, the water is decanted and the washed, acid-form resin is slurried with a minimal amount of water and poured into a 2 x 46 cm. chromatographic tube. The column is connected to a reservoir containing water that is allowed to flow slowly in a dropwise manner through the resin. After 24 hours, the resin is ready for use. If the column is not to be used for some time, the slow efflux of water is continued, thereby, continually washing from the resin any soluble products which may form with standing. The column is never allowed to become dry. A column of this size will remove the cations from 60 ml. of Krebs-Ringer phosphate solution.

The resin is regenerated after each use by removing it from the tube and boiling it as described above with 2 N HCl. Finally, the resin is boiled with distilled water and after it has settled the water is decanted. The resin is slurried with water and returned to the chromatography tube. The column is then connected to the water reservoir at least 24 hours prior to its use.

(b) Charcoal (Nuchar-C 190, a highly activated vegetable carbon): The charcoal is suspended in 6 N HCl and boiled for 30 minutes with mechanical stirring. Care will prevent serious bumping. The suspension is centrifuged down and the acid decanted. This procedure is repeated twice. Finally, the charcoal is boiled for 30 minutes with water, then centrifuged and decanted.

This acid-washed charcoal is stored in a bottle covered by water. In preparing a charcoal column, the washed charcoal is suspended and enough poured into a chromatographic tube with diameter of 1.8 cm. to fill it to a height of 1 cm. A reservoir is attached and one liter of 5% (v/v) ammonia water (ammonia water being a solution of 28-30% NH_3 in water) is allowed to pass dropwise through the column. This is followed by a liter of 1.8 N HCl and, finally, the column is connected to a reservoir of water. Water is passed through dropwise for at least 24 hours prior to use or until the column is needed. After each use, the charcoal is discarded and a new column prepared as described in this paragraph.

(c) Silica Gel (100 - 200 mesh, Grade 923): A suspension of silica gel is brought to boiling with 6 N HCl, using mechanical stirring to avoid severe bumping, then allowed to settle, and the acid is decanted. This is repeated two more times. The silica gel is suspended in 5% ammonia water and the suspension brought to boiling; after the suspension settles, the ammonia water is decanted. The silica gel is next suspended in 1.8 N HCl and brought to boiling. After settling, the hydrochloric acid solution is decanted. Finally, the silica gel is boiled with distilled water and allowed to settle. The water is decanted. The silica gel is slurried with water and poured into a tube

with a diameter of 2 cm. to a height of 44 cm. The tube is then connected to a reservoir of water and washed with a drop-wise flow for at least 24 hours before using.

The silica gel is rewashed after use by removing it from the tube and successively boiling it, as described above, with 6 N HCl, 5% ammonia water, 1.8 N hydrochloric acid and, finally, with distilled water. After refilling the tube, a drop-wise flow of water is maintained for at least 24 hours before it is used.

(d) Whatman #1 chromatographic paper: One step of the isolation procedure involves the use of chromatographic paper which is washed in the following manner. First, the Chromatocab is washed with soap and water, rinsed and wiped dry. The paper is suspended in the Chromatocab and water is allowed to pass through it, descending, for 24 hours. After the paper is air-dried, it is again suspended from the trough in the Chromatocab and MBBW solvent (prepared from reagent-grade solvents) is allowed to pass descending through it for 24 hours. The paper is again dried in still air away from possible contamination by dust and stored in a dust-free location.

2. Quantitative colorimetric methods

(a) Glucuronide by the naphthoresorcinol method (72):

(1) Solutions required:

i. 0.35% W/v naphthoresorcinol in 0.1 N H_2SO_4, freshly prepared and filtered.

ii. 15 N H_2SO_4.

iii. 1% W/v aqueous chloramine-T.

(2) Special apparatus:

i. 6 x 5/8-Inch test tubes with ground-glass stoppers.

ii. Bausch and Lomb Spectronic spectrophotometer.

(3) Procedure:

Two milliliters of glucuronide solution are introduced into a test tube. Next, 2.0 ml. of the naphthoresorcinol solution are added and then 3.0 ml. of 15 N H_2SO_4. Finally, 0.2 ml. of the chloramine-T solution are added, and the tube is gently shaken. The test tube is covered with a marble and heated in a boiling water bath for 2 hours. It is then taken from the bath and cooled in ice for 10 minutes. Ten ml. of anhydrous ethyl acetate are added and the tube is stoppered and shaken ten seconds, then inverted ten times. After standing for 10 minutes to permit separation of the liquid layers, the ethyl acetate layer containing the purple pigment is transferred by means of a pipette to a Lumetron tube and read in the spectrophotometer at 565 mμ against a similarly treated blank. Each time an unknown is analyzed, a series of known concentrations of glucuronic acid is run. In this manner, any abnormalities occurring in the analysis will become apparent in the standard curve.

(b) Glucuronide by the carbazole method (73):

(1) Solutions required:

i. Concentrated sulfuric acid.

ii. 0.1% Alcoholic solution of carbazole.

(2) Special apparatus:

i. Evelyn colorimeter.

(3) Procedure:

One milliliter of the glucuronide solution is mixed with 6.0 ml. of sulfuric acid in an Evelyn tube. It is added slowly while the tube is cooled in an ice-bath. The tube is then covered with a marble

and heated for twenty minutes in a boiling water bath. After cooling to room temperature, 0.2 ml. of the carbazole solution are added with shaking. A pink color will develop within a few minutes and continue to darken for two hours. After two hours but not more than three hours, the optical density of the solution is read against a similarly treated blank. If the nature of the glucuronide is such that color is formed by heating it with concentrated sulfuric acid, then a correction can be obtained by running two tubes containing the glucuronide, adding carbazole solution to one and using the other for the blank. With every analysis, as a check on the procedure, a standard curve is obtained with known concentrations of glucuronic acid.

(c) Glucuronide by the ferricyanide method following hydrolysis with β-glucuronidase:

(1) Solutions required:

i. Ferric iron-gum ghatti solution (74): A saturated solution of gum ghatti is prepared by suspending the "tears" in a copper screen at the top of a tall container filled with water. About 18 hours should be allowed for saturation and 20 g. of gum ghatti should be used per liter of water. The solution is filtered and 7.0 g. of ferric sulfate hydrate dissolved in 75 ml. of 85% phosphoric acid are added per liter. Finally, small amounts of 1% $KMnO_4$ are added until a trace of pink persists for 15 minutes. This solution should be kept in a dark place.

ii. Cyanide-carbonate buffer solution (74): Four grams of Na_2CO_3 dissolved in 20 to 25 ml. of water are added to 0.75 g. of NaCN dissolved in 75 ml. of water. The combined solutions are then diluted to 500 ml. with water.

iii. Potassium ferricyanide solution (74): Two hundred and

fifty milligrams of $K_3Fe(CN)_6$ are dissolved in 500 ml. of water. This solution is kept in a dark place.

iv. Dilute tungstic acid solution (74): 13.4 ml. of 0.84 N sulfuric acid and 15 g. of sodium tungstate are added separately to 477 ml. of water with shaking. This solution is 1 2/3 times as concentrated as the usual dilute tungstic acid solution.

v. β-Glucuronidase-buffer solution (24): One milligram (6,150 units) of bacterial β-glucuronidase (Sigma Chemical Company) is dissolved in one ml. of 0.15 M acetate buffer, pH 6.0.

(2) Special apparatus:

i. Dubnoff shaker.

ii. Klett-Summerson colorimeter.

(3) Procedure:

One milliliter of the glucuronide solution is added to a 20-ml. beaker. Next, one ml. of the β-glucuronidase-buffer solution is added. Ten lambda of reagent-grade chloroform are added to prevent bacterial action. The beaker is covered with a watch-glass and incubated with gentle shaking at 37° in the Dubnoff incubator for 24 hours.

At the end of the incubation period, the beaker is removed from the incubator and 3.0 ml. of the dilute tungstic acid solution are added to precipitate the enzyme. The precipitate is removed by centrifugation.

One milliliter of the supernatant (duplicate samples may be run) is pipetted into a Klett tube which is calibrated at 5.0 ml. One ml. of the potassium ferricyanide solution is added and the tube heated for 15 seconds in a boiling water bath. The tube is removed from the bath and one ml. of the carbonate-cyanide buffer is added. The tube is then covered with a marble and placed in the boiling water bath for

exactly 15 minutes. At the same time, a control tube containing water and a thermometer are placed in the bath. At the end of 15 minutes, both tubes are placed in an ice-bath and the marble is removed from the sample tube. When the temperature in the control tube reaches 30°, one ml. of the iron-gum ghatti solution is added to the sample tube. The solution is diluted to 5.0 ml. and shaken. After 40 minutes, the color is read, using a 660 filter, in the Klett-Summerson colorimeter. The reagent blank is treated in the same manner except that distilled water is used in place of glucuronide solution. A series of known concentrations is run simultaneously.

In the case of glucuronides of easily-oxidized phenolic groups (such as the 8-hydroxyl of xanthurenic acid), some of the potassium ferricyanide will be reduced by liberated phenol. The amount of color formed is directly proportional to the concentration of either the glucuronic acid or the xanthurenic acid and is additive when both compounds are present together. In the presence of xanthurenic acid, a correction must be applied to obtain the true glucuronic acid concentration. The details involved in making the correction are discussed in a later section.

(d) Xanthurenic acid by absorption at 330 mμ:

(1) Solution required:

i. M/15 phosphate buffer, prepared using secondary sodium phosphate and primary potassium phosphate, pH 7.4.

(2) Special apparatus:

i. Beckman spectrophotometer.

(3) Procedure:

One milliliter of the xanthurenic acid conjugate is pipetted

into thick-walled, 15-ml. centrifuge tubes. This is followed by one ml. of concentrated hydrochloric acid. The tube is shaken and then sealed. The sealed tube is heated at 100° in an oven for 6 hours (for safety the tube is wrapped in a cloth). The tube is then cooled and the seal is broken. The tube is placed in a vacuum desiccator. Bumping, which seems to be caused primarily by dissolved gases in the solution, is avoided in the following manner. The tube is left in the vacuum desiccator over calcium chloride for 12 hours. Then a vacuum is produced with a water aspirator and this mild vacuum is maintained for another 12 hours. Finally, using a gas train consisting of a drying tower containing sodium hydroxide, two towers of calcium chloride, and a dry-ice trap, the desiccator is connected to an oil-pump which will maintain a vacuum of about 15 mm. of mercury. After the contents of the tube have been taken to dryness, two ml. of distilled water are added and the evaporation is repeated. Two ml. of distilled water are added again and the evaporation procedure is repeated for the third time. This removes all detectable traces of hydrochloric acid.

The contents of the tube are then taken up in two ml. of phosphate buffer. The tube is shaken and the solution is stirred with a glass rod to ensure complete solution of the residue. The contents are transferred to a quartz cuvette and read at 330 mμ in the Beckman spectrophotometer against an identically treated blank. Standard xanthurenic acid solutions are run simultaneously.

If the conjugate is a glucuronide of xanthurenic acid, a small amount of the absorption at 330 mμ will be due to the presence of glucuronic acid. The absorption at 330 mμ is linear with concentration in both the case of xanthurenic acid and in the case of glucuronic acid.

When the two compounds are present together their absorption is additive. In order to obtain the true xanthurenic acid concentration after hydrolysis of a xanthurenic acid glucuronide, it is necessary to apply a correction for the glucuronic acid. Other details of the analysis will be given in a later section along with the results which were obtained.

(e) Xanthurenic acid by the iron-chelate method (75):

(1) Solutions required:

i. Sodium bicarbonate solution: 6.9 g. of sodium bicarbonate are dissolved in 100 ml. of water.

ii. Ferric ammonium sulfate stock solution: 0.769 g. of $Fe_2(NH_4)_2 (SO_4)_4 \cdot 24H_2O$ are dissolved in 100 ml. of water. This solution is kept for one week in the refrigerator, after which it is filtered.

iii. Dilute ferric ammonium sulfate solution: One milliliter of the stock solution is mixed with 4.0 ml. of water.

(2) Special apparatus:

i. Beckman spectrophotometer.

(3) Procedure:

The hydrolysis of the xanthurenic acid conjugate is carried out by the method described in Part III, Section B-2d. After the hydrolysis, xanthurenic acid is determined by the iron-chelate method. A blank containing distilled water instead of the xanthurenic acid conjugate and a series of known concentrations of xanthurenic acid should also be run.

Each of the tubes, i.e., unknown, reagent blank, and standard tubes, should be read in the spectrophotometer against a simultaneously prepared blank. This is necessary because, after standing for 5 minutes,

the dilute iron solution becomes turbid. The reading must be made within this time, after addition of the reagents. The reagents are added in the following manner. To the sample tube, which contains the dried residue of the hydrolyzate and to an empty test tube are added 1.5 ml. of water. The sample tube is shaken to ensure solution of the residue. Then 0.125 ml. of freshly diluted iron solution are added. Finally, 0.5 ml. of the sodium bicarbonate solution are added, the tube is shaken again, and the contents are transferred to a Beckman, 1.[illegible] ml., quartz cuvette (light path 1.0 cm.). Exactly two minutes after mixing, the optical density is read at 620 mμ. After all the tubes have been read, including a reagent blank which has been carried through the hydrolysis and evaporation procedure, a standard curve is drawn and the concentration of the unknown is determined.

(f) Amino acid by the ninhydrin method (76):

(1) Reagents required:

i. Ninhydrin.

ii. Hydrindantin: This is prepared by the reduction of ninhydrin with ascorbic acid. 800 Mg. of ninhydrin are dissolved in 20 ml. of water at 90°. 800 Mg. of ascorbic acid dissolved in 4 ml. of water at 40° are added to the ninhydrin solution with stirring and the solution is allowed to cool to room temperature. Crystallization begins immediately. The preparation is cooled with tap water for one hour. The crystals are filtered and washed with water. After drying in a vacuum desiccator, the crystals are stored in an amber bottle.

iii. Sodium acetate buffer: A 4 N sodium acetate buffer, pH 5.5, is prepared and stored at 4°.

iv. Methyl cellosolve, peroxide-free.

v. Diluent: A diluent is prepared by mixing equal amounts of ethanol and distilled water.

vi. Ninhydrin solution: 200 mg. of ninhydrin and 30 mg. of hydrindantin are weighed out and dissolved in 7.5 ml. of methyl cellosolve. The solution is stirred without aeration until all the solid has dissolved. 2.5 Ml. of acetate buffer are then added and the solution gently gassed with a stream of nitrogen. The ninhydrin solution is made just before use and is kept in a stoppered, green-glass container.

(2) Special apparatus:

i. Bausch and Lomb Spectronic spectrophotometer.

(3) Procedure:

One milliliter of the conjugate solution is hydrolyzed by the method described in Part III, Section B-2d. To the unsealed tube, which has been evaporated to dryness three times as described earlier, is added one ml. of distilled water. The tube is shaken to ensure solution of the residue. One ml. of the ninhydrin solution is added and the top of the tube is covered with a square of aluminum foil. After shaking the tube for 10 seconds, it is placed in a boiling water bath for exactly 15 minutes. At the end of this time, the tube is removed and placed in an ice-bath for 5 minutes. Five ml. of diluent are added to the cooled solution. The contents are mixed and transferred to a Lumetron tube. Fifteen minutes after the tube has been removed from the boiling water bath, it is read in the spectrophotometer at 560 mμ. During the analysis the tubes are protected from strong sunlight. A blank and a series of serine solutions of known concentrations are run along with the unknown. It should be noted that the optical density is linear with concentration of amino acid in the

range of 10 to 20 gamma of serine per milliliter, although this is not clearly shown in Figure 58. The optical density is not affected by equimolar amounts of glucuronic acid or of xanthurenic acid.

C. Results

1. Reasons for abandoning earlier chromatographic procedures

As soon as some of the tests described above were applied to the XI eluate, it was evident that information concerning the amino acid and glucuronic acid composition could not be obtained. First of all, the ninhydrin color obtained on the eluate, before any hydrolysis, was too high to read in the colorimeter. In addition, the naphthoresorcinol reaction for glucuronic acid did not resemble at all the reaction obtained with standard glucuronic acid, either in color, time of formation, or solubility of precipitate.

The presence of some unknown contaminant causing the anomalous reaction with naphthoresorcinol was indicated. Whether or not the fluorescent compound was a glucuronide could not be tested until this contaminant was removed. Other solvent systems were tried in an effort to get rid of the interfering material. One such system was 95% ethanol. XI barely moved from the origin after ethanol had been run through the chromatograms for several days. XI was again prepared using this solvent in conjunction with the others previously described, the chromatographic purification with ethanol occurring between the BAW step and the final MBBW step. Ethanol was run through the papers for four days, during which time much of the ninhydrin-positive material was eluted from the sheets. The XI prepared in this manner still gave the anomalous naphthoresorcinol test and a strong ninhydrin color. The

spectrum of the material obtained upon reaction with naphthoresorcinol is shown in Figure 38 in comparison with that obtained with glucuronic acid.

The paper was considered as the source of the contaminant interfering with the naphthoresorcinol test. Water was run descending through a sheet of Whatman 3MM paper and collected. The spectrum of the material obtained when this eluate was treated with naphthoresorcinol is also shown in Figure 38.

Attempts were made to purify the paper by washing with various dilute and concentrated hydrochloric acid solutions, as well as with ether, methanol and saturated versene solutions, but the preparation of chromatographic paper which would not contaminate eluates was not achieved.

A two-inch wide strip of Whatman 3MM paper was washed by a descending flow of water for 24 hours in the Chromatocab. For the next 24 hour period, the water was collected as it dripped off the bottom of the strip. The ultraviolet absorption spectrum of this eluate was obtained using a distilled water blank (Figure 39). The peak at 270 mμ may be responsible for the shoulders obtained in the ultraviolet absorption spectra of KI, XI, TA and AXA as shown in Figure 29. When such an eluate was lyophilized to dryness, a viscous, yellow oil, poorly soluble in water, remained. This material gave a positive thiocyanate test for iron. The results obtained by different washing procedures suggested that the interfering material in the paper consisted of an organic material associated with ferric iron. Ferric iron is able to catalyze oxidation and polymerization of naphthoresorcinol and may, therefore, be partly responsible for the anomalous reactions.

Figure 38. Absorption spectra obtained from the reaction of naphthoresorcinol with (a) XI after elution from paper, (b) water after elution through paper, (c) 8-hydroxyquinoline glucuronide and (d) standard glucuronic acid

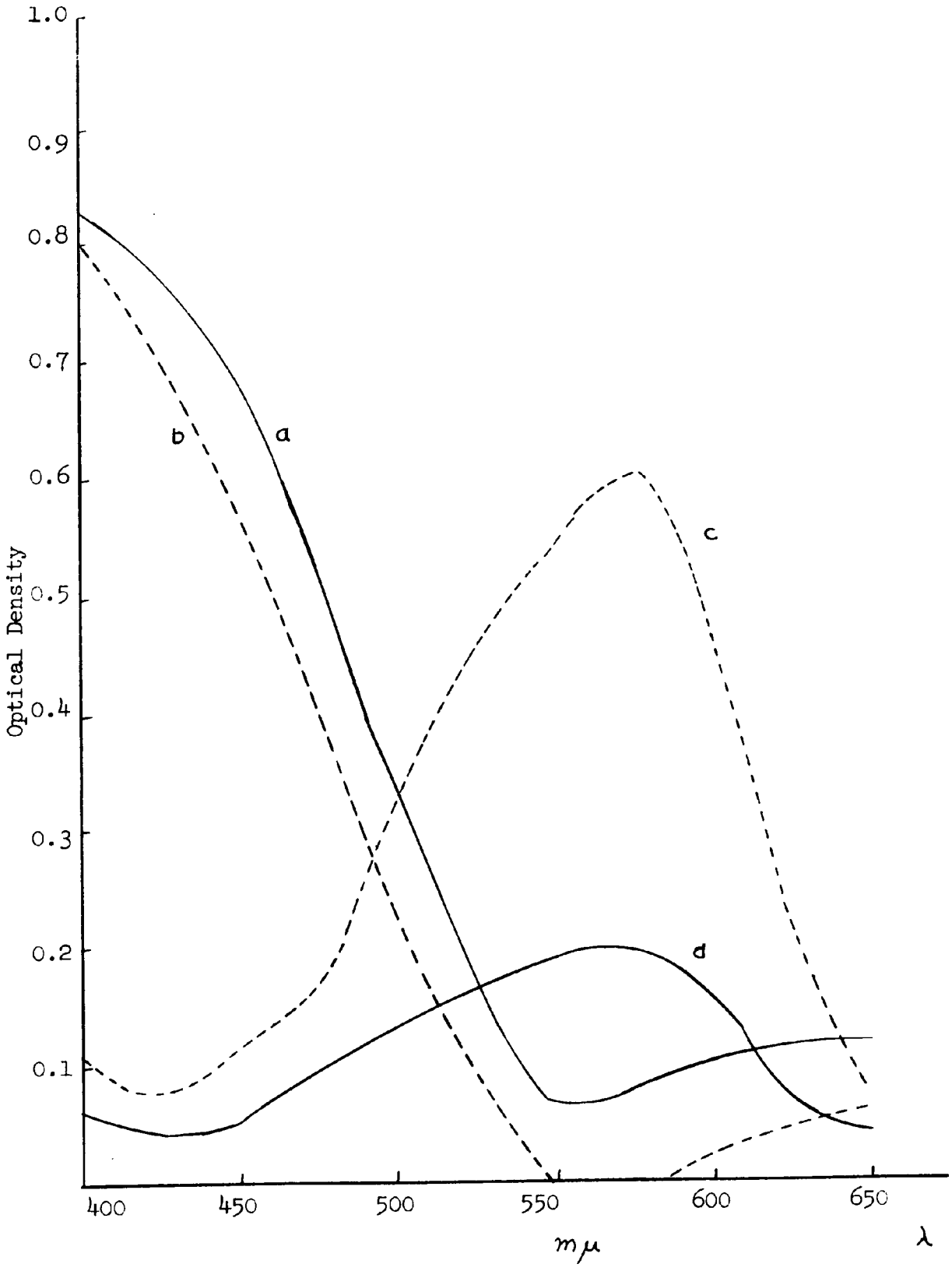
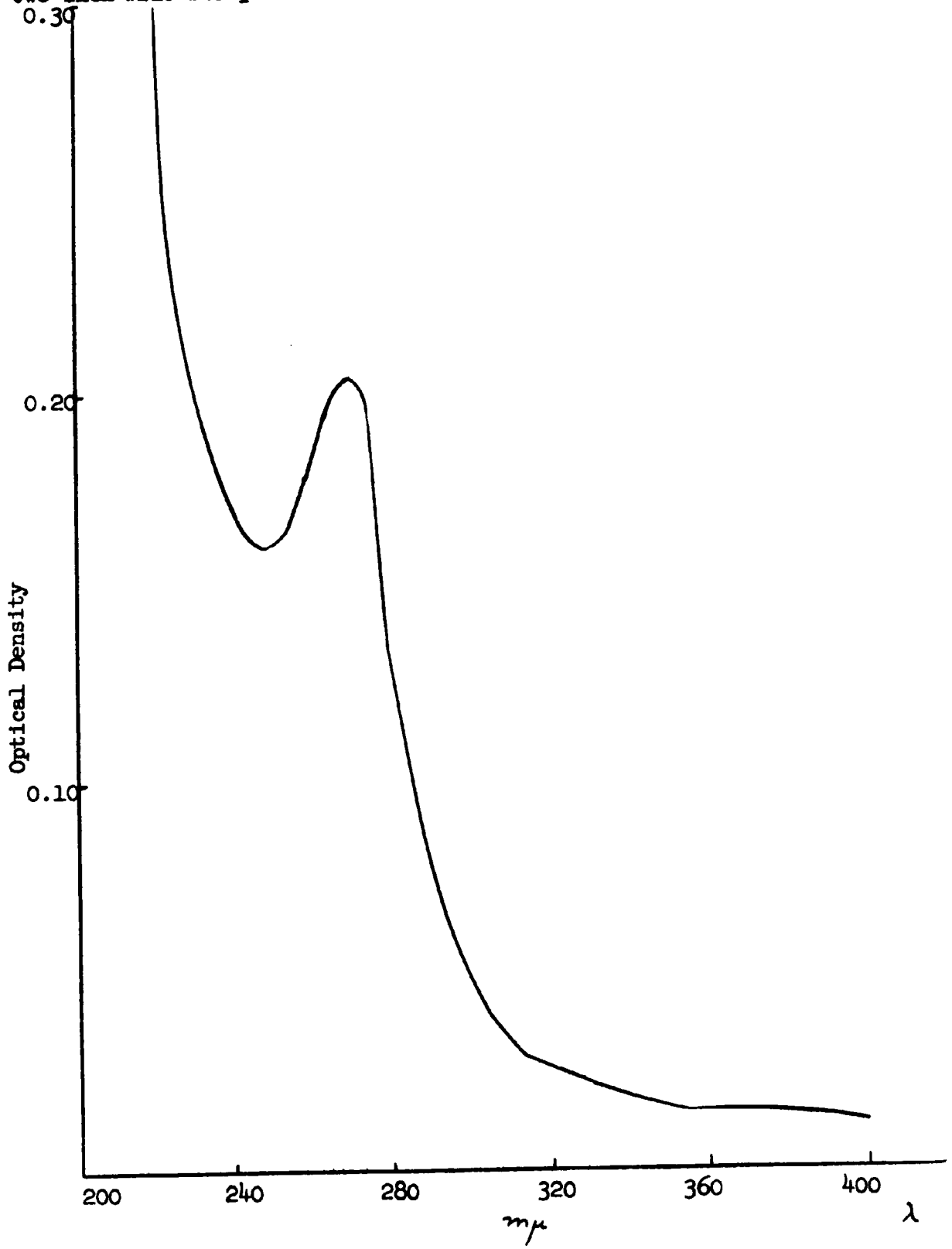


Figure 39. Absorption spectrum of water that had passed through a two-inch wide strip of Whatman 3MM paper



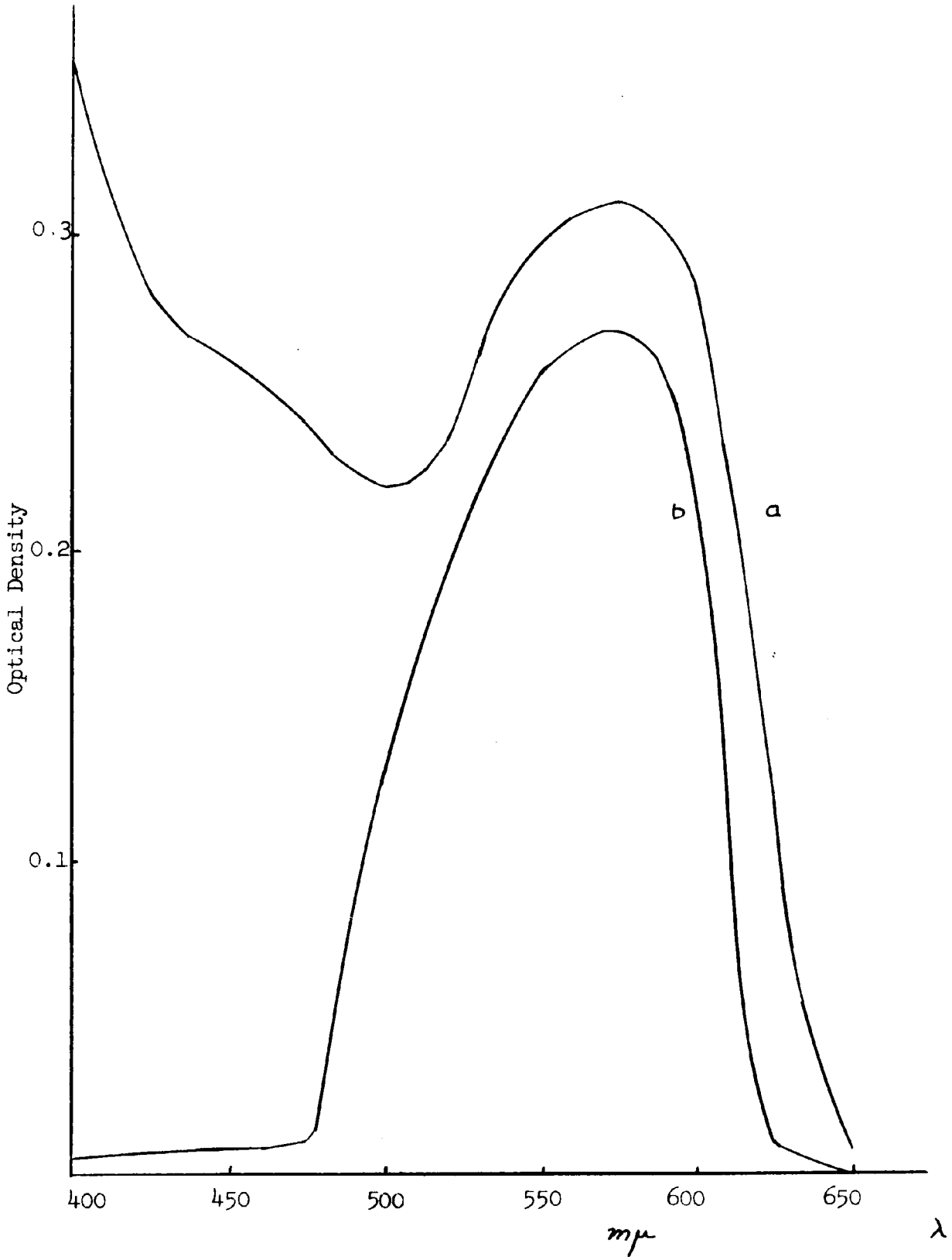
Some of the attempts to purify the paper sheets resulted in their disintegration, so further attempts were made using a 68 x 2.5 cm. column of Whatman, ashless, cellulose powder. The column was prepared and water allowed to flow dropwise through it for 24 hours prior to use. A portion of the XI preparation was then passed through this column. The XI eluate, after passage through this column, still gave an off-color with naphthoresorcinol, but the red color due to glucuronic acid was evident for the first time. The spectrum of the color obtained is shown in Figure 40. This preparation gave only a very faint color with the ninhydrin reagent. Attempts to wash the cellulose powder in order to remove completely the interfering materials were unsuccessful.

2. Isolation of XI by column chromatography

At this point, the attempt to purify XI by paper chromatography alone was dropped and other chromatographic media investigated. The purification was limited to aqueous solvents because the fluorescence of XI was quenched by various organic solvents and this made it difficult to locate the fractions containing the material. Chromatographic media which were investigated but not eventually utilized were, in addition to cellulose powder, acid-alumina and Dowex-1. The fluorescent compound could not be eluted from the acid-alumina with water, and did not elute from Dowex-1 unless 5 M ammonium formate was used (it started trailing at around 3 M, but even at 5 M the elution was not sharp). The large volume of highly concentrated salt solution obtained using Dowex-1 made this method of purification impractical.

The method of isolation as finally developed will now be described with reasons for the various steps given. Careful purification of the chromatographic media as described in the experimental section was

Figure 40. Absorption spectra obtained from the reaction of naphthoresorcinol with (a) XI eluate after an additional purification step using a cellulose chromatographic column and (b) standard glucuronic acid



found to be absolutely necessary if contaminants were not to be introduced from the media during the isolation of XI.

Considering that ferric iron might be the cause of the anomalous reaction with naphthoresorcinol, Dowex-50 was a logical chromatographic medium to employ. In addition, it could be used to initiate the desalting of the incubation mixture. Another property of Dowex-50 was discovered, namely, that xanthurenic acid adheres to this resin and is not removed by water. On the other hand, XI was barely retarded in its movement through the column. Dowex-50 was used to remove both the unreacted xanthurenic acid and the Krebs-Ringer cations from the incubation mixture.

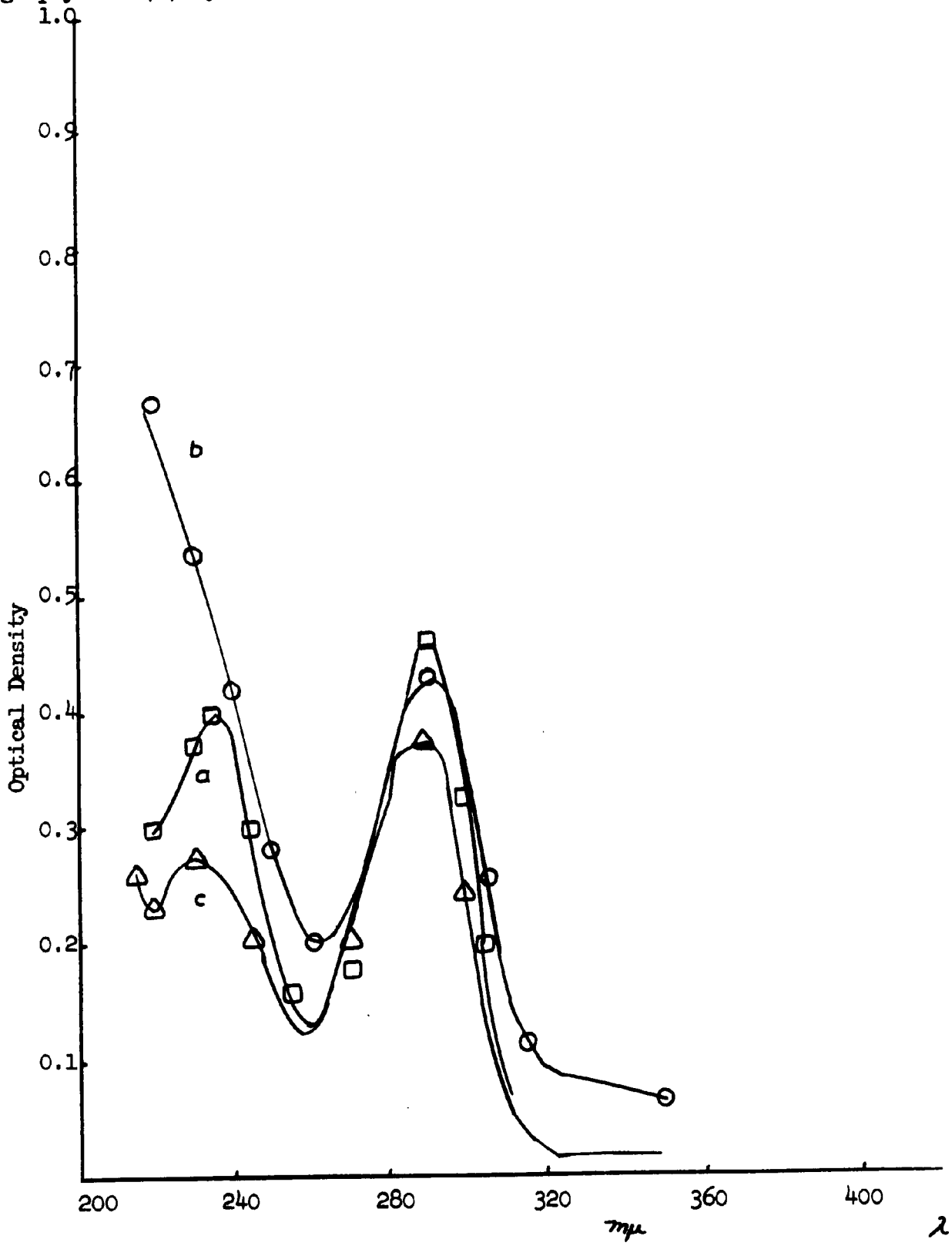
The incubation of xanthurenic acid with kidney slices was carried out as described in Part II of this thesis except that radioactive sulfate was not used. The incubation mixture was deproteinized before pouring it onto the Dowex-50 column because proteins may clog the column, make resolution poor, and make the resin difficult to clean. Deproteinization by precipitation was avoided because of the possible loss of XI by adsorption and coprecipitation. Instead, the incubation mixture (60 ml.) was dialyzed against 600 ml. of water overnight in the refrigerator using a magnetic stirrer. The 600 ml. of protein-free solution were then lyophilized to dryness, and taken up in about 15 ml. of water. The salts which did not dissolve were removed by centrifugation. The solution was applied to the Dowex-50 column and elution with water was begun. Fractions of 60 drops were collected. XI, located by its fluorescence, came off in tubes 17 to 29 (20 to 29 in the second trial). The pH value of the combined eluates from these tubes was 4.3. The solution was left overnight in the refrigerator, during

which time a precipitate of fine, white crystals formed. This material was centrifuged down and redissolved in water. The ultraviolet absorption spectrum of this solution (Figure 41) resembles closely that of authentic uric acid.

The solution containing XI was neutralized with 0.1 N NaOH, using a pH-meter, and then lyophilized to dryness. The residue was taken up in about 5 ml. of water and poured onto the charcoal column. Charcoal was used because the aromatic nature of the conjugate causes it to be strongly adsorbed while contaminants are washed away. Water was allowed to flow dropwise through the column for 24 hours (25 hours on the second trial). Previously, 5% pyridine, which has a pH value of 8.6, had been found to displace XI from the charcoal almost immediately. At the end of 24 hours of elution with water, elution with 5% pyridine was begun. Sixty-drop fractions were collected. Tubes 4 to 6 were found to contain the fluorescent material (3 to 4 in the second trial). To remove the pyridine, the contents of these tubes were lyophilized to dryness, dissolved in about 5 ml. of water, and then lyophilized to dryness again. At this stage, there was little residue left in the lyophilizing flask, but with the addition of water a very intensely fluorescent solution was obtained.

The residue was taken up in 4 ml. of water and applied by alternately streaking and drying to one sheet of Whatman #1 paper, washed as described earlier. Paper chromatography was used at this stage because a trace of fluorescent material in the charcoal was found to be eluted by pyridine. This fluorescent material had been found to travel near the solvent front when chromatographed on paper with MBBW. After application of the XI solution, the sheet was developed by the

Figure 41. Absorption spectra of (a) the compound that crystallizes out of the eluant obtained by passing the dialyzed incubation mixture through Dowex-50, (b) the interfering contaminant isolated using paper chromatography and (c) synthetic uric acid



descending method using MBBW (prepared from reagent-grade solvents) in a freshly washed Chromatocab. A fluorescent band, 1 7/8-inches wide, was found at an R_f value of 0.40. The band was cut out and eluted with water overnight (16 hours) in the Chromatocab. The eluate was lyophilized to dryness and taken up in 5 ml. of water.

The solution of XI as obtained from the Whatman #1 paper was then applied to the regenerated Dowex-50 column and eluted with water. Dowex-50 was used this second time to remove any iron which might have been picked up during the paper chromatographic step. Fractions of 60 drops were collected. The XI came off in a sharp and symmetrical fashion in tubes 13 to 18. The contents of these tubes were lyophilized.

The lyophilized material was taken up in 5 ml. of water and applied to the silica gel column. At this stage, there was still a barely perceptible yellow cast to the solution. The silica gel step reduced to a negligible value the ultraviolet absorption of a water blank which had been put through the Dowex-50, charcoal, paper and Dowex-50 steps. Silica gel was also chosen because of its ability to absorb lipoid materials which might have passed some of the other purification stages.

XI was eluted from the silica gel with water. The highly fluorescent band could be followed easily with an ultraviolet lamp as it developed down the translucent, silica gel column. Sixty-drop fractions were collected until the fluorescent material began to emerge from the column in the tenth tube. All of the XI was collected in a volume of about 20 ml. in this tube. The contents of the tube were lyophilized. The residue was taken up in a few milliliters of

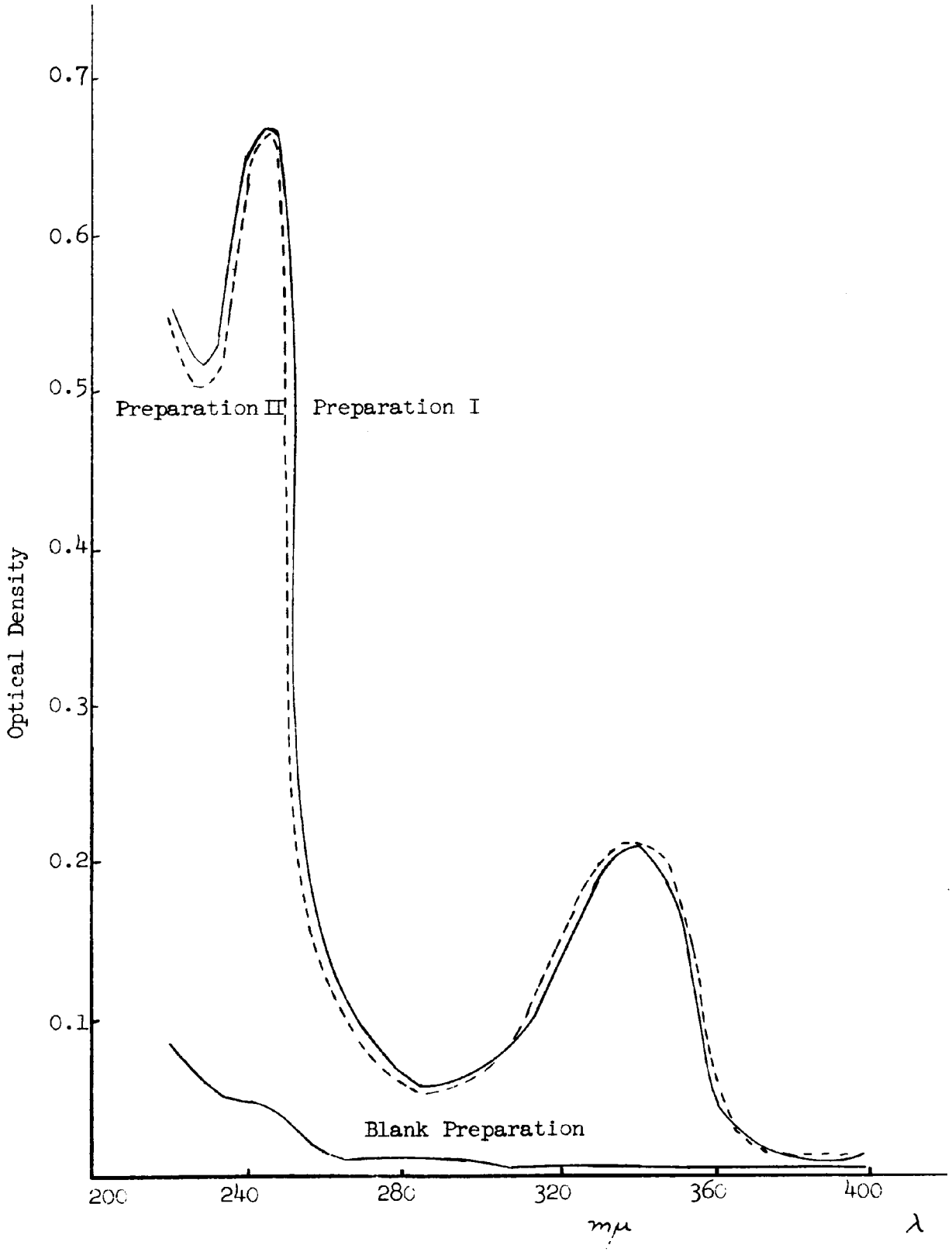
water and centrifuged to remove insoluble silica gel. The aqueous solution of XI was lyophilized again to dryness. The residue was a barely-visible, fine, light-yellow powder. The powder was very soluble in water and gave a colorless solution with an extremely intense, blue-white fluorescence. The residue in the lyophilizing flask was taken up in 15.0 ml. of water.

After the procedure had been worked out as just described, it was checked for its ability to remove contaminants by using it to prepare a blank solution which was subsequently analyzed. The kidneys of five rats were sliced and incubated as described previously but with no xanthurenic acid present. This incubation mixture was deproteinized by dialysis against 600 ml. of water as before. However, after the dialysis, the 30 mg. of xanthurenic acid were added, there being no possibility of XI being produced at this stage. From this point on, the procedure as described for XI was followed, except that, in the absence of any fluorescence, the chromatographic fractions that were retained were chosen on the basis of the preparation of XI. A second preparation of XI was also carried out exactly as described for the first preparation (designated Preparation I). All of these preparations (Preparations I and II, and the blank preparation) were stored at approximately -15° until used in the tests to be described.

3. Determination of some of the properties of purified XI

The ultraviolet absorption spectra of Preparations I and II and of the blank preparation are shown in Figure 42. Both preparations of XI have substantially identical spectra with maxima at 245.5 mμ and 339 mμ. Absorption by the blank was low over most of the ultraviolet range. The isolation procedure appeared to be reproducible

Figure 42. Ultraviolet absorption spectra of XI obtained of two preparations and of a blank preparation[a]



[a]Preparations diluted six-fold with distilled water.

judging from the similarity of the results of the two preparations. The fluorescence spectra of XI are shown in Figure 43. XI had a fluorescence activation maximum at 355 mμ and an emission maximum at 450 mμ.

Enough of the XI preparation was spotted on paper to give a bright fluorescence and the sheets were developed with MBBW. Spraying the dried sheets with Pauly's, Ehrlich's, ammoniacal silver nitrate, or ninhydrin reagents revealed no positive reactions (see Appendix III).

One milliliter of XI was hydrolyzed by the procedure described in Part III, Section B-2d. After the three-fold evaporation to dryness, the material was taken up in M/15 phosphate buffer and the fluorescence spectra were obtained. A comparison of the spectra of the product of hydrolysis of XI with those of xanthurenic acid is shown in Figure 44, furnishing additional evidence that XI yields xanthurenic acid upon hydrolysis. The ultraviolet absorption curves of Preparations I and II (dissolved in phosphate buffer) after acid hydrolysis, are shown in Figure 45 together with the spectrum obtained with the blank preparation.

In order to demonstrate that the product obtained by the isolation procedure was identical with the product formed by the slices, i. e., that no degradation of the compound had occurred during the isolation process, the following experiment was run. In one beaker, xanthurenic acid was incubated with kidney slices as before, and, in another, a blank incubation was run without the xanthurenic acid substrate. After the period of incubation, each of three sheets of Whatman #1 paper were spotted with (a) XI (Preparation II) alone, (b) XI together with the blank incubation mixture, (c) the xanthurenic acid incubation mixture, and (d) XI together with the xanthurenic acid incubation mixture. The R_f values obtained with three different solvents are

Figure 43. Fluorescence spectra of XI

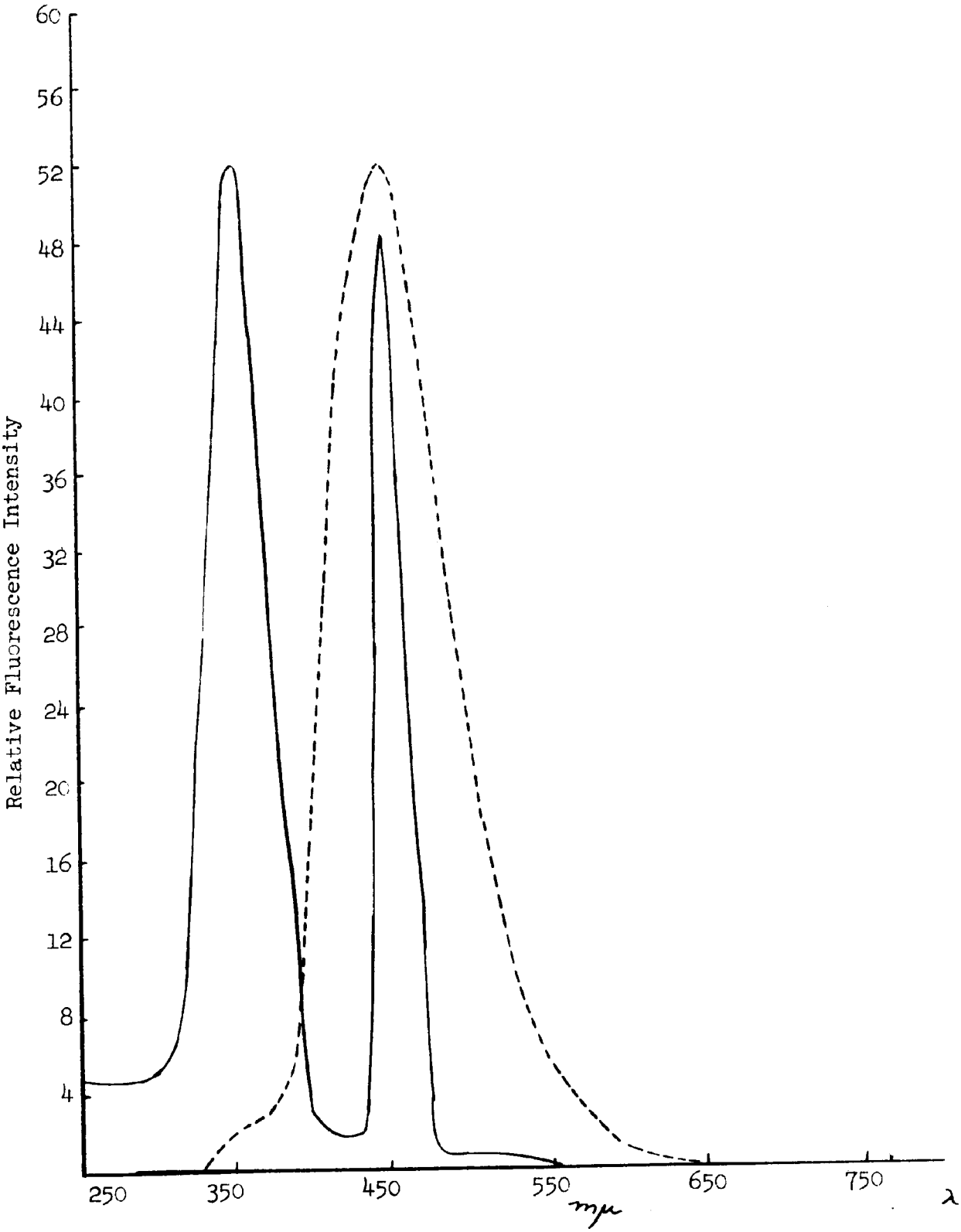


Figure 44. Fluorescence spectra of hydrolyzed XI compared with xanthurenic acid

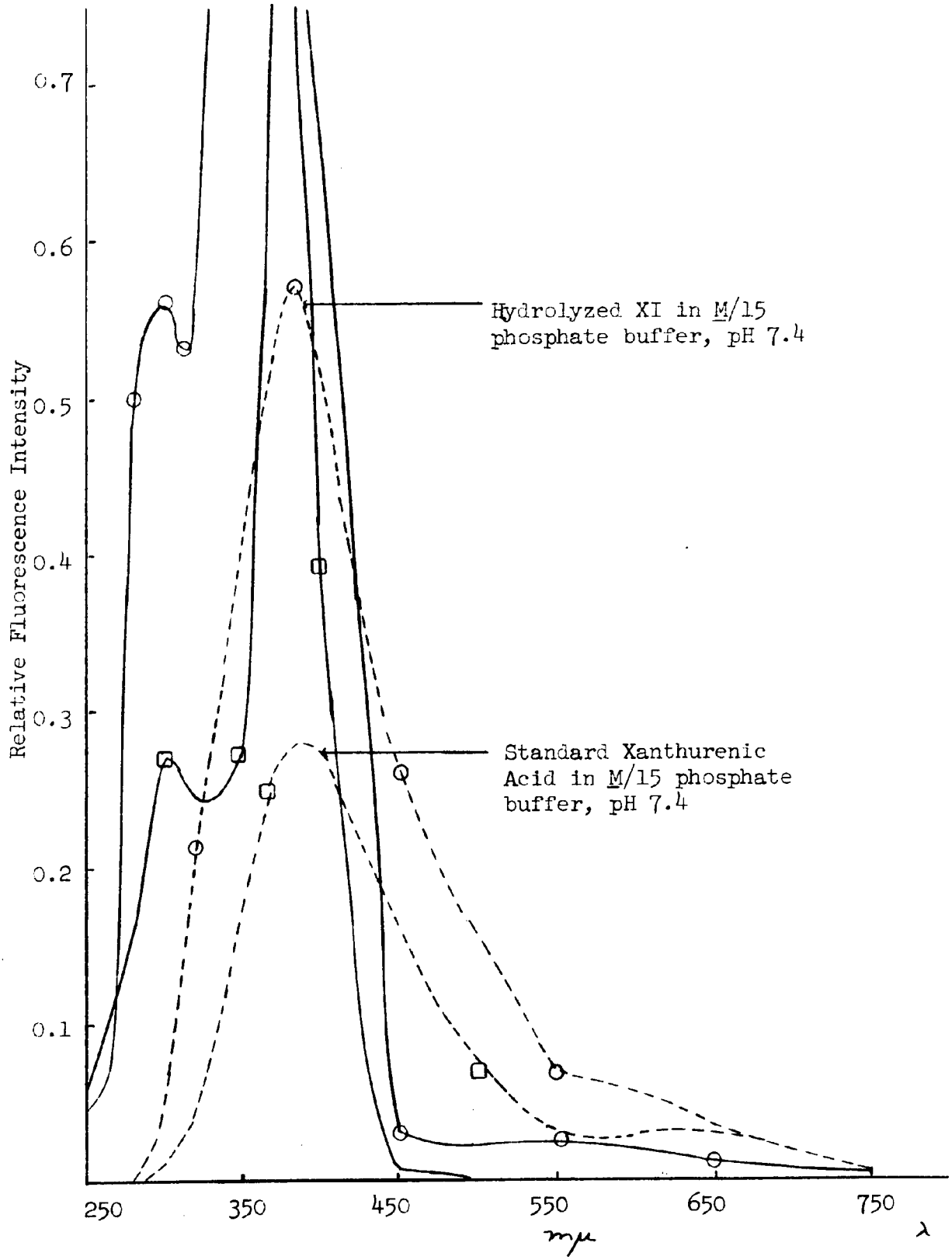
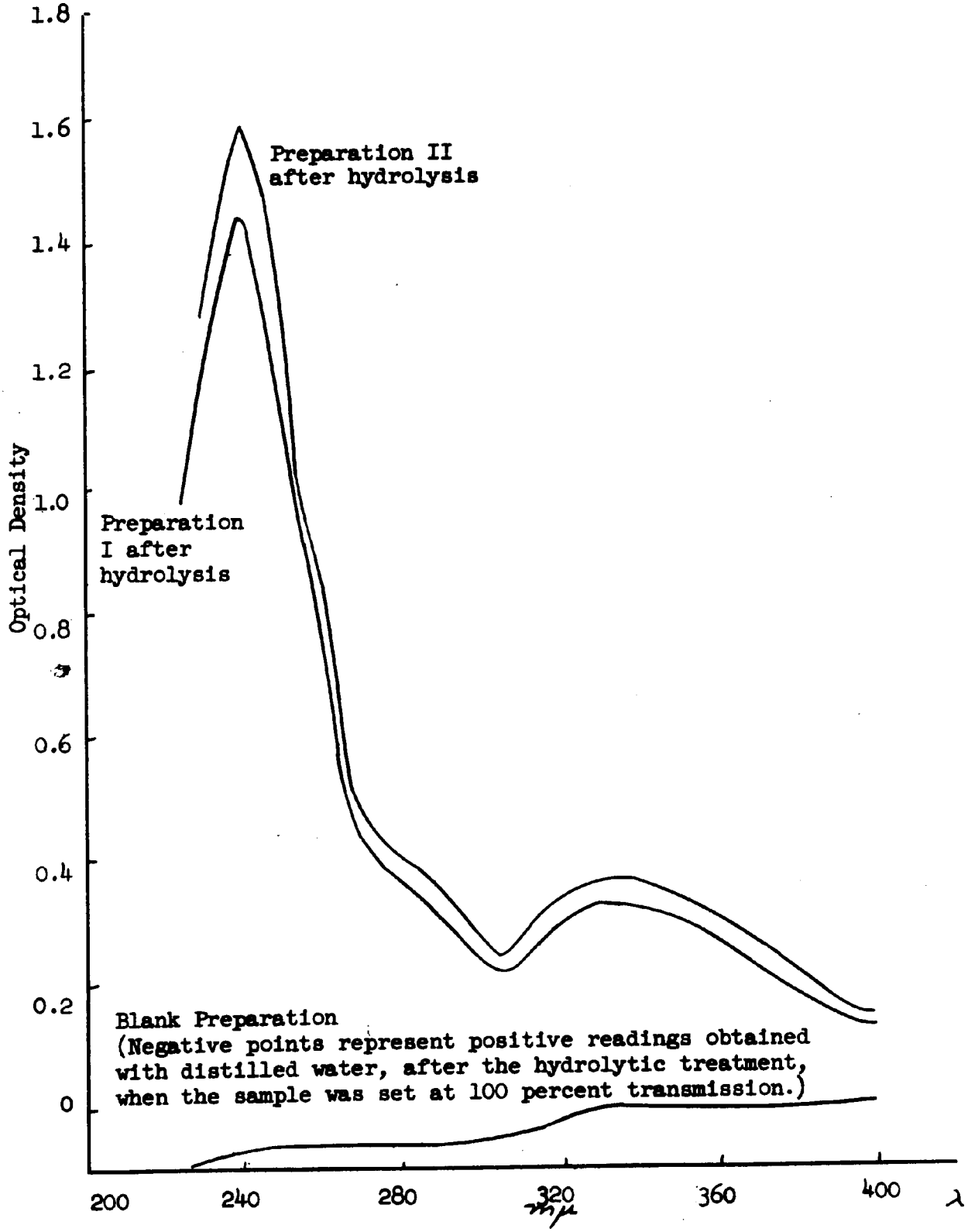


Figure 45. Ultraviolet absorption spectra of XI after acid hydrolysis, obtained on preparations I, II and on the blank preparation



tabulated in Table 14. These results show no difference between the isolated conjugate and the fluorescent material in the crude incubation mixture.

4. Quantitative analyses of hydrolysis products of XI

(a) Determination of the concentration of glucuronic acid formed from XI using the naphthoresorcinol method: The standard glucuronic acid was a synthetic product. It was labeled to have an $\left[\alpha\right]_{\underline{D}}^{24}$ of 34.4° and to be 86.47% in the acid form, so its molecular weight was taken as 192. It melted at 165°, reported 165°. A sample of glucuronolactone (M.W. 176) supplied by Corn Products Co. (a gift of Dr. S. Roseman) was also used. The results obtained with the two materials were substantially the same. Every time XI was analyzed, a series of glucuronic acid standards was included. The standard curves obtained are shown in Figure 46, and were obtained simultaneously with two different analyses of XI. The points obtained using the Corn Products Co. glucuronolactone, adjusted to correspond to the weight of glucuronic acid-glucuronolactone used as standard, are also given, i. e., the readings obtained with a given weight of glucuronolactone were multiplied by 176/192.

The probable error, defined in Appendix IV, was calculated for all the quantitative procedures. The interpretation applied to the probable error is that if the experiment is repeated under the same conditions with the same care, then half of the results will fall within a range of $\pm$ r, and that there is only one chance in one hundred of obtaining a random error exceeding $\pm$ 4r.

The spectrum obtained with XI in the naphthoresorcinol method is compared with that obtained from glucuronic acid, in Figure 47, and the two are seen to be essentially identical. The results obtained by

Table 14

Comparison of R_f values obtained for purified XI with those of the original fluorescent product in the crude incubation mixtures

Material Chromatographed	R_f value of blue-white, fluorescent product using		
	KCl	BAW	MBBW
XI (Prep. II)	0.53	0.17	0.29
XI (Prep. II) cochromatographed with a blank incubation mixture	0.35[a]	0.18	0.24
Fluorescent product in crude incubation mixture	0.35	0.18	0.25
XI (Prep. II) cochromatographed with incubation mixture in which xanthurenic acid was substrate	0.35	0.19	0.26

[a]Note the drop in R_f value of XI due to the presence of salts.

Figure 46. Standard curves for the naphthoresorcinol method for glucuronic acid

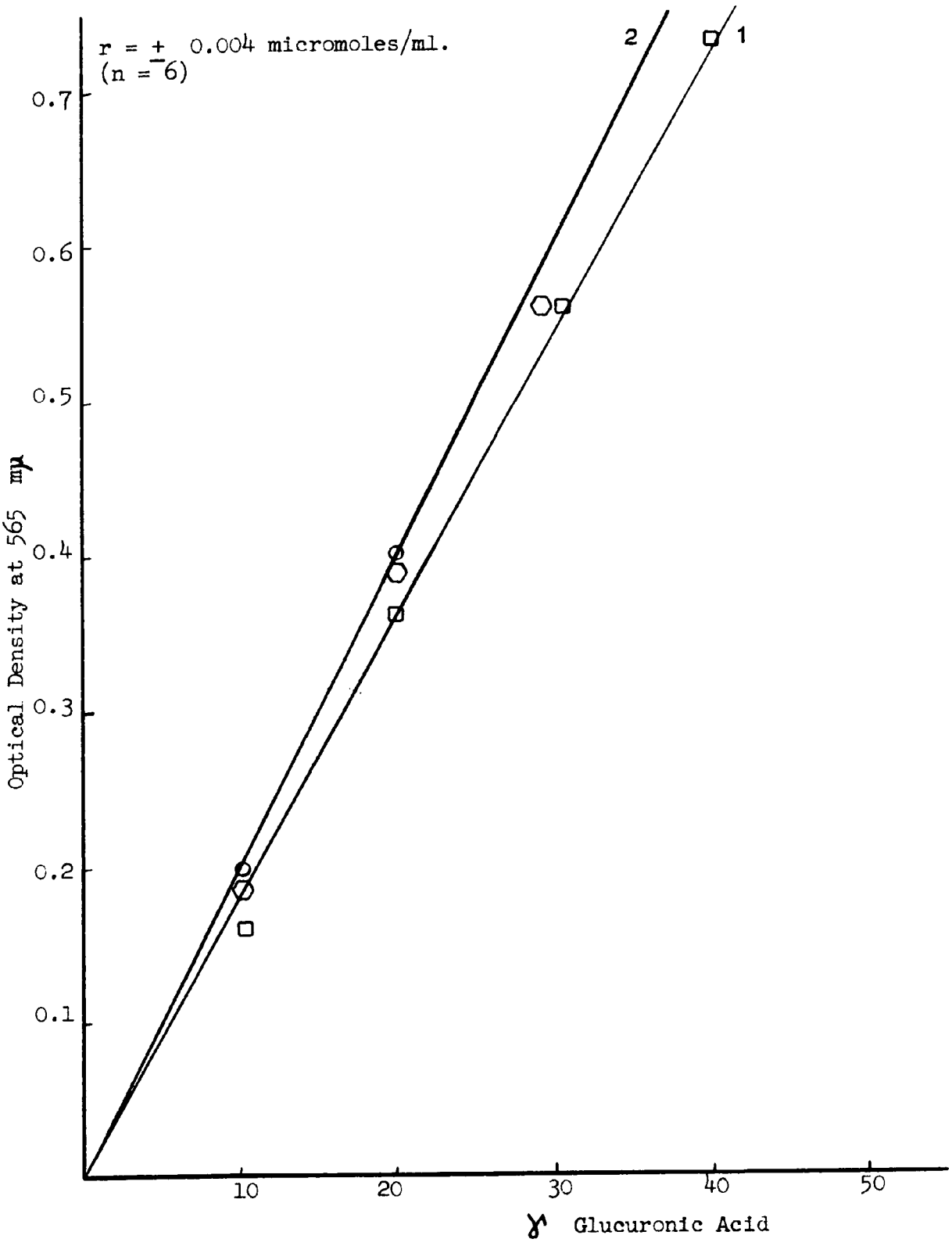
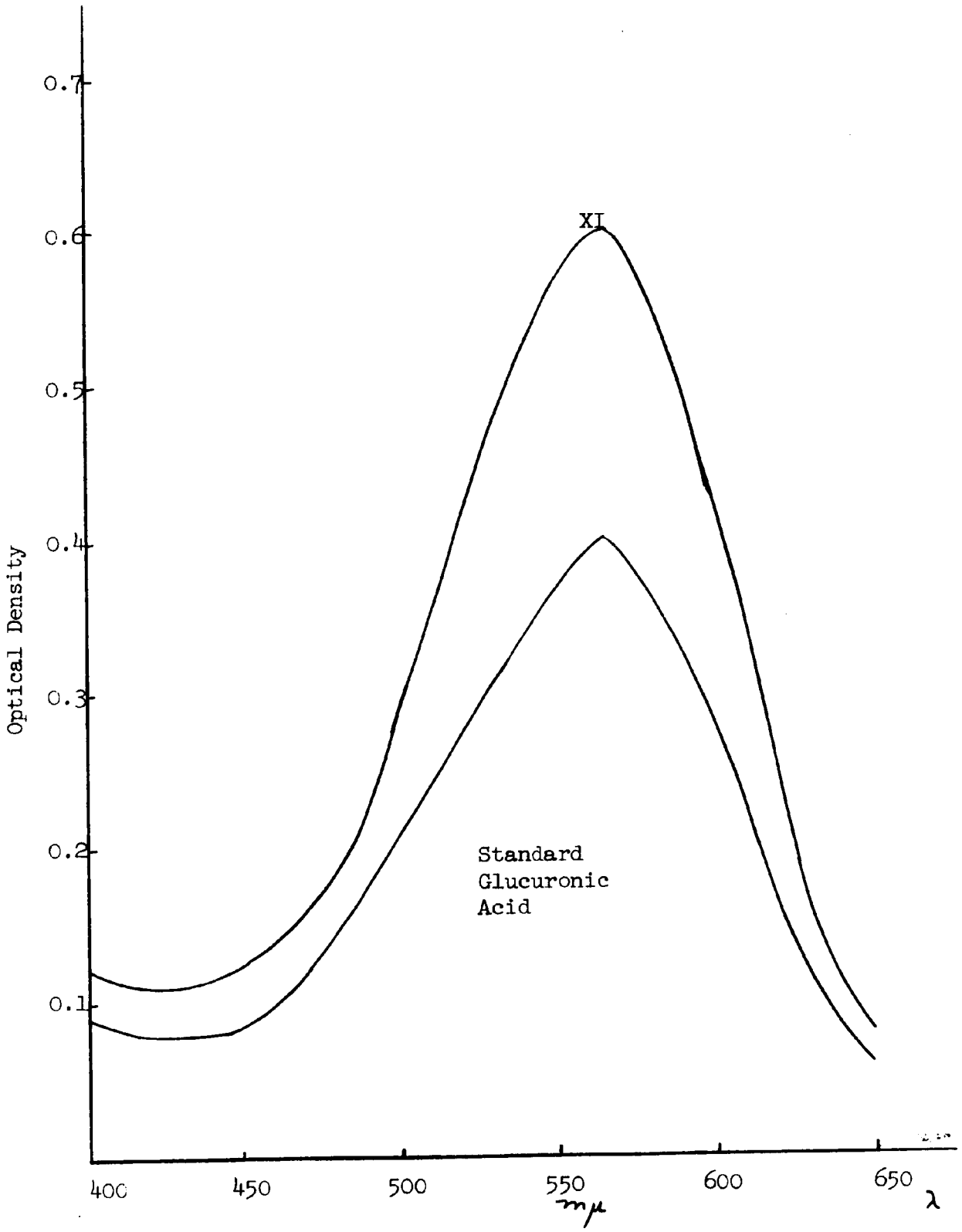


Figure 47. Absorption spectra obtained from the reaction of naphthoresorcinol with XI and with standard glucuronic acid



this method in the analyses of XI and the blank preparation, have been tabulated in Table 15, and, along with the results obtained by other methods, in Table 21.

(b) Determination of the concentration of glucuronic acid formed from XI using the carbazole method: The spectra obtained by this method from one ml. of XI solution and from standard glucuronic acid are shown in Figure 48. The spectra are essentially identical. XI was found to give a light yellow color when heated with concentrated sulfuric acid before the carbazole was added. In order to correct the values obtained after addition of carbazole for the presence of this yellow color, one sample (Preparation II) was run without addition of carbazole. This value was subtracted from all the other samples of XI (Preparations I or II) which had been run without such a blank being provided. The results obtained with standard glucuronic acid solution are shown in Figure 49. The data obtained in the analyses of XI and the blank preparation are given in Table 16, and in Table 21 in comparison with other constituents of XI.

(c) Determination of the concentration of glucuronic acid formed from XI by hydrolysis with β-glucuronidase, using the ferricyanide method: The micromethod for blood glucose (74) was modified for these experiments as described in the experimental section. The spectrum of the color obtained from XI following hydrolysis is substantially identical with that obtained with glucuronic acid as can be seen from Figure 50. Thus the color can be assumed to be due to the reduction of the ferricyanide and the formation of Prussian blue.

Xanthurenic acid was also found to be oxidized by ferricyanide. Therefore, before the method could be used for analysis of glucuronic

Table 15

Data obtained in the analysis of XI using the naphthoresorcinol method

Curve number in Figure 46	Sample	K value of curve, O.D./γ	Optical Density (per 1 ml. sample)	Micrograms glucuronic acid/ml.	Micromoles glucuronic acid/ml.
1	Prep. I	0.180	0.27	15.0	0.08
2	Prep. I	0.200	0.30	15.0	0.08
1	Prep. II	0.180	0.29	16.1	0.08
1	Blank Prep.	0.180	0.00	-	-

Figure 48. Absorption spectra obtained from the reaction of carbazole with XI, Preparations I and II, and with standard glucuronic acid

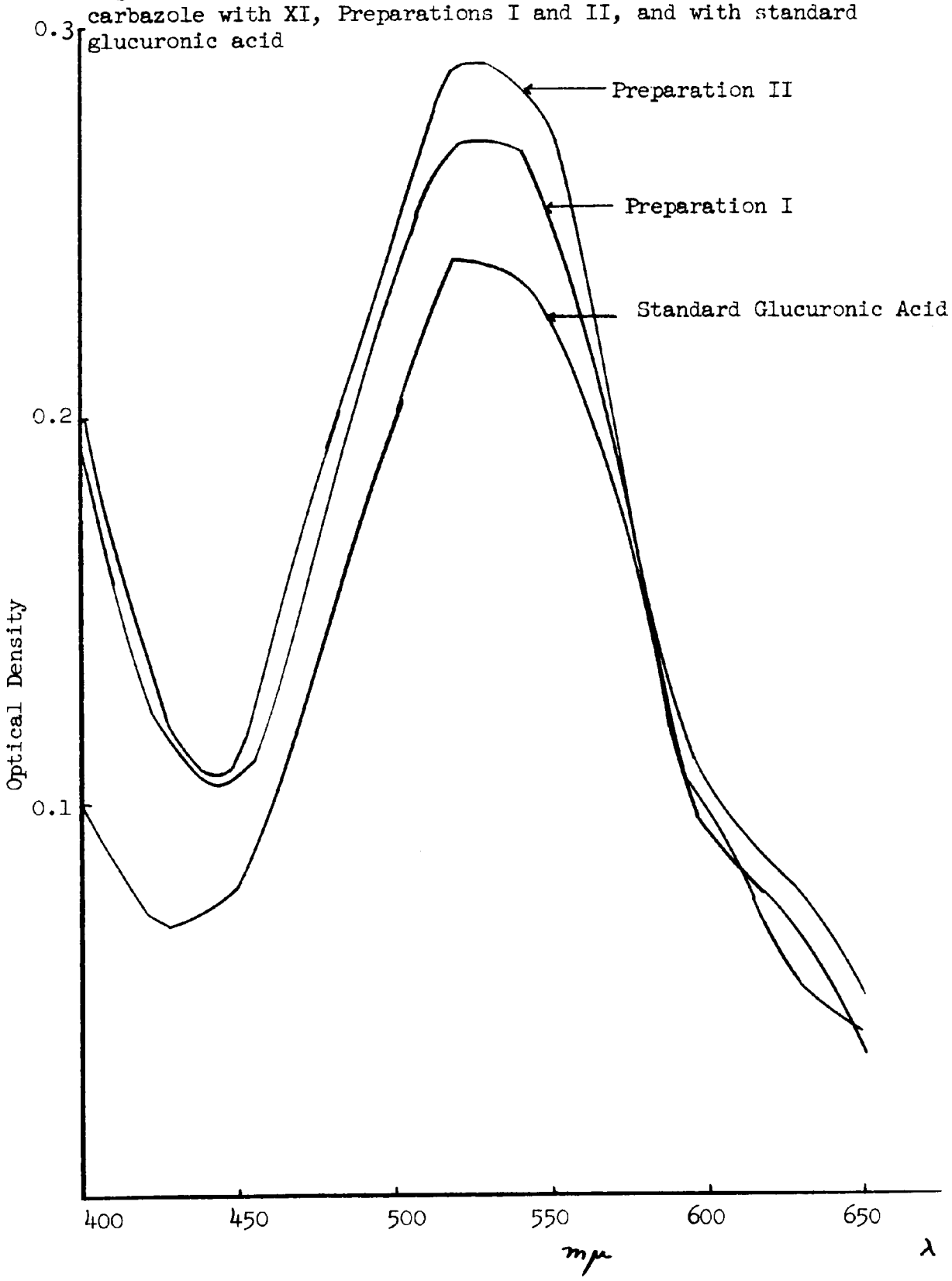


Figure 49. Standard curves for the carbazole method for glucuronic acid

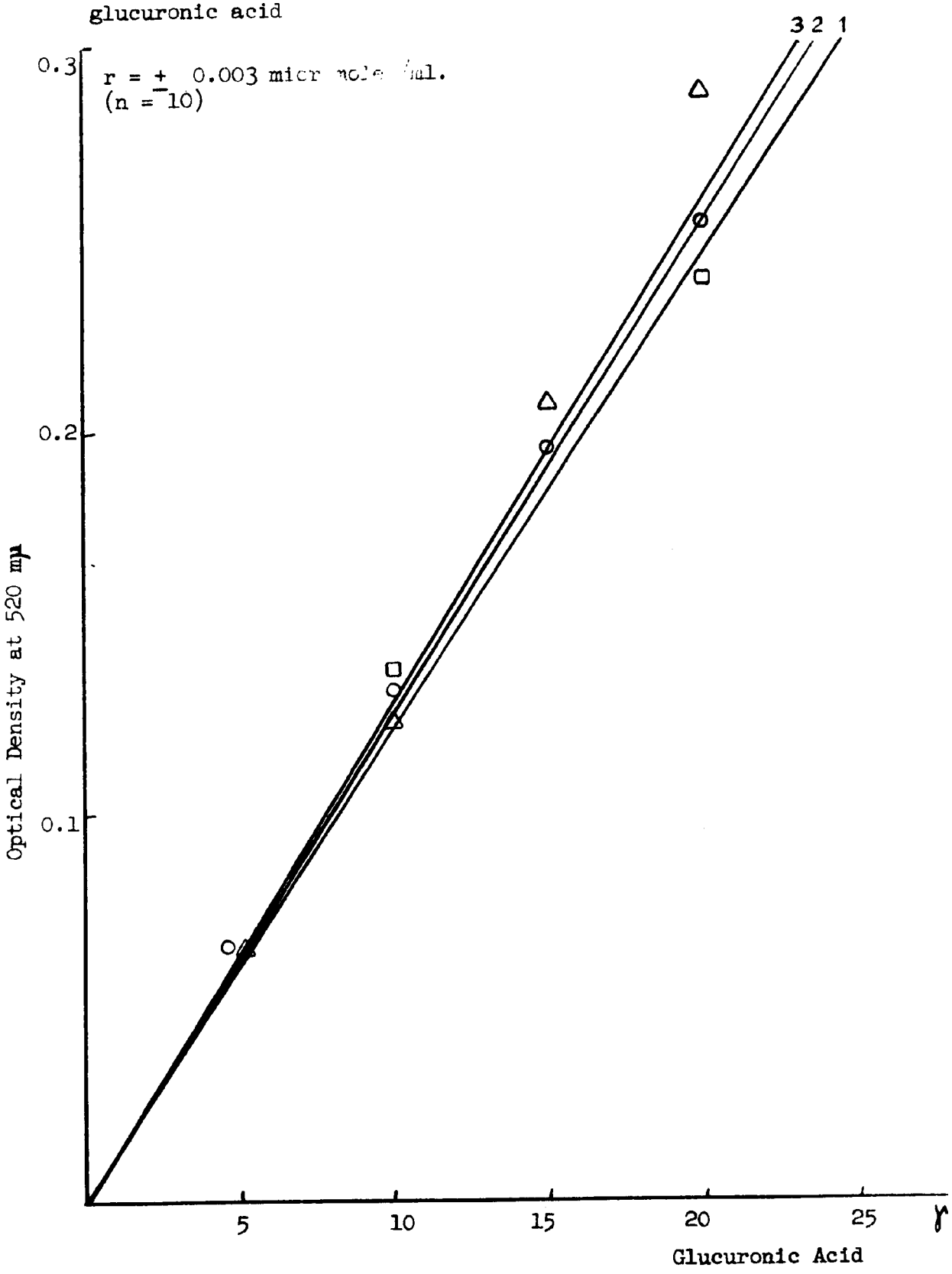


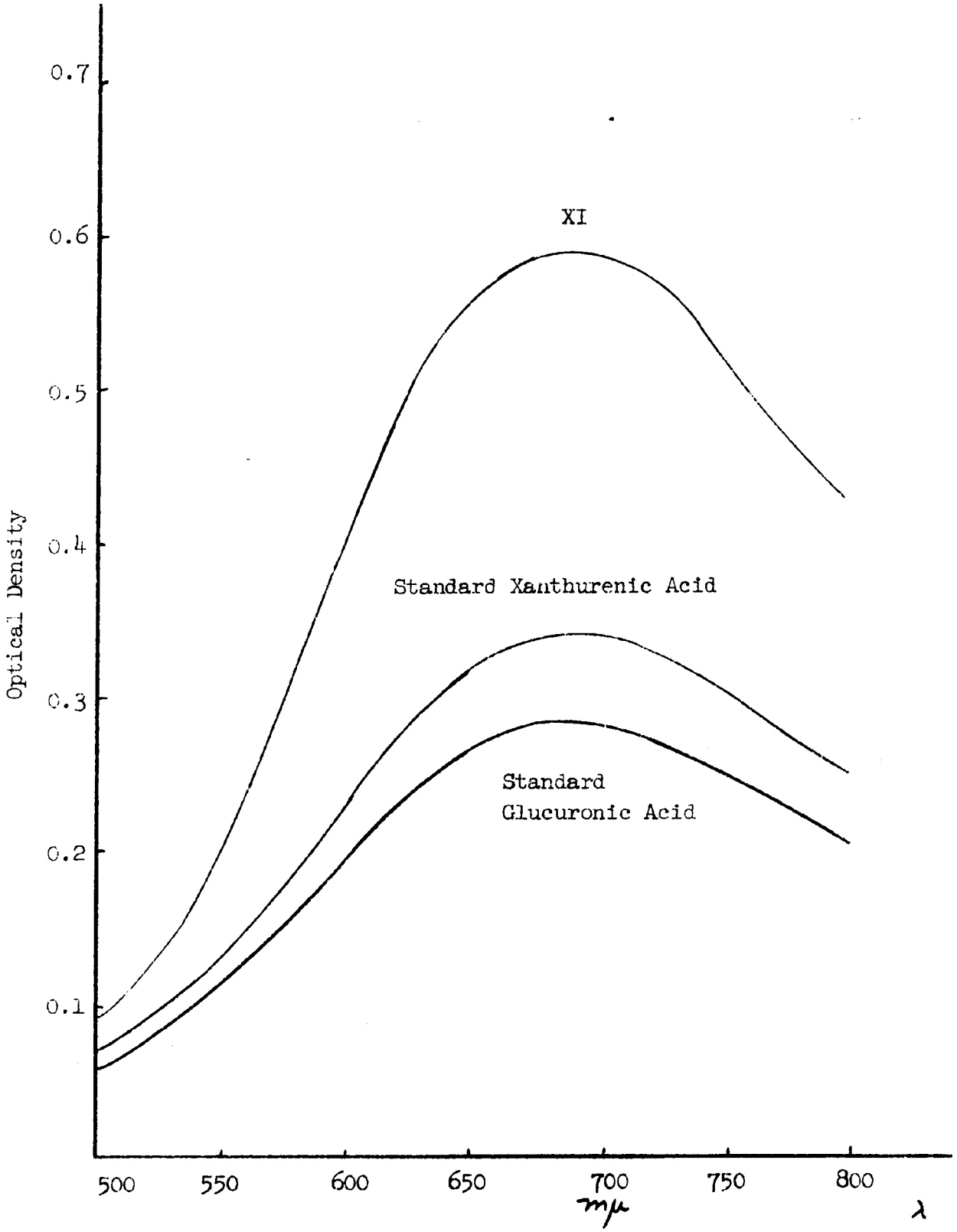
Table 16

Data obtained in the analysis of XI using the carbazole method

Curve Number in Figure 49	Sample	K value of curve, O.D./γ	Optical Density[a] (per 1 ml. sample)	Micrograms glucuronic acid/ml.	Micromoles glucuronic acid/ml.
3	Prep. I	0.0133	0.239	18.0	0.09
1	Prep. II	0.0123	0.221	18.0	0.09
1	Prep. II	0.0123	0.221	18.0	0.09
2	Prep. II	0.0127	0.174	13.7	0.07
3	Prep. II	0.0133	0.218	16.4	0.09
3	Blank Prep.	0.0133	0.032	-	-

[a]These readings were corrected for color produced by the action of hot, concentrated acid on XI (see page 66).

Figure 50. Absorption spectra obtained from the reaction of ferricyanide with XI (after enzymatic hydrolysis), with standard xanthurenic acid and with standard glucuronic acid



acid, an investigation was necessary to learn whether the color produced with xanthurenic acid in the presence of glucuronic acid was directly proportional to the concentration of xanthurenic acid and independent of glucuronic acid concentration. In Figure 51, the optical densities of three series of determinations are given (these determinations follow the entire experimental procedure, including incubation with β-glucuronidase). The determinations consist of one series of standard xanthurenic acid solutions, a series of standard glucuronic acid solutions, and a series of standard solutions containing equal weights of glucuronic acid and xanthurenic acid. These graphs show that the optical densities of the two compounds are additive and that, if the level of xanthurenic acid in a mixture is known, a correction can be applied so that the amount of glucuronic acid can be determined. The correction was based on the xanthurenic acid concentration determined by ultraviolet absorption at 330 mμ, which will be described in Section C-4d.

In Figure 51, a series of standard glucuronic acid curves can be seen. All points represent solutions which were carried through the complete experimental procedure. In the case of duplicate determinations of aliquots of the same sample, both of the optical density values obtained are included. The amount of glucuronic acid released by the action of β-glucuronidase on XI is determined from the standard curve which was obtained simultaneously with the analysis of XI. The amount of glucuronic acid released is given for XI (Preparations I and II) and the blank preparation in Table 17, and in Table 21 along with values obtained by the other methods. The values obtained for XI (Preparations I and II) represent only one complete analysis of each

Figure 51. Standard curves for the ferricyanide method for glucuronic acid

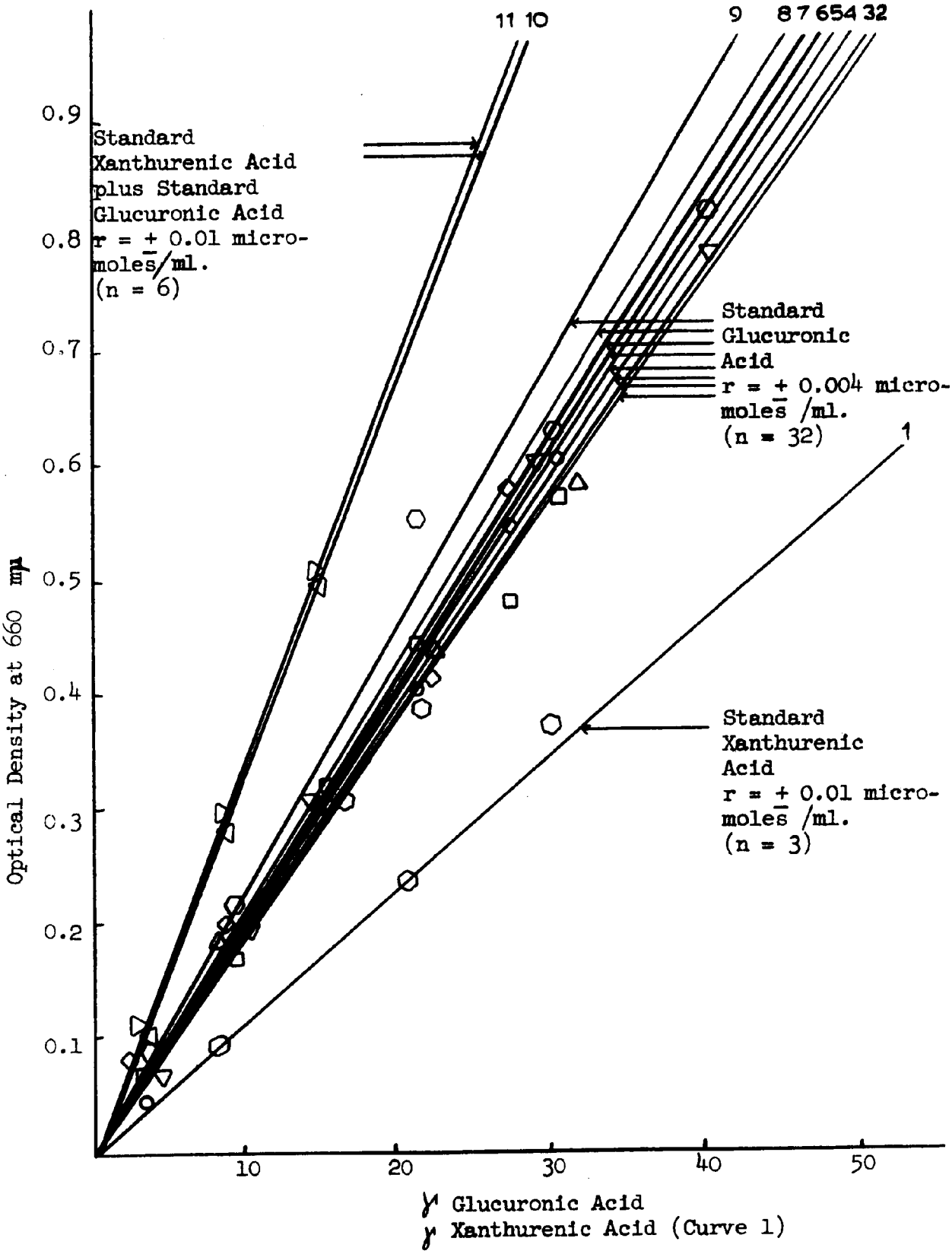


Table 17

Data obtained in the analysis of XI using the ferricyanide method after treatment with β-glucuronidase

Curve number in Figure 51	Sample	K value of curve, O.D./γ	Optical Density[a] (per 1 ml. sample)	Micrograms glucuronic acid/ml.	Micromoles glucuronic acid/ml.
2	Prep. I	0.0187	0.369	19.7	0.10
3	Prep. I	0.0188	0.395	21.0	0.10
5	Prep. I	0.0200	0.446	22.3	0.11
2	Prep. II	0.0187	0.374	20.0	0.10
3	Prep. II	0.0188	0.375	20.0	0.10
5	Prep. II	0.0200	0.366	18.3	0.09
3	Blank Prep.	0.0188	0.015	-	-
4	XI plus denatured enzyme	0.0194	0.024	-	-

[a]The optical density due to xanthurenic acid as determined from Curve 1 was first subtracted, using the xanthurenic acid concentration determined spectrophotometrically at 330 mμ.

preparation, the triplicate values being obtained on three aliquots of the same deproteinized filtrate. However, these aliquots were not all treated with ferricyanide at the same time, so that the analytical values are based on three standard curves.

One sample was run in which β-glucuronidase solution was added to the XI solution after the dilute tungstic acid had been added so that there was no chance of enzymatic hydrolysis. The solution was centrifuged and the supernatant fluid reacted with ferricyanide as usual. There was essentially no reduction of the ferricyanide as can be seen in Table 17.

(d) Determination of the concentration of xanthurenic acid formed from XI, using the ultraviolet absorption of xanthurenic acid at 330 mμ: That XI yields xanthurenic acid upon hydrolysis has been shown by means of paper chromatography (see Part II) and with fluorescence and ultraviolet absorption spectra obtained on acid hydrolyzates (see Part III, Section 3).

During the development of this procedure, it was found that glucuronic acid, when put through the hydrolysis procedure, absorbs slightly in the ultraviolet region. Unless the presence of glucuronic acid liberated from XI during the hydrolysis is considered, the values obtained for xanthurenic acid will be significantly too high. In Figure 52, spectra of xanthurenic acid, glucuronic acid and XI are compared as they are obtained after hydrolysis and solution in phosphate buffer. In Figure 53, the ultraviolet absorption curves obtained with a solution of known xanthurenic acid concentration, with a solution of known glucuronic acid concentration, and with a solution containing a known amount of both xanthurenic and glucuronic acids are shown. From

Figure 52. Absorption spectra of XI, standard xanthurenic acid and standard glucuronic acid (after acid hydrolysis, evaporation and solution in phosphate buffer, pH 7.4)

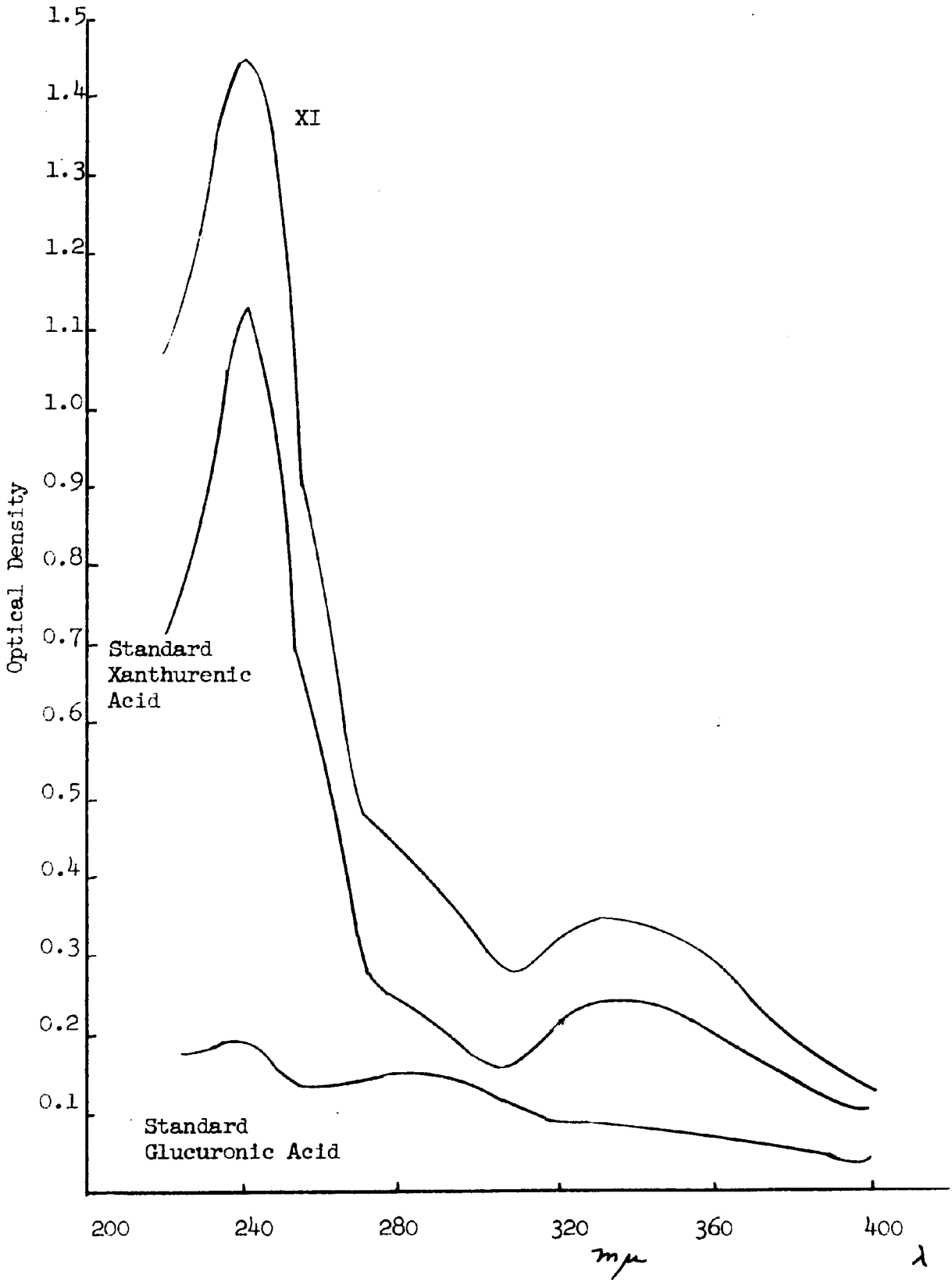
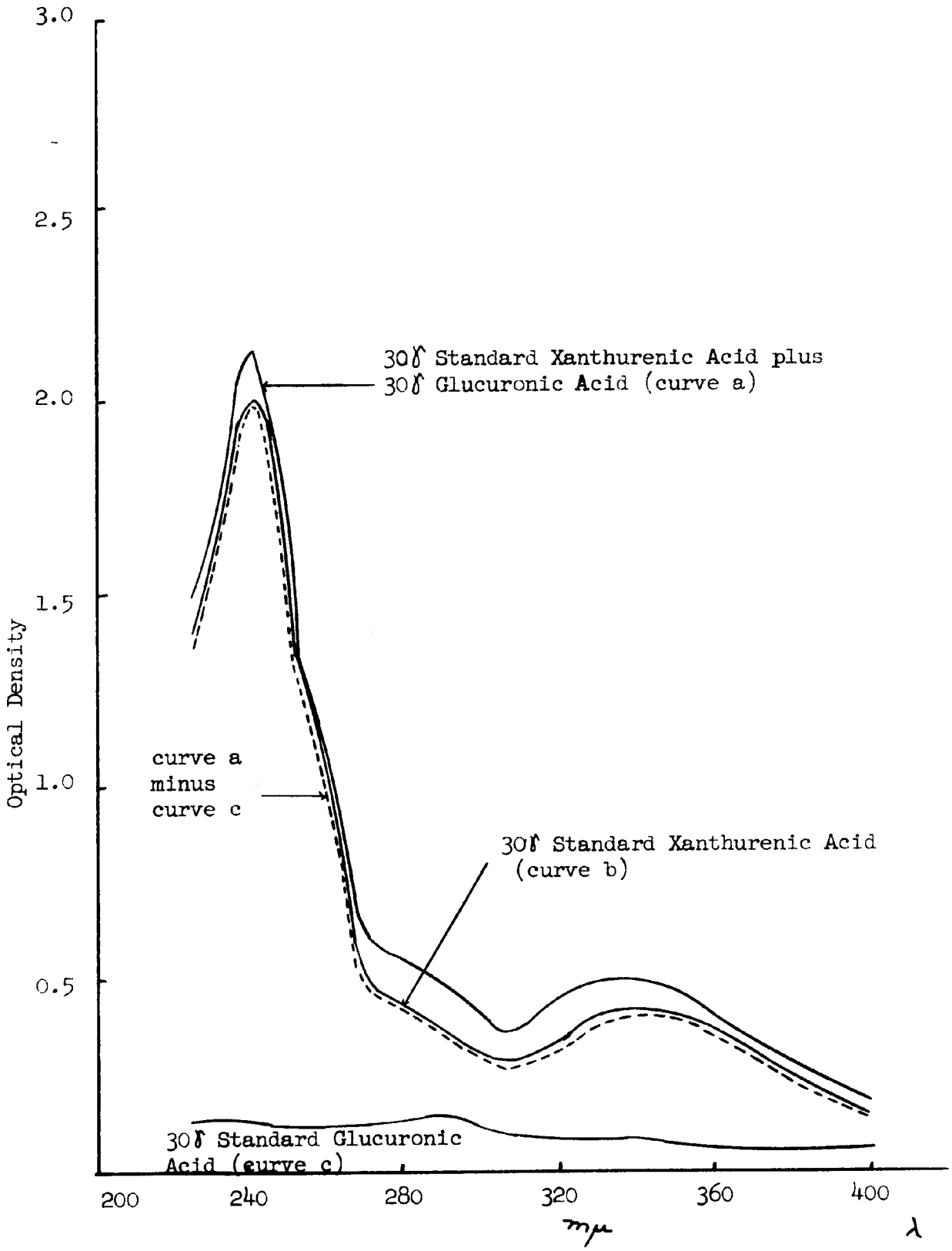


Figure 53. Absorption spectra of standard xanthurenic acid, of standard glucuronic acid and of standard xanthurenic and glucuronic acids combined (after acid hydrolysis, evaporation and solution in phosphate buffer, pH 7.4)



the figure, one can see that it is possible to subtract the absorption produced by the glucuronic acid alone from the absorption produced by a mixture of glucuronic acid and xanthurenic acid and, thereby, obtain the absorption produced by xanthurenic acid alone.

In Figure 54, the optical density at 330 mμ is plotted against glucuronic acid concentration, and, in Figure 55, the optical density at 330 mμ is plotted against xanthurenic acid concentration. Using the values of glucuronic acid in XI (obtained by the naphthoresorcinol method), the small absorption caused by this amount of glucuronic acid is determined from Figure 54. These values (obtained for Preparations I and II) are subtracted from the optical density readings obtained at 330 mμ after hydrolysis of XI, and the concentration of xanthurenic acid is determined using Figure 55. The values obtained for XI and the blank preparation are given in Table 18, and in Table 21 along with the values obtained by the other methods.

(e) Determination of the concentration of xanthurenic acid formed from XI using the iron-chelate method: The chelate formed with xanthurenic acid has a distinctive green color, and its specificity has made this a convenient method of analysis when large amounts of xanthurenic acid are involved. The method has been modified for measurement of the low concentrations of xanthurenic acid in the XI hydrolyzates and serves as an independent and specific check on the xanthurenic acid concentration.

In Figure 56, the spectral curves of standard xanthurenic acid and XI after hydrolysis are given. Both curves are characteristic of the xanthurenic acid-ferric iron-chelate. In Figure 57, the standard curve is plotted and the amount of xanthurenic acid liberated during hydrol-

Figure 54. Optical density of glucuronic acid at 330 mμ after acid treatment, evaporation and solution in phosphate buffer, pH 7.4

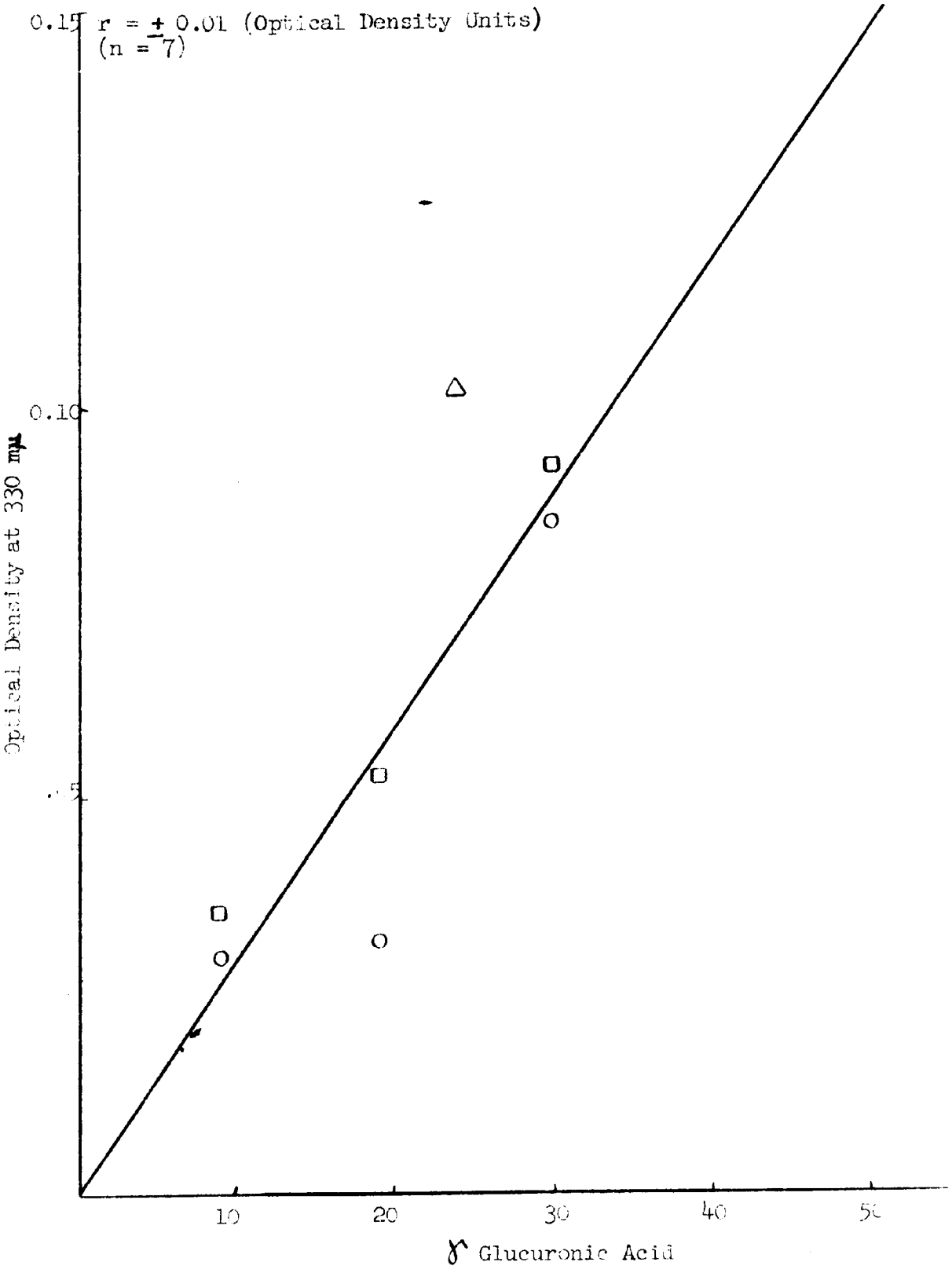


Figure 55. Standard curves for the spectrophotometric determination of xanthurenic acid

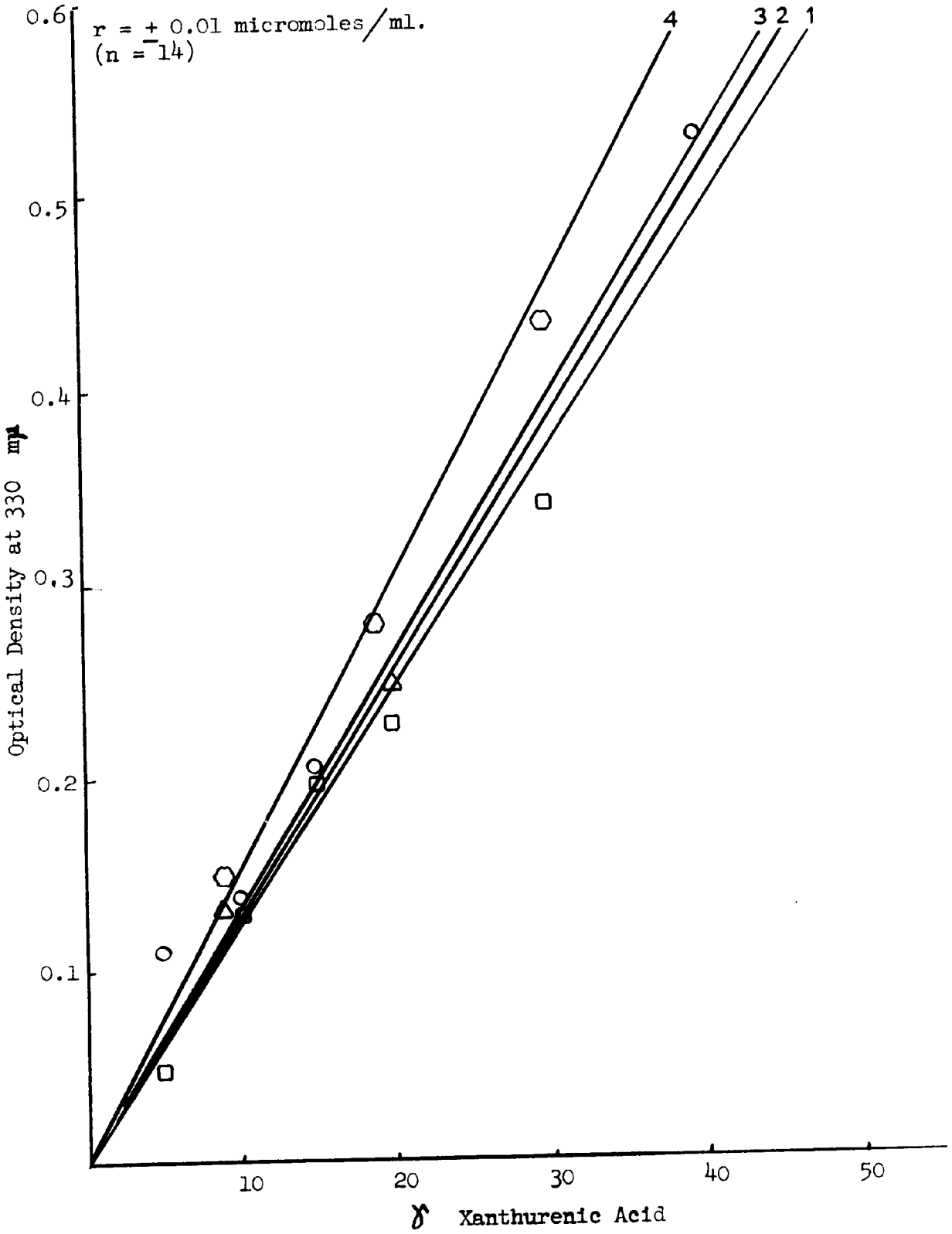


Table 18

Values obtained in the analysis of XI using the spectrophotometric method at 330 mμ following acid hydrolysis

Curve number in Figure 55	Sample	K value of curve, O.D./γ	Optical Density[a] (per 1 ml. sample)	Micrograms xanthurenic acid/ml.	Micromoles xanthurenic acid/ml.
1	Prep. I	0.0122	0.305	25.0	0.12
2	Prep. I	0.0126	0.341	27.0	0.13
2	Prep. I	0.0126	0.347	27.6	0.13
3	Prep. I	0.0130	0.308	23.7	0.12
1	Prep. II	0.0122	0.341	28.0	0.14
2	Prep. II	0.0126	0.344	27.3	0.13
2	Prep. II	0.0126	0.332	26.4	0.13
1	Blank Prep.	0.0122	-0.017[b]	-	-
2	Blank Prep.	0.0126	0.016	-	-

[a]The optical density due to glucuronic acid as determined from Figure 54 was first subtracted, using the glucuronic acid concentration as determined by the naphthoresorcinol method. For curves 1 and 2, the optical density was also corrected for that due to the blank preparation.

[b]A water sample which had undergone the analytical procedure was read against the blank preparation set at 100% transmission.

Figure 56. Absorption spectra obtained from the reaction of ferric ammonium sulfate with XI (after acid hydrolysis) and with standard xanthurenic acid

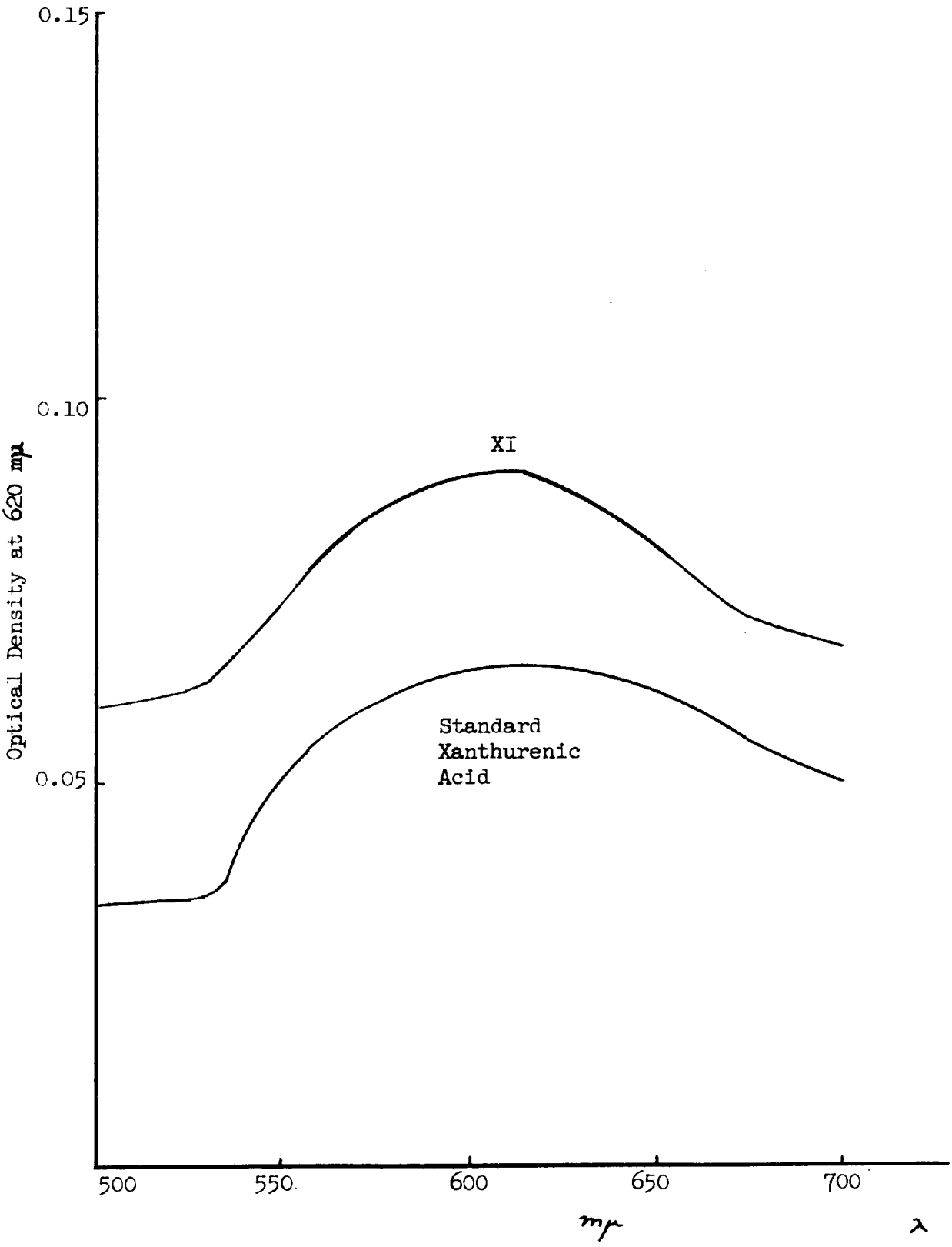
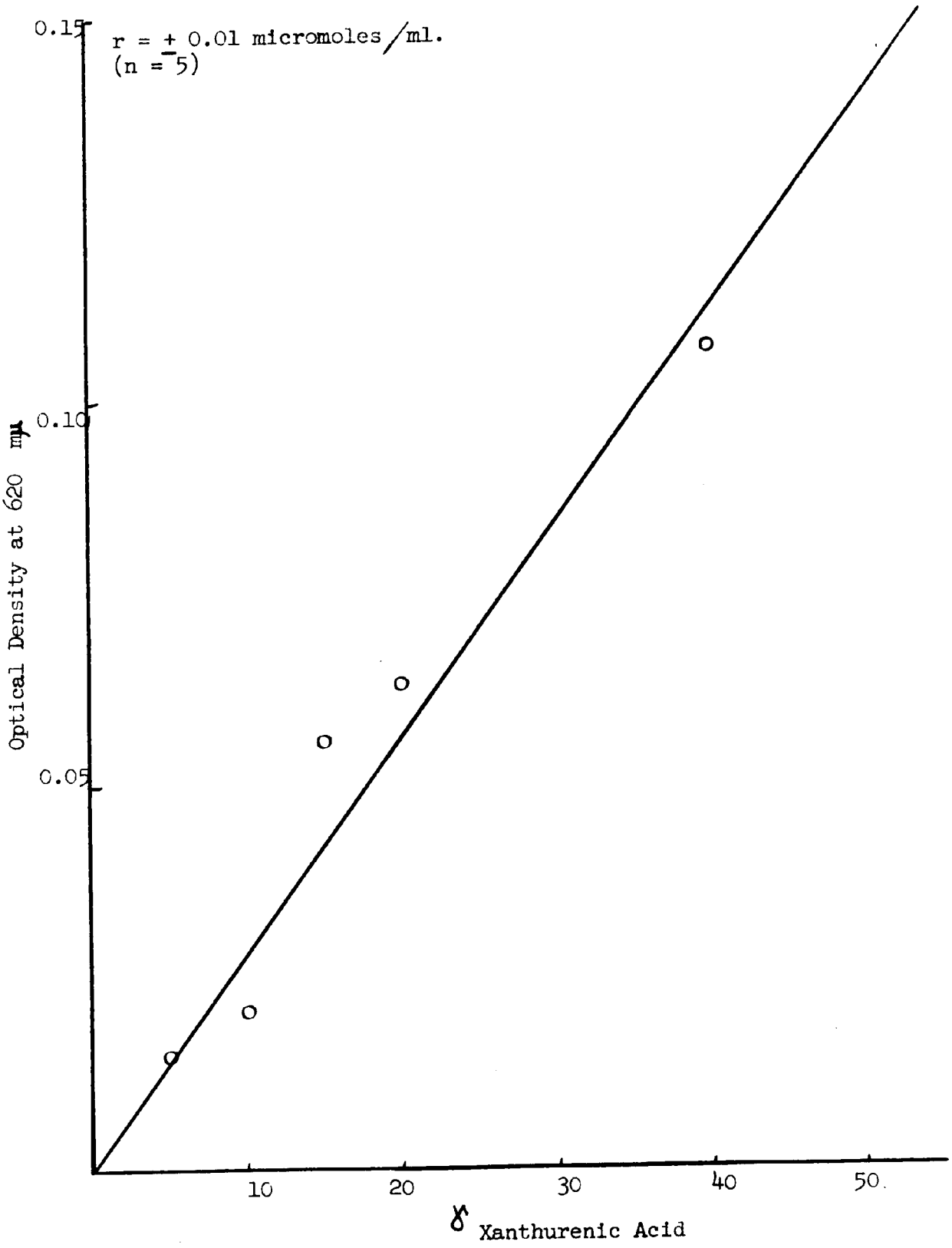


Figure 57. Standard curve for the iron-chelate method for xanthurenic acid



ysis can be read from the curve. This curve, like the others, contains all the experimental points that were obtained. The results are given in Table 19, and in Table 21 along with results obtained by the other methods.

(f) Determination of the absence of amino acid in hydrolyzates of XI: A plot of values obtained with standard serine solutions is shown in Figure 58. Table 20 shows the values obtained with XI (Preparation I). Also included are solutions containing known amounts of xanthurenic acid and glucuronic acid in addition to the serine. The recovery of serine is not affected by the presence of these other materials. XI is seen to be free of amino acid and no amino acid is liberated by hydrolysis; consequently, XI is not an amino acid conjugate.

5. Determination of the ultraviolet absorption spectra of kynurenic acid, xanthurenic acid and XI at various pH values and a determination from these spectra of the pK' values of ultraviolet-absorbing, dissociable groups in these molecules

The ultraviolet absorption spectra were run using the Cary recording spectrophotometer. Dilute aqueous solutions of kynurenic acid and xanthurenic acid were prepared. One milliliter of XI (Preparation I) was diluted with 4.0 ml. of water. By means of a very fine capillary pipette, extremely small drops of 18 N KOH, 1.0 N KOH, 0.1 N KOH, 1.0 N HCl, 0.1 N HCl, or 12 N HCl were added to each of the solutions in a small beaker, the solutions were then mixed and the pH values of the solutions measured using the Beckman pH-meter. Then each of the solutions was transferred to a cuvette and the spectrum of the solution obtained. By such additions, curves were obtained for solutions differing by about one pH unit from about a pH value of 2 to 12. For the

Table 19

Values obtained in the analysis of XI using the iron-chelate method

Sample	K value of curve, O.D./ γ	Optical Density (per 1 ml. sample)	Micrograms xanthurenic acid/ml.	Micromoles xanthurenic acid/ml.
Prep. I	0.00278	0.083[a]	29.8	0.15
Prep. II	0.00278	0.062[a]	22.3	0.11
Blank Prep.	0.00278	0.004	-	-

[a]The optical density due to the blank preparation has been subtracted from these values.

Figure 58. Standard curve for the ninhydrin method for amino acids

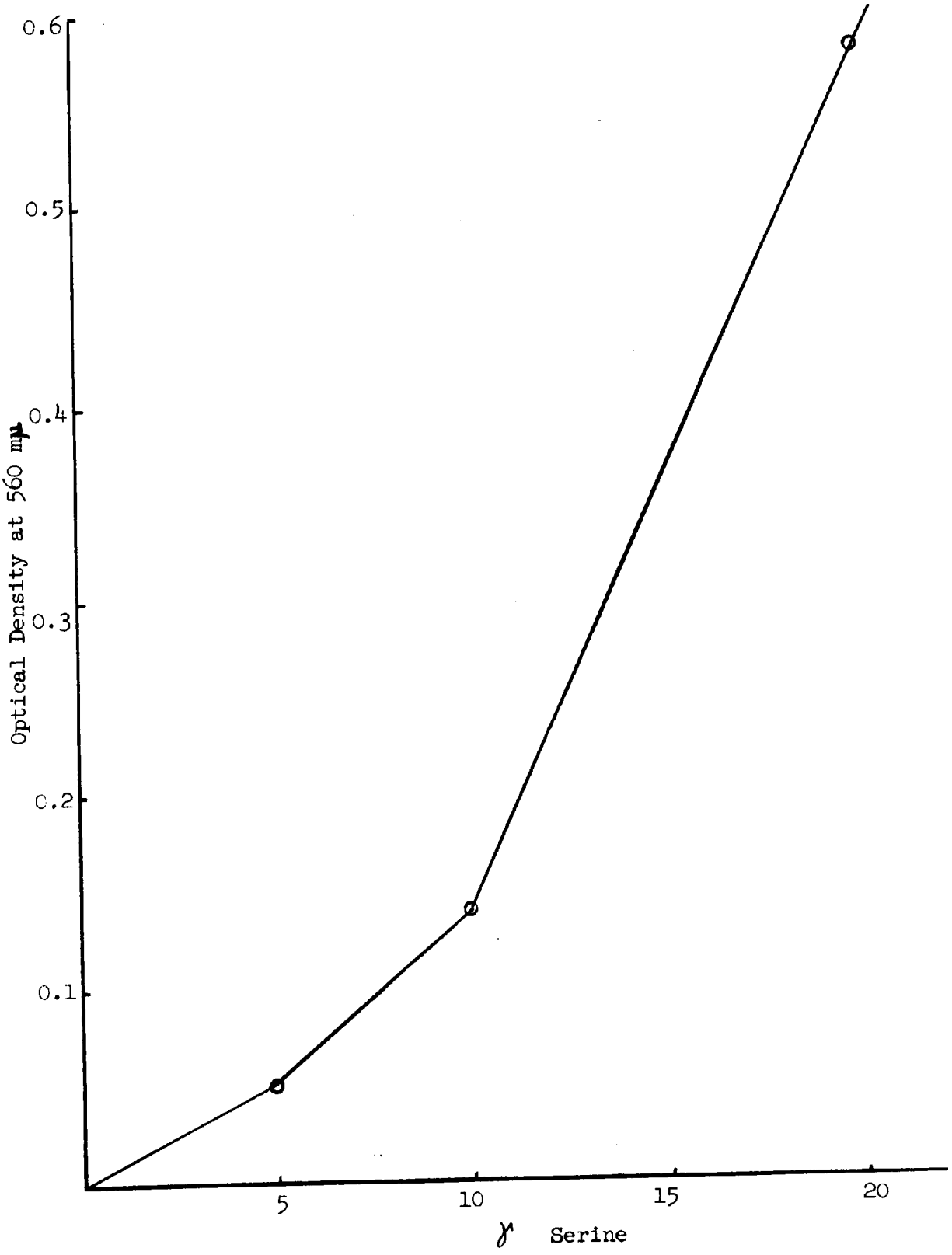


Table 20

Data obtained in the analysis of XI using the ninhydrin method

Sample	Optical Density
XI (Prep. I)	0.023
XI (Prep. I)	0.028
17γ Serine/ml.	0.480
17γ Serine/ml. plus 25γ Xanthurenic Acid/ml. plus 25γ Glucuronic Acid/ml.	0.520
17γ Serine/ml. plus 25γ Xanthurenic Acid/ml. plus 25γ Glucuronic Acid/ml.	0.650

Table 21

Quantitative determination of the micromoles of glucuronic acid and xanthurenic acid present per milliliter of XI solution

Xanthurenic Acid (micromoles /ml.)

Optical Density at 330 mμ method of analysis			Iron-chelate method		
Prep. I	Prep. II	Blank Prep.	Prep. I	Prep. II	Blank Prep.
0.12 0.12 0.13 0.13	0.13 0.13 0.14	0.00	0.15	0.11	0.01
r = ± 0.01			r = ± 0.01		

Glucuronic Acid (micromoles /ml.)

Naphthoresorcinol method[a]			Carbazole method[a]			Ferricyanide method		
Prep. I	Prep. II	Blank Prep.	Prep. I	Prep. II	Blank Prep.	Prep. I	Prep. II	Blank Prep.
0.08 0.08	0.08	0.00	0.09	0.07 0.09 0.09 0.09	0.01	0.10 0.10 0.11	0.10 0.10 0.09	0.00
r = ± 0.004			r = ± 0.003			r = ± 0.01		

[a] Examining the results, the values obtained for glucuronic acid by the naphthoresorcinol method and the carbazole method seem to be significantly lower than the values obtained by the other methods. Apparently, the strong acid solutions used in these two methods cause a small loss of glucuronic acid. This is in agreement with the results obtained by the authors of the carbazole method who reported obtaining values which were 15% low in the case of menthol glucuronide. A correction of this size would essentially bring the results of the three methods of analysis of glucuronic acid into agreement and would indicate a ratio of glucuronic acid to xanthurenic acid in a range of 1:1 to 1:1.5 (1:1 to 1:1.8 without the correction). The possibility that XI is a novel type of conjugated derivative consisting of more than one xanthurenic acid residue to each glucuronic acid residue is extremely remote, so the above data have been interpreted to indicate a 1:1 ratio.

higher pH values, drops of alkali were also added to the blank. With this procedure, only a small sample is required and no appreciable error is introduced by the volume of alkali or acid added. For the curves run in 6 N HCl or at pH values near 0, 1.0, 13 and 14, equal volumes of kynurenic acid or xanthurenic acid were added to 12 N HCl, 2.0 N HCl, 0.2 N HCl, 0.2 N NaOH and 2.0 N NaOH. Blanks were prepared in the same manner using distilled water. For XI, known amounts of 18 N KOH were added and a blank prepared in the same manner. In these cases, a dilution factor (determined spectrophotometrically) was used to convert these readings into those which would be obtained with solutions of the same concentration as the ones whose spectra were obtained in the pH range of 2 to 12.

The optical density readings obtained at two different wavelengths (chosen where the differences in spectra appeared to be clearly defined) were plotted against the pH values of the solutions on which these readings were obtained. These plots are shown in Figures 59-61. Those pH values at which changes in the spectral curves occur can be seen from the changes in optical density with pH. These changes are caused by ionizing groups which are involved in the absorption of ultraviolet light. The shape of the plot in the region where a group is undergoing ionization is sigmoid and the pK' value for the group can be estimated by the midpoint of the inflection. All the points obtained from the different spectral curves are shown. Complete ultraviolet absorption spectra of kynurenic acid, xanthurenic acid and XI are given in Figures 62-64, at those pH values where a given ionic species appears to be all in one form.

The plots of kynurenic acid show practically no change excepting

Figures 59, 60 and 61. The optical density at specified wavelengths plotted against pH value for solutions of XI, kynurenic acid and xanthurenic acid

Figure 59.

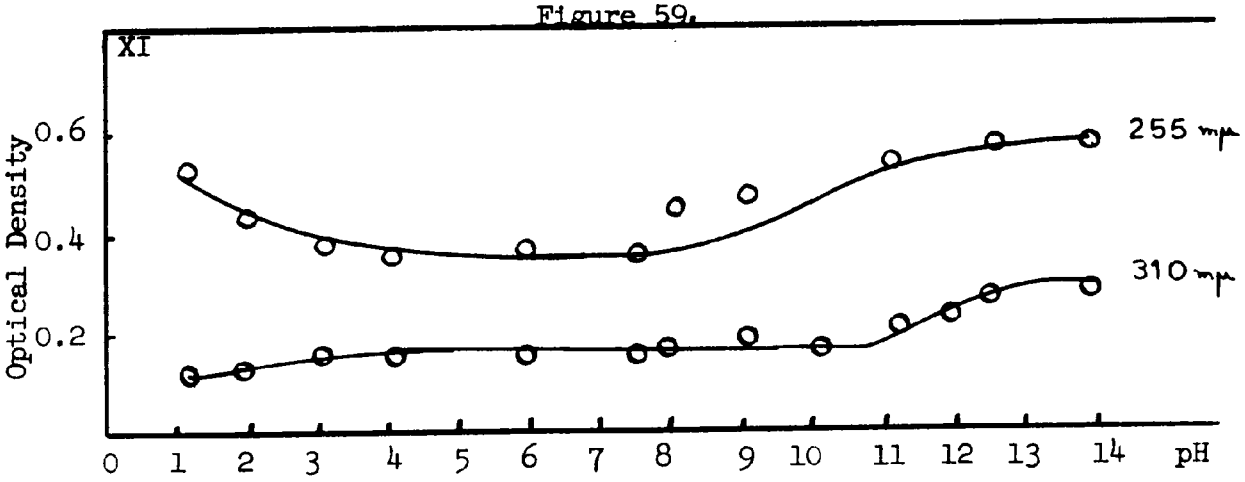


Figure 60.

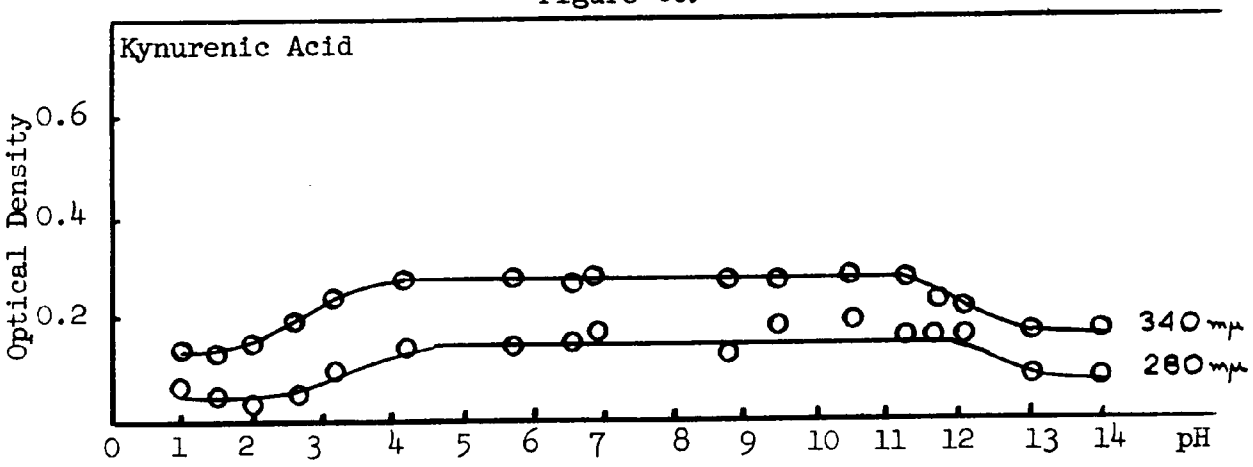
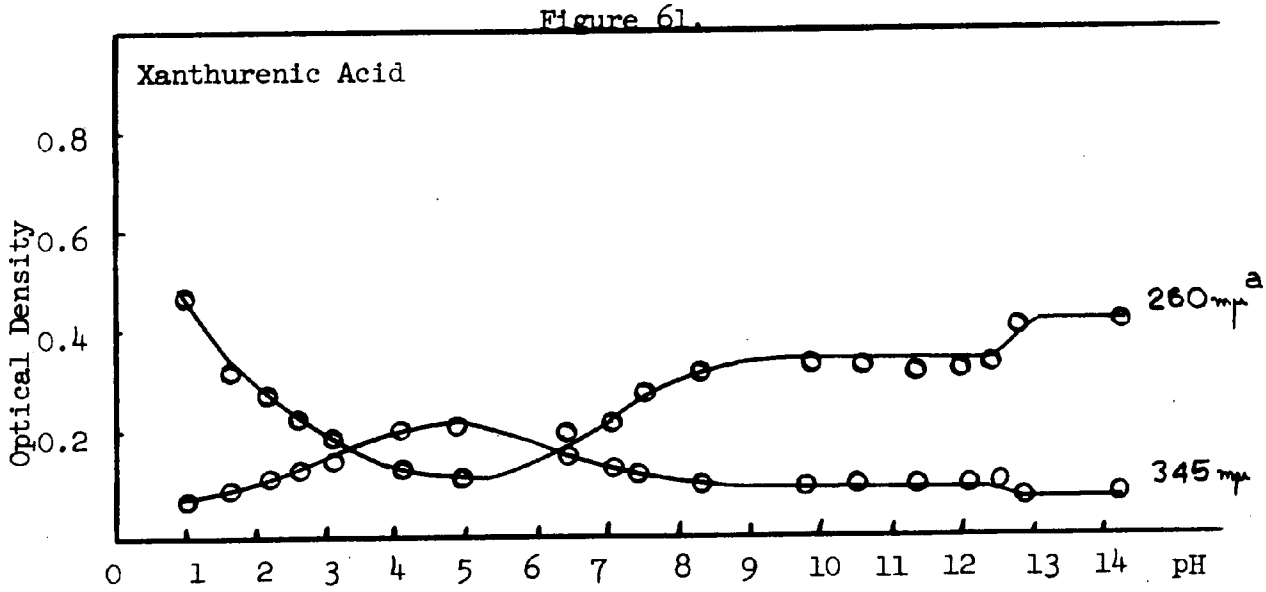


Figure 61.



[a]Optical density values divided by one-half.

Figure 62. Ultraviolet absorption spectra of four ionic forms of kynurenic acid

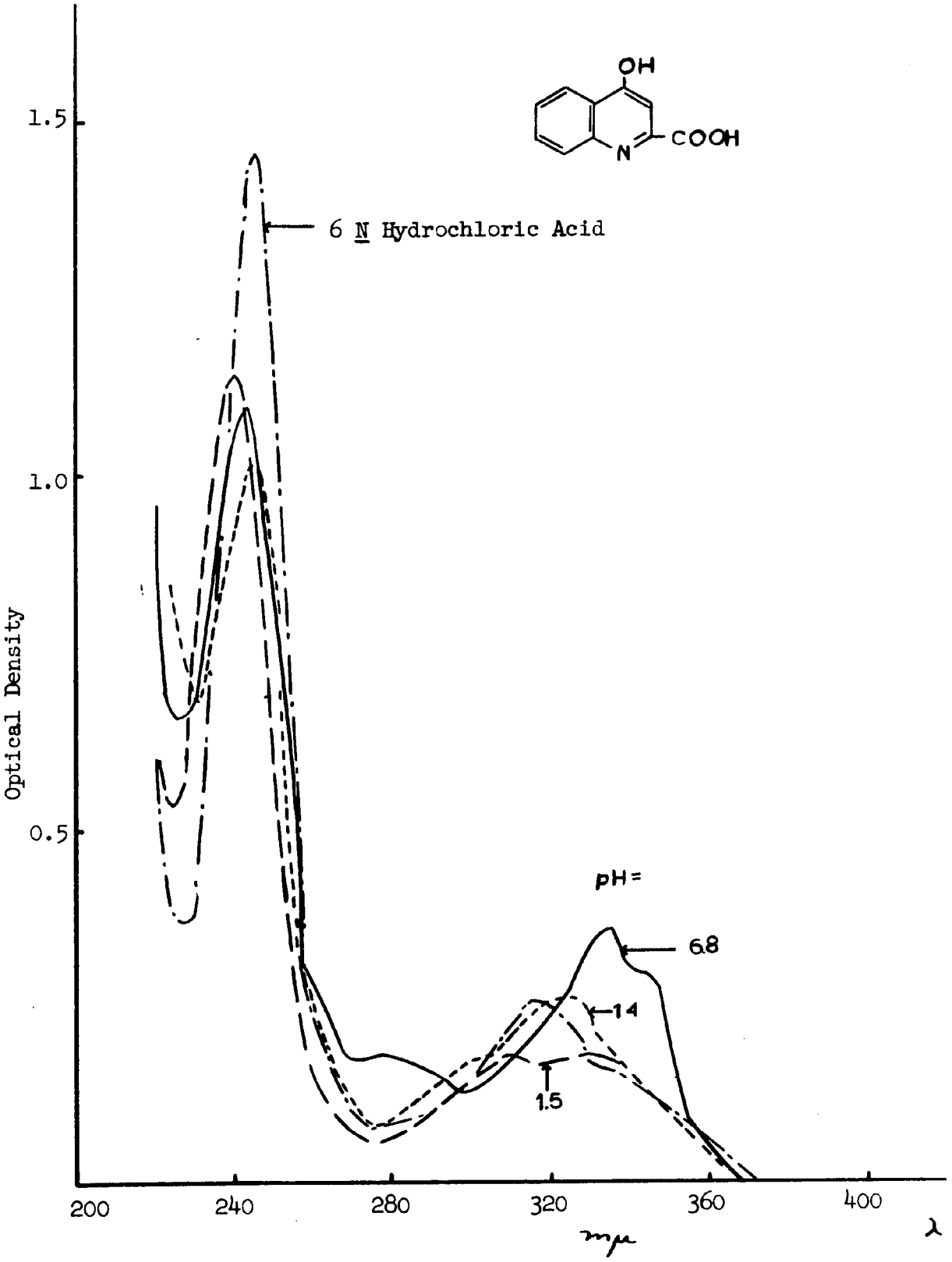


Figure 63. Ultraviolet absorption spectra of five ionic forms of xanthurenic acid

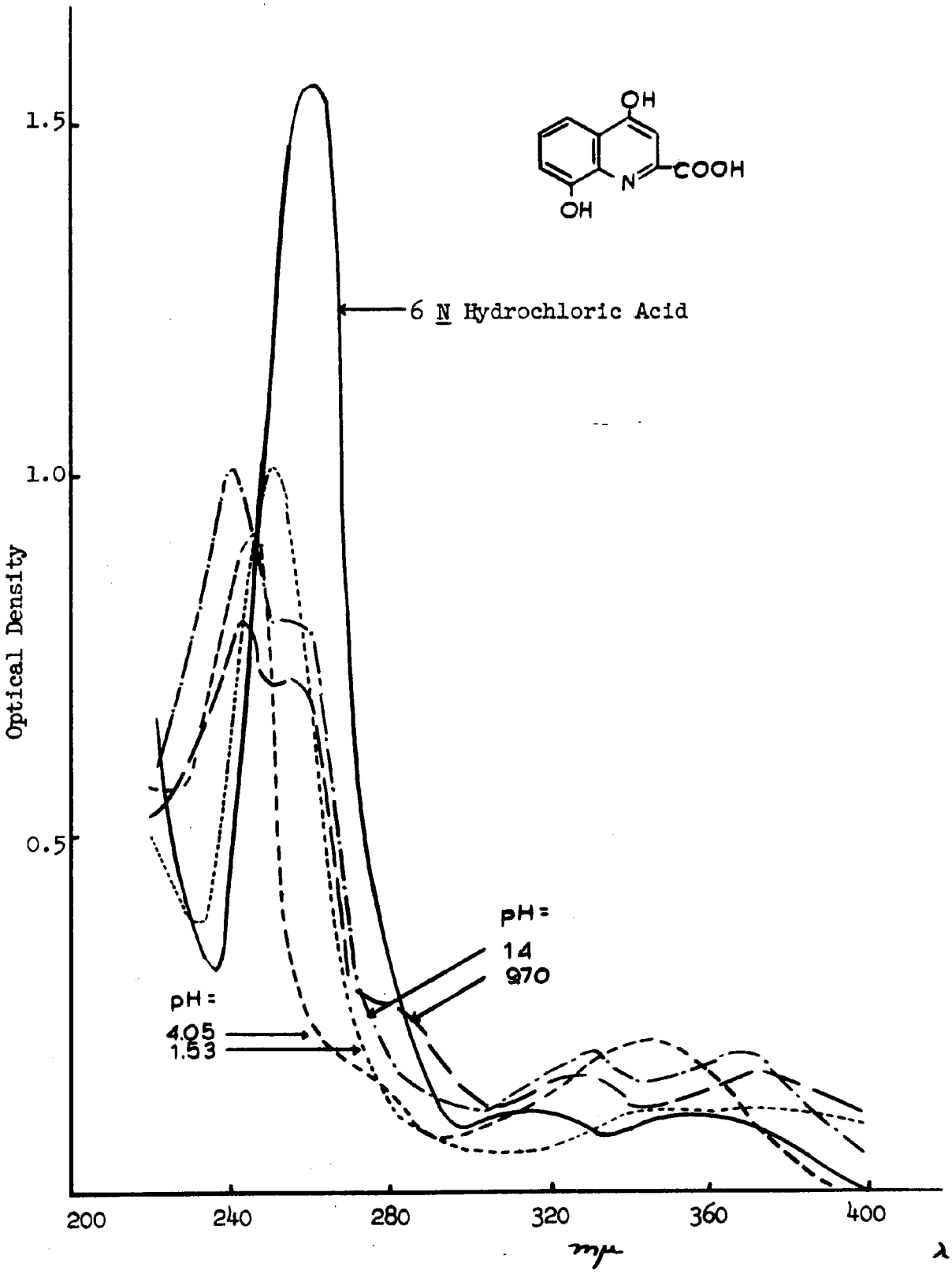
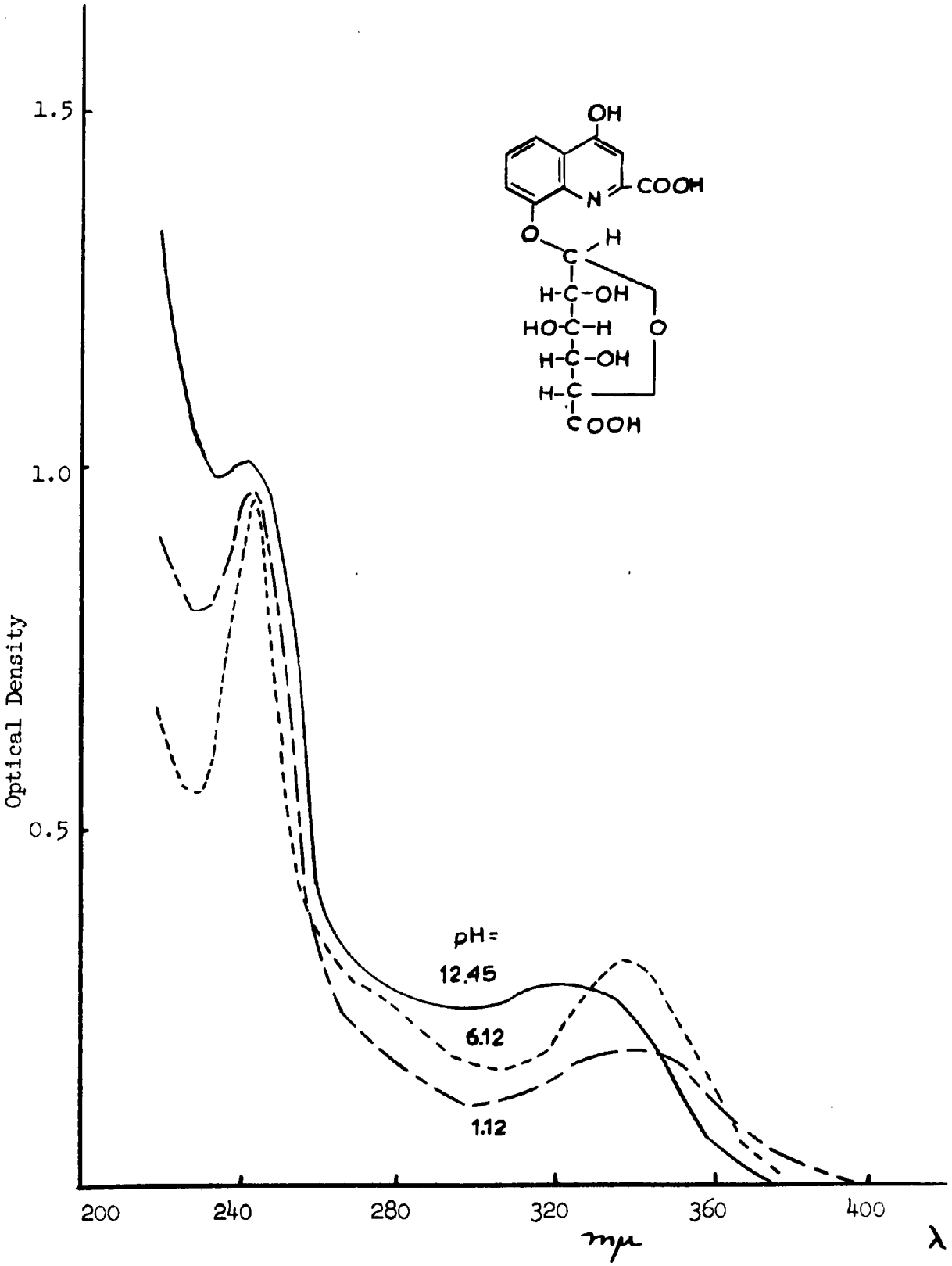


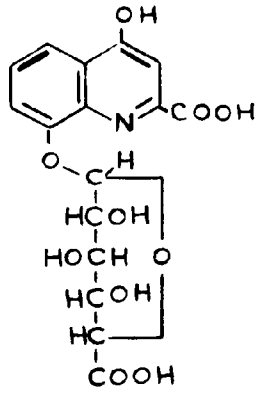
Figure 64. Ultraviolet absorption spectra of three ionic forms of XI



at a low pH value owing to dissociation of the 2-carboxyl group, and at a high pH value owing to dissociation of the 4-hydroxyl group. Xanthurenic acid, on the other hand, shows in addition a dissociation around a value of 6.5, corresponding to the 8-hydroxyl group. The plot of XI resembles that of kynurenic acid, except that the pK'_2 value is somewhat lower. The dissociation of a phenolic group, such as is present in xanthurenic acid, is not found for XI. The pK' values obtained by inspection of Figures 59-61, are tabulated in Table 22.

6. Conclusion as to the structure of XI

The structure for XI which would account for all the observed properties, and in particular for the fact that only xanthurenic and glucuronic acids have been found as components of this compound (in equimolar amounts) is β-(8-xanthurenyl) glucosiduronic acid. This compound does not reduce ferricyanide until it has been hydrolyzed with β-glucuronidase (although both free xanthurenic and glucuronic acids are reducing agents); hence, these acids must be joined by means of their reducing groups.



D. Summary

1. Chromatography paper was found to contain an impurity which could not be removed and which interfered with analyses for glucuronic

Table 22

pK' Values of ultraviolet light-absorbing, dissociable groups in kynurenic acid, xanthurenic acid and XI

Group assumed to be associated with this pK' value	Estimated pK' value of		
	Kynurenic Acid	Xanthurenic Acid	XI
2-Aromatic carboxyl group	$pK'_1 = 2.6$	$pK'_1 = 2.5$	$pK'_1 = 2.0$
8-Phenolic group	-	$pK'_2 = 6.5$	-
4-Phenolic group	$pK'_2 = 12.5$	$pK'_3 = 12.5$	$pK'_2 = 10.6$

acid.

2. A chromatographic method of isolating XI free of interfering materials was described.

3. Isolated XI was shown to behave on paper chromatograms identically with unisolated XI.

4. The behavior of XI toward Pauly's, Ehrlich's, ammoniacal silver nitrate and ninhydrin sprays was found to be negative.

5. The ultraviolet absorption spectrum of hydrolyzed XI was obtained. The spectrum was shown to have the same maxima as that of xanthurenic acid.

6. The fluorescence spectra of XI were obtained. The fluorescence spectra of hydrolyzed XI were shown to have the same maxima as those of xanthurenic acid.

7. The presence of glucuronic acid in XI was demonstrated by its reaction with naphthoresorcinol and carbazole.

8. XI was shown to be hydrolyzed by β-glucuronidase.

9. The ultraviolet absorption spectra of kynurenic acid, xanthurenic acid and XI were determined for a wide range of pH values.

10. The pK' values of dissociable groups in kynurenic acid, xanthurenic acid and XI were estimated from the spectral curves obtained in solutions having different pH values.

11. Two analytical methods for xanthurenic acid conjugates and three for glucuronic acid conjugates were described and the probable errors to be expected for these methods were determined. An analytical method for amino acid conjugates was described.

12. XI was analyzed by these methods and found to contain xanthurenic acid, glucuronic acid and amino acid in the ratios of 1:1:0.

13. On the basis of the analytical results, XI was assigned a structure, β-(8-xanthurenyl) glucosiduronic acid.

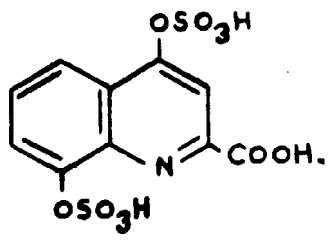
DISCUSSION

Fluorescent products formed *in vitro* from kynurenine and xanthurenic acid, and those formed *in vivo* from tryptophan and xanthurenic acid, were shown to be identical on the basis of the similarities of their R_f values in several solvent systems, their ultraviolet absorption spectra, their fluorescence spectra, their behavior on hydrolysis and also on the basis of the uniformly negative results obtained with radioactive sulfate, and with Pauly's, Ehrlich's, ammoniacal silver nitrate and ninhydrin sprays.

The properties of the compound are similar to those of one of the unidentified, fluorescent derivatives discovered by Dalgliesh *et al* (54) in the urine of tryptophan-loaded rats, and by Kotake *et al* (53) and Makino *et al* (35) in the urine of humans. Its properties also coincide well with those described by Kotake and Nogami (22) for the compound (designated F_1) they isolated from the urine of xanthurenic acid-loaded rats using paper chromatography. The properties of F_1 as described by these authors were the following:

1. It had an R_f value of 0.28 using MBBW and of 0.08 using BAW on Toyo #52 paper.
2. It had a dark purplish-blue fluorescence.
3. It was negative to Pauly's, silver nitrate and ninhydrin sprays.
4. It had an ultraviolet absorption spectrum with maxima at 246 mμ and 336 mμ in M/15 phosphate buffer, pH 7.4.
5. It yielded xanthurenic acid and inorganic sulfate following acid hydrolysis.

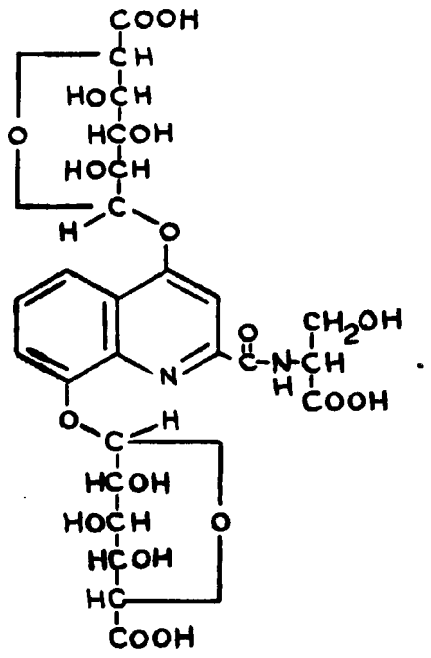
The structure assigned to F_1 by Kotake and Kato (23) was the following:



The properties of the compound studied in this thesis also resemble closely those of a compound (designated B) isolated by Rothstein and Greenberg (24) from the urine of xanthurenic acid-loaded rats using paper chromatography. The properties of B as described by these authors were the following:

1. It had an R_f value of 0.24 using MBBW (descending) and of 0.21 using BAW (descending).
2. It had a bright blue fluorescence.
3. It was negative to Pauly's, Millon's, ninhydrin and ammoniacal silver nitrate sprays.
4. It had an ultraviolet absorption spectrum with maxima at 245 mμ and 340 mμ in water solution.
5. It yielded xanthurenic acid and serine following strong acid hydrolysis.
6. It gave a strongly positive reaction with naphthoresorcinol.
7. It was hydrolyzed by β-glucuronidase.

The structure assigned to B by Rothstein and Greenberg was the following:



In comparison, XI was seen to have the following properties:

1. It had an R_f value of 0.29 using MBBW and of 0.17 using BAW.
2. It had a bright, blue-white fluorescence.
3. It was negative to Pauly's, ammoniacal silver nitrate and ninhydrin sprays.
4. It had an ultraviolet absorption spectrum with maxima at 245.5 mμ and 339 mμ in water solution.
5. It yielded xanthurenic acid and glucuronic acid following acid hydrolysis.
6. The purified material gave the naphthoresorcinol test for glucuronic acid.
7. It was hydrolyzed by β-glucuronidase to xanthurenic acid.

These results suggest that F_1, B and XI may be the same compound. If this is the case, then the earlier structure assignments would be incorrect. However, those features of the preceding structures which differ from the assignment we propose are understandable in the light

of the difficulties we encountered with the serine and inorganic sulfate contaminants and with the naphthoresorcinol-reacting impurities obtained from the paper.

These impurities and the additional difficulties which can be encountered in the presence of uric acid and by the formation of fluorescent spots caused by chelation or absorption, may also explain the spectral properties of the fluorescent product obtained by Kotake et al (21) by incubation of xanthurenic acid with rat and rabbit liver and kidney extracts which they interpreted to indicate a breakdown of the pyridine ring of xanthurenic acid by "xanthurenicase". We have not found the rat kidney slice to form such breakdown products, although some of the spectra obtained on impure preparations of XI have resembled theirs closely.

The intense fluorescence of XI, by giving an erroneous impression of abundance, was the main cause of the difficulties encountered in the isolation and assignment of structure to this compound. Whereas approximately only three percent conversion (calculated on the basis of 15 ml. of solution containing 52γ of XI per milliliter obtained from 30 mg. of xanthurenic acid) was obtained in the production of XI from xanthurenic acid in the kidney slice experiments, it will be seen in Appendix II that XI fluoresces in solution with about five-hundred times greater intensity than an equal weight of xanthurenic acid. In the absence of other means of judging the concentration, the low level of the product and the lack of progress in purification were not evident, so that impurities were retained or were added during chromatographic isolation procedures.

Uric acid was troublesome because it migrated with XI in MBBW and

has a strong ultraviolet absorption which completely masked the spectrum of XI. Serine and inorganic sulfate were troublesome because they have R_f values similar to that of XI in both MBBW and BAW and could easily be mistaken to be chemically associated with the fluorescent material. The naphthoresorcinol-reacting impurities present in the paper were troublesome because they masked the reaction of the glucuronide and, since they arose from the paper, they could not be removed by repeated paper chromatography. Although uric acid was removed by the second chromatographic step using BAW, serine and inorganic sulfate were not. The trace amounts of XI actually present made these impurities of such relatively great importance as to prevent the determination of its structure in their presence.

As soon as the chromatography paper was recognized as the source of contaminants causing the anomalous naphthoresorcinol reaction, the need for another method of isolation was apparent. The procedure developed satisfied the following conditions: (1) it permitted the fluorescence to be observed and followed, (2) it did not cause the degradation of the compound, (3) it provided for the elimination of a variety of contaminants, (4) it did not appear to involve large losses of material, (5) it did not introduce impurities which would interfere with the characterization of the unknown. The procedure involved three different types of chromatographic media, which were capable of being made quite free of contaminants, in addition to the use of paper chromatography. By means of this procedure, it was found possible to run an incubation mixture (plus added xanthurenic acid but containing no conjugate) through the separation steps and end with practically no interfering residue as judged by the low absorption in the ultraviolet

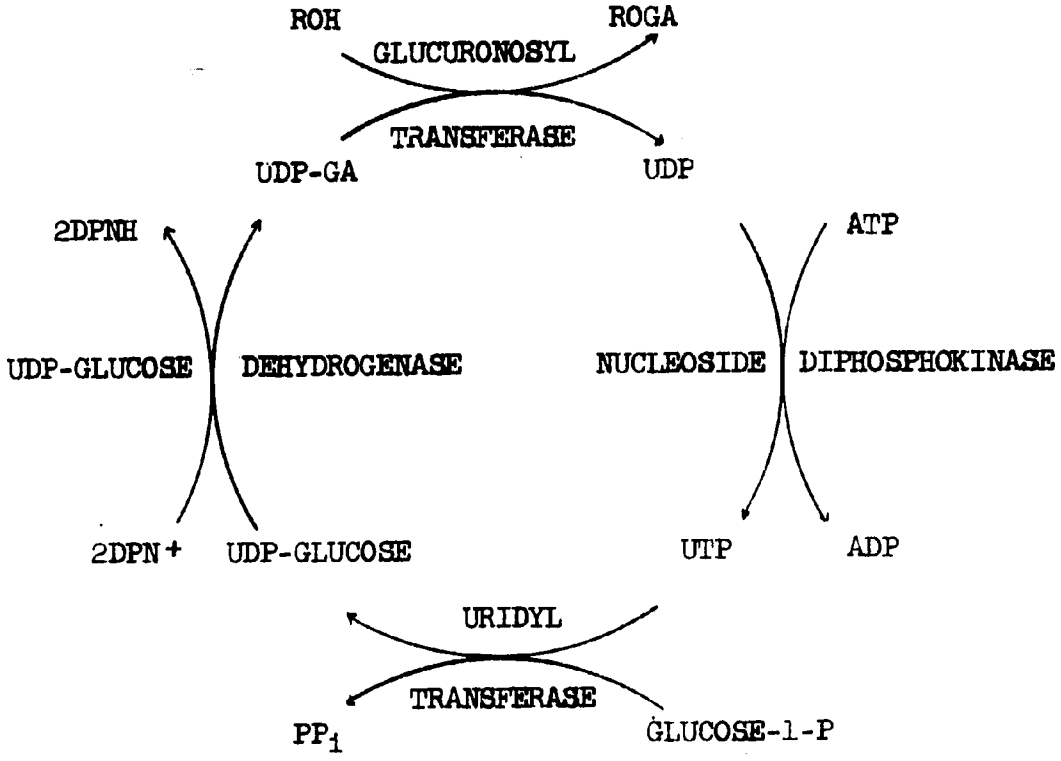
and by the absence of any color reactions in the various quantitative tests. The mildness of the procedure was proved by the fact that no separation took place when the isolated material was chromatographed in several solvents from incubation mixtures also containing the unisolated material.

Once the compound was obtained free from interfering materials, a comparison of the molar ratios of the products obtained by hydrolysis was possible through the use of sensitive analytical methods. The compound was found to be β-(8-xanthurenyl) glucosiduronic acid.

The fact that the 4-hydroxyl group was not also involved in a synthetic reaction may be a result of the very different properties of the 8-hydroxyl and the 4-hydroxyl groups. The 4-hydroxyl group is mesomeric with the ketone form owing to the influence of the para pyridine-nitrogen atom and for this reason does not give the usual phenolic color reactions, is not readily oxidized and has a high pK' value of 12.5 Furthermore, data presented in Appendix II will show that the fluorescing ability of quinoline is modified quite differently by the introduction of a 4-hydroxyl or an 8-hydroxyl group. Various 4-hydroxyquinoline analogues which do not have the 8-hydroxyl group, including kynurenic acid, were apparently not conjugated as judged by their failure to form fluorescent derivatives. On the other hand, several 8-hydroxyquinoline derivatives appeared to form fluorescent derivatives. This probably occurs by the same reactions involved in the formation of the glucuronide of xanthurenic acid, since glucuronide conjugation of phenolic compounds appears to be of fairly low substrate specificity.

The scheme presented in Figure 65 has been shown to operate for

Figure 65. The conjugation of phenols

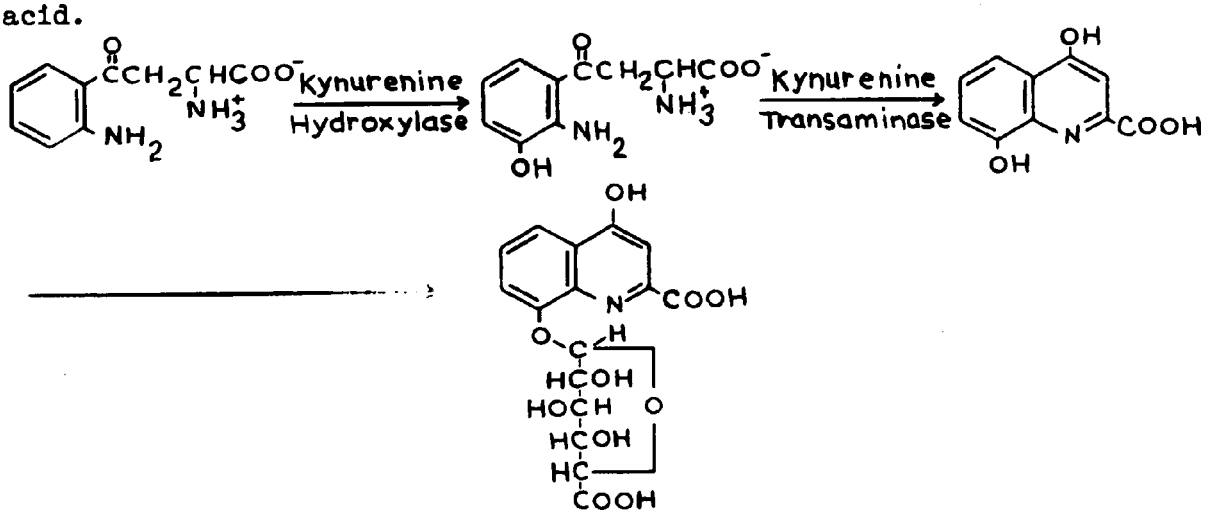


Note:

The abbreviations used are: a general phenolic compound (ROH), a general phenol-glucuronic acid conjugate (ROGA), uridine 5'(pyro)-diphosphate (UDP), uridine 5'(pyro)-triphosphate (UTP), adenosine 5'(pyro)-diphosphate (ADP), adenosine 5'(pyro)-triphosphate (ATP), glucose 1-phosphate (GLUCOSE-1-P), inorganic pyrophosphate (PP_i), uridine diphosphate glucose (UDP-GLUCOSE), uridine diphosphate glucuronic acid (UDP-GA), diphosphopyridine nucleotide (DPN^+), reduced diphosphopyridine nucleotide (DPNH).

other conjugations involving glucuronic acid (77). Further work on the enzymology of this particular reaction should reveal whether such a series of reactions is operating in this case, and if so, the preparation of this conjugate in cell-free systems should be possible.

The following sequence of reactions probably occurs in the kidney, resulting in the final formation of β-(8-xanthurenyl) glucosiduronic acid from either L-kynurenine, L-3-hydroxykynurenine, or xanthurenic acid.



Each step in the sequence has now been shown to occur either with kidney slices or kidney mitochondria. Whether the in vivo formation of the conjugate occurs in the kidney by this pathway remains to be determined.

The question naturally arises as to whether any deliterious or beneficial effects might occur in the organism from the production of this conjugate. The plausible but still inconclusively proven proposal of Kotake et al (46-52) is that xanthurenic acid acting long enough can cause diabetes by producing atrophy of the beta cells of the pancreas by its action as a zinc chelator. If this is the case, then the conjugation of the 8-hydroxyl group with a glucuronyl moiety (which is probably more complete when the trace amounts of xanthurenic acid

normally present are involved) would prevent this diabetogenic action.

The work of Szent-Györgyi (78, 79) (see Appendix II) has emphasized that great biological importance may be associated with the fluorescent properties of molecules, and that, in a living system, the concentration of such excitable molecules may greatly affect the chemical environment. In this fashion, a small amount of an intensely fluorescent compound may produce effects altogether out of proportion to its weight. R. Mason (80) has suggested a mechanism whereby carcinogenesis by fluorescent molecules might occur. Boyland, et al (81) have shown that the 8-methoxyxanthurenic acid is a natural carcinogen. Because of the similarities of structure and of fluorescent properties of 8-methoxyxanthurenic acid and 8-xanthurenyl glucosiduronic acid, the latter compound should also be tested for carcinogenicity. If it is carcinogenic, then this compound should be considered along with the other tryptophan metabolites, which include in addition to 8-methoxyxanthurenic acid, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, as a factor in the etiology of bladder cancer in humans.

CONCLUSION

Kynurenine, 3-hydroxykynurenine and xanthurenic acid upon incubation with rat kidney slices all formed a blue-white, fluorescent compound (XI), which was identified as β-(8-xanthurenyl) glucosiduronic acid. The conversion of L-kynurenine to L-3-hydroxykynurenine, of L-3-hydroxykynurenine to xanthurenic acid, and of xanthurenic acid to XI was demonstrated, so the following sequence of reactions was postulated to explain the formation of XI from kynurenine in kidney slices:

L-kynurenine ⟶ L-3-hydroxykynurenine ⟶ xanthurenic acid ⟶ β-(8-xanthurenyl) glucosiduronic acid.

These reactions can apparently operate *in vivo* since XI was also isolated from the urines of tryptophan and xanthurenic acid-loaded rats. In addition to xanthurenic acid, several other 8-hydroxquinoline derivatives were tentatively shown to be converted into fluorescent products by kidney slices. Other quinoline analogues, some containing the 4-hydroxyl group (but not the 8-hydroxyl group) did not form fluorescing derivatives under the same conditions. β-(8-Xanthurenyl) glucosiduronic acid was found to be about one-thousand times as intense in its fluorescence as an equivalent amount of xanthurenic acid.

A chromatographic method was developed for the isolation of XI free of interfering contaminants and the purified preparations were analyzed by specially modified, colorimetric procedures in order to determine the structure. In attempting to isolate this compound using paper chromatography, difficulties were encountered with the formation of fluorescent artifacts in the presence of the salts, with contamination with uric acid, serine and inorganic sulfate, and with contaminants present in the paper. These contaminants necessitated the use of Dowex-

50, charcoal and silica gel in conjunction with paper in order to obtain the product in a state suitable for analysis. The possibility of the operation of these factors in causing misinterpretations of data in other studies of tryptophan metabolism was discussed.

In the process of identifying the compound as β-(8-xanthurenyl) glucosiduronic acid, its ability to react with Pauly's, Ehrlich's, ammoniacal silver nitrate and ninhydrin reagents was tested and its R_f values in several solvent systems were determined. Its behavior on acid hydrolysis and with β-glucuronidase was studied. The ultraviolet absorption spectrum and its variation as a function of pH value, and the fluorescence spectra of the compound were obtained; the pK' values of its ultraviolet-absorbing, dissociable groups were determined.

In connection with this work, substrates were prepared as described in Appendix I. The phosphorescent and fluorescent properties of a number of related compounds were studied as described in Appendix II. Conjugates of the phenolic compounds studied were found to have greater intensities of fluorescence and longer phosphorescent afterglows than the unconjugated phenols from which they were derived. The fluorescence emission maxima of conjugated phenols were shifted to longer wave lengths in comparison to the maxima of unconjugated phenols. The N-acetylation of o-, m- and p-aminobenzoic acids produced compounds with diminished fluorescence. Several implications of these studies were discussed.

APPENDIX I. PREPARATION OF SUBSTRATES USED IN THE INVESTIGATION OF TRYPTOPHAN METABOLISM IN THE KIDNEY

A. Experimental

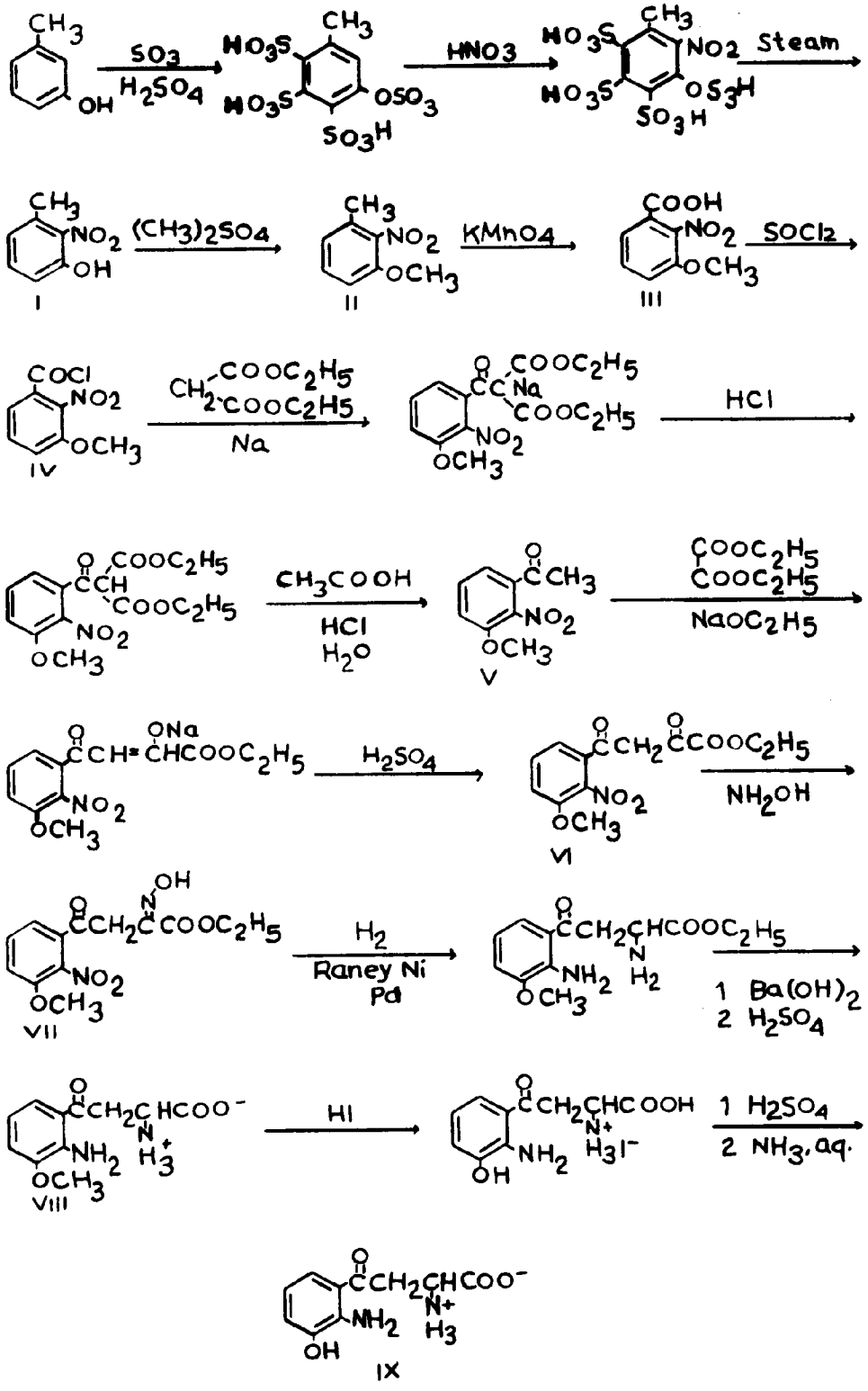
When this investigation was initiated, the required substrates were not readily available and a synthesis of compounds was necessary. The compounds which were synthesized for this work included L-kynurenine, L-3-hydroxykynurenine, D-3-hydroxykynurenine, xanthurenic acid and 4, 8-dihydroxyquinoline. Chemical intermediates involved in the syntheses of the above compounds, e.g. 8-methoxyxanthurenic acid, were also used. In this section of the thesis, the chemical methods used in these preparations and the chemical properties of the products will be described.

1. Synthesis of DL-3-hydroxykynurenine

Of the syntheses reported, that of L-3-hydroxykynurenine is by far the most intricate. This procedure was used following unsuccessful attempts to use a method published by M. Kotake *et al*. The procedure is unlike some of the other published methods (82, 83, 84) in that (1) it gives fuller experimental details than the note by Butenandt and Hallmann upon which the synthesis is partly based; (2) it uses Warnell's method of nitrating the benzene ring (85); (3) it involves other intermediates than those used by Butenandt and Hallmann, e.g. methoxy derivatives instead of benzyloxy ones; (4) certain purification steps are followed by paper chromatography; (5) the by-products of the synthesis are identified in certain cases; (6) the optical isomers are resolved using paper chromatographic technique. The steps involved are shown in Figure 66.

(a) **Preparation of 2-nitro-3-hydroxytoluene, (I):** The procedure

Figure 66. The synthesis of 3-hydroxykynurenine



written here generally follows and derives from that given by Warnell (85, 86). 930 Ml. of 7½% oleum (SO_3/ H_2SO_4) were prepared by mixing 698 ml. of concentrated sulfuric acid with 232 ml. of 30% fuming sulfuric acid. The solution was cooled to 0°. 216 Ml. of m-cresol (Eastman Kodak Co., practical) were slowly added. The temperature rose to 70° and the solution turned a clear-brown color. After standing 1 hour, the mixture was cooled to 10° and 100 ml. of concentrated nitric acid in 300 ml. of 7½% oleum were added while maintaining a temperature of 10-20°. The addition took about 1 hour. The solution was allowed to stand for 24 hours.

An equal volume of water was added cautiously to the solution. The temperature rose to about 110°. The solution was steam-distilled using dry steam and a heated flask. To avoid violent foaming the steam distillation should be run as slowly as possible at first. There was some foaming until the temperature reached 110°. The distillation flask was heated gently with a mantle up to 130°. Some foaming occurred. At a temperature of 130°, the steam was kept on while external heating of the flask was discontinued until the foaming had subsided, then the heating was continued slowly. The viscous, yellow oil was distilled at a temperature of 130° to 170° during a period of two hours. 251 G. of product were obtained, representing a yield of 81.5%.

(b) Preparation of 2-nitro-3-methoxytoluene, (II), (see 85): All of I was dissolved in 500 ml. of cold 2 N NaOH and 1.34 moles of dimethyl sulfate were added in the hood with stirring over a period of about 1 hour. The solution was refluxed for 5 hours, maintaining strong alkalinity by additions of NaOH as required. The solution was then cooled in an ice-bath and crystals of II were filtered

off. One mole of NaOH and one mole of dimethyl sulfate were added to the filtrate, and the solution was again refluxed for 5 hours. The solution was then cooled and extracted with ether. About 25 ml. of unreacted material were recovered from the extracted solution upon acidification. The ethereal solution was evaporated under vacuum. The oil remaining was redistilled using a short fractionating column. Distillate was collected in the temperature range of 100 - 160° at 45 mm of Hg pressure. The yield of thin, yellow oil was 74%. This material was not further purified. The pure material has a reported melting point of 49°.

(c) Preparation of 2-nitro-3-methoxybenzoic acid, (III), (see 85): Sixty grams of II were added to 4 liters of water containing 1.5 moles of NaOH. The solution was heated to reflux and 64 g. of $KMnO_4$ were added. The refluxing was continued until the solution decolorized; approximately 2 hours were required. An additional 64 g. of $KMnO_4$ were added. Refluxing was continued with caution, since the bulky MnO_2 precipitate caused some bumping. After refluxing 6 hours, the decolorized solution was cooled and the MnO_2 was filtered off. The precipitate was washed with dilute NaOH. The filtrate and washings were acidified with 50% sulfuric acid and III precipitated out in large, flaky, white crystals upon standing overnight. The melting point was 256°, reported 256°. Four batches of II were oxidized with an average yield of 43.5%; 103.4 g. of III were obtained.

(d) Preparation of 2-nitro-3-methoxybenzoyl chloride, (IV), (see 85): 45.3 G. of twice recrystallized, Norite-clarified, and finely pulverized III were gently refluxed with 55 g. of freshly

distilled thionyl chloride until all of the III had dissolved. This took about 2 hours. The excess thionyl chloride was removed by vacuum distillation leaving a red oil which solidified (IV). The temperature was kept below 50° because of the explosive nature of nitroacylhalides. IV was stored under vacuum until used in the next step.

(e) Preparation of 2-nitro-3-methoxyacetophenone, (V), (see 85):

6.4 G. of freshly cut sodium were dissolved in 114 ml. of anhydrous alcohol. [The anhydrous alcohol was prepared by distillation from magnesium methoxide (87).] 45.8 Ml. of diethyl malonate (Eastman Kodak Co.) were added. The solution was cooled to 0° and 45.3 g. of IV were added. Anhydrous conditions were maintained. The suspension was shaken in an ice-bath for 1 hour, then at room temperature for 4 hours. The precipitated material was filtered off and the ethanol solution was concentrated under vacuum to a thick oil which solidified. This sodium diethyl 2-nitro-3-methoxybenzoyl malonate was suspended in 69 ml. of water and acidified with 5 N HCl. The solution was then extracted with three portions of benzene; a total of 229 ml. of benzene was used. The benzene solvent was distilled off under vacuum with gentle heating, leaving 2-nitro-3-methoxybenzoyl diethyl malonate in the form of a red oil.

This compound was hydrolyzed by dissolving it in a mixture of 366 ml. of glacial acetic acid, 229 ml. of water and 64 ml. of HCl, and refluxing for 4 hours. Another 64 ml. of HCl were added and the solution refluxed another 4 hours. The solution was cooled and crystals formed upon standing overnight. These were filtered off and the filtrate evaporated as long as possible with an aspirator and then evaporated to dryness at 0.03 mm Hg pressure with gentle heating. The precipitate

and residue were combined and dissolved in 500 ml. of 4 N NaOH and allowed to stand in an ice-bath for one hour. The undissolved 3-methoxy-2-nitroacetophenone was filtered off. The air-dried material was dissolved in about 400 ml. of 95% alcohol on a steam-bath and clarified with Norite. The cooled solution and the mother liquor yielded a total of 19.4 g. of V, m.p. 130°, reported 128°. The yield from III was 39%.

(f) Preparation of 2-nitro-3-methoxybenzoyl pyruvic acid ethyl ester, (VI), (see 85): 2.28 G. of freshly cut sodium were dissolved in 136 ml. of anhydrous alcohol and added to 48.5 ml. of diethyl oxalate. The solution was cooled to 0° and 19.4 g. of V were added. Anhydrous conditions were maintained throughout. The solution was stirred for 4 hours at room temperature. It turned dark green and solidified into a thick, white paste. The mixture was filtered and the 2-nitro-3-methoxybenzoyl pyruvic acid sodium enolate ethyl ester was suspended in 194 ml. of water and acidified with 50% sulfuric acid. VI was extracted with 1,160 ml. of benzene. The benzene solution was dried with sodium sulfate and filtered. The benzene solvent was removed with vacuum distillation using gentle heating. The residue was taken up in 400 ml. of boiling ethanol and clarified with Norite. The solution was filtered and refrigerated overnight. VI crystallized out and was filtered off and the mother liquor was treated again in the same way. A total of 22.9 g. of VI was obtained. It had a melting point of 113°, reported 113-113.5°. The yield was 78%.

(g) Preparation of 2-nitro-3-methoxybenzoyl pyruvic acid α-oxime ethyl ester, (VIII): In a mortar, 5.4 g. of hydroxylamine hydrochloride were triturated with 6 g. of anhydrous sodium acetate and 1.5

ml. of water. The mixture was dissolved in one liter of anhydrous alcohol. A precipitate of sodium chloride was filtered off. The filtrate was dropped directly into a solution of VI in one liter of anhydrous alcohol. Anhydrous conditions were maintained as far as possible. The solution was stored for 10 hours at room temperature. It was then concentrated under an atmosphere of nitrogen using an aspirator and at a temperature of 30 - 45° until the volume reached about 50 ml. and no more alcohol distilled off. An equal volume of ether was added to the remaining oil and the precipitated inorganic salt filtered off. The solution was again reduced to a volume of about 50 ml. and an equal volume of water was added. A white precipitate formed which was filtered off. No further recovery was obtained from the mother liquor. The precipitate was dried and dissolved in about 100 ml. of hot benzene. After clarification of the solution with Norite, the addition of 200 ml. of petroleum ether brought an oil out of solution which solidified into a white, hard cake, VII. After it was pulverized and dried, VII melted at 113°. The preparation weighed 7.2 g. representing a yield of 83.5%.

(h) Preparation of 3-methoxykynurenine, (VIII): The Parr hydrogenation apparatus was calibrated (1) for temperature of the solution in the reaction vessel versus Variac voltage setting of the heating element and (2) for pressure gauge reading versus moles of hydrogen in the reaction vessel. This latter calibration was accomplished by reducing known amounts of maleic acid to succinic acid and recording the drop in gauge reading following the uptake of hydrogen in the reduction process.

Raney nickel was prepared (88). A suspension equivalent to 1.80 g. and 0.72 g. of palladium-black were used. These and 7.2 g. of VII were added to 180 ml. of anhydrous alcohol in the reaction vessel. The mixture was placed under about 15 pounds of pressure with hydrogen gas, and was shaken until four equivalents of hydrogen had reacted. The temperature was then raised to 60° and shaking continued until three more equivalents had reacted. The total shaking time was about 6 hours.

The reaction mixture was filtered through an asbestos mat in a Gooch crucible and concentrated under nitrogen at about 30° by means of an aspirator to a volume of 36 ml. This solution was diluted with 720 ml. of water and 5.0 g. of $Ba(OH)_2.8H_2O$ were added. The mixture was shaken at room temperature for 12 hours. About 10 ml. of 50% sulfuric acid were added and the precipitated barium sulfate was filtered off. The precipitate was washed about five times with both hot alcohol and hot water. The filtrate and washings made a total of about 2 liters. It was concentrated with an aspirator under an atmosphere of nitrogen at a temperature of approximately 30° to a volume of about 500 ml. At this volume, some material precipitated out. It was saved and the solution was treated further in order to obtain the fraction of the mixture which contained VIII. The pH value of the solution was adjusted to 5.5 with 50% ammonia water (ammonia water being a 28-30% solution of NH_3 in water). A gelatinous precipitate formed and was filtered off. The filtrate was concentrated by vacuum distillation to a volume of 400 ml. A yellow precipitate formed and, after refrigerating the solution overnight, the precipitate was collected.

The highly fluorescent, yellow solution was concentrated with a rotating evaporator to a volume of about 150 ml. A yellow precipitate

formed and, after refrigerating the solution overnight, it was collected. The filtrate was again evaporated and more yellow precipitate formed as well as some tarry material. At a volume of 25 ml., needles of ammonium sulfate were obtained, and the final residue, after evaporation to dryness, consisted mostly of ammonium sulfate.

By chromatographing samples from the various fractions obtained during the concentration of the solution on Whatman #1 paper with MBBW, BAW and KCl, it was possible to identify certain of the by-products of the reaction, since these same compounds had been obtained as intermediates in the synthesis (which will be described later) of xanthurenic acid. In the first material which precipitated upon concentrating the solution was found methoxyxanthurenic acid and methoxyxanthurenic acid ethyl ester. Two further unidentified compounds were found to be present in the tarry residue. Nearly all the 3-methoxykynurenine was found in the precipitate which had formed when the solution had reached a volume of 150 ml. A total of 0.656 g. of material was obtained. The yield obtained was 12%. VIII had a melting point of 217°.

(i) Preparation of DL-3-hydroxykynurenine, (IX): 600 Mg. of VIII were dissolved in 12 ml. of 48% hydrobromic acid in a 50-ml., ground-glass, round-bottom flask fitted with a gas-inlet tube. The solution was refluxed for 10 hours while a stream of nitrogen gas was bubbled through the solution. This step was carried out under the hood. After cooling, the solution was adjusted to a pH value of 5.5 with dilute NH_3 water (a solution containing approximately 15% NH_3 dissolved in water). Alcohol was added to make a 60% alcoholic solution. It was treated with a small portion of Norite and filtered. Approximately

2.0 ml. of ether were added and 1.5 equivalents of sulfuric acid. The solution was seeded with a sample of 3-hydroxykynurenine which was a gift from Dr. L. M. Henderson. After standing at approximately -15° for about one month, all of the 3-hydroxykynurenine had precipitated and was shown to be identical with that obtained from Dr. L. M. Henderson. The remaining solution contained another compound which was not identified. A yield of 0.358 gm. of DL-3-hydroxykynurenine was obtained, representing 44%. The overall yield from m-cresol was 0.34%. The melting point was 235°. The reported melting point is 238° (83). It had ultraviolet absorption maxima at 230, 265 and 375 mμ in water, while reported values are (1) 223, 260, 369 mμ at a pH value of 2.0; (2) 228, 267, 368 mμ at a pH value of 7.4; and (3) 248, 286, 393 mμ at a pH value of 12.0 (64). The R_f values of the synthesized material agreed with those obtained with the DL-3-hydroxykynurenine obtained from Dr. L. M. Henderson in three solvents. These values are listed in Table 23. The synthetic material gave a positive reaction with both ninhydrin and Folin's reagents.

2. Resolution of DL-3-hydroxykynurenine

Twenty milligrams of IX were dissolved in a minimum amount of water and streaked along the margin of two sheets of Whatman 3MM paper. The sheets were developed descending with MBBW. The isomers separated into two bands on the paper and these bands were cut out and eluted. The eluates were lyophilized and then taken up in 1% hydrochloric acid solution for determination of optical rotation. Both solutions were optically active; at room temperature, L: $[\alpha]_D$ = +26° and D: $[\alpha]_D$ = +10°, but impurities, probably arising from the paper, were also found present in the solutions. The solutions were then dialyzed to remove tarry materials (the solutions darken on standing) and evaporated to dryness

Table 23

Comparison of R_f values of synthesized 3-hydroxykynurenine with R_f values of an authentic sample

Source of 3-hydroxykynurenine	R_f values obtained using the following solvents		
	MBBW	BAW	KCl
Synthesized	0.28[a] 0.34	0.28[a] 0.33	0.57
Gift from Dr. L. M. Henderson	0.26[a] 0.33	0.29[a] 0.33	0.56

[a]The optical isomers separate on paper.

in a vacuum desiccator. During the evaporation long needles of 3-hydroxykynurenine crystallized. The optical isomers obtained in this way were used in the incubation experiment, and they gave only one spot on paper chromatographic development.

3. Synthesis of L-kynurenine

Figure 67 shows the sequence of reactions involved in this synthesis. In order to obtain the L-isomer of kynurenine, the L-isomer of tryptophan was used as the starting material. The initial acetylation follows the procedure of du Vigneaud and Sealock which does not cause racemization (89). The ozonolysis and the hydrolysis steps followed the procedure of Warnell and Berg (90). Since nothing new is offered in these syntheses, the detailed procedures will not be described.

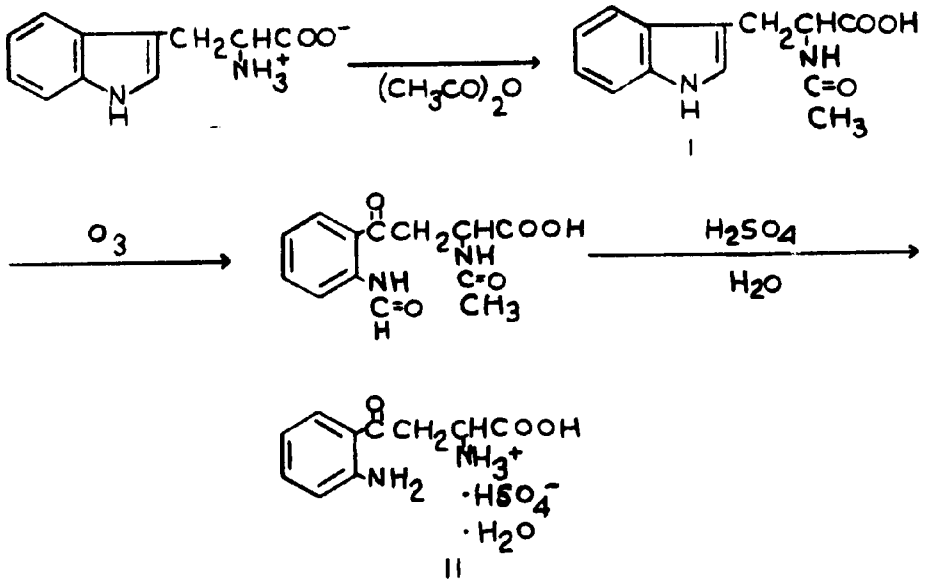
(a) Preparation of N-acetyl L-tryptophan, (I): Ten grams of L-tryptophan were used as the starting material. The acetylated derivative was obtained in 62.5% yield. It had a melting point of 183°, reported 189 - 90°, and an $[\alpha]_D$ of +28° at room temperature, reported $[\alpha]_D^{31}$ of +29°.

(b) Preparation of L-kynurenine, (II): 7.5 G. of I were used as the starting material. It was ozonized and the resulting formyl L-kynurenine was hydrolyzed with acid and II was isolated as the sulfate monohydrate. The yield was 31%. II had a melting point of 176° (decomp.), reported 178° (90).

4. Syntheses of xanthurenic acid, methoxyxanthurenic acid ethyl ester and methoxyxanthurenic acid

The synthesis of xanthurenic acid was carried out by Mr. W. Dempsey using the method described by Furst and Olsen (91). The

Figure 67. The synthesis of L-kynurenine



reaction sequence involved is shown in Figure 68. The reactions are described to make clear the origin of several products used in this thesis (especially xanthurenic acid).

(a) Preparation of ethyl 8-methoxy-4-hydroxyquinaldate, (methoxyxanthurenic acid ethyl ester), (I): Ethyl oxalacetate is condensed with o-anisidine to form diethyl α-(o-methoxyanil) succinate. This product is isolated and then added to refluxing Dowtherm A whereupon it cyclizes to form I.

(b) Synthesis of xanthurenic acid, (II): I is refluxed with a mixture of KI and phosphoric acid. Upon cooling, II crystallizes out of solution. This compound was checked by paper chromatography and was free of methoxyxanthurenic acid. (The first batch prepared contained a trace amount of the uncleaved ether.) The product was positive to Pauly's reagent; it melted at 292°, reported 286°.

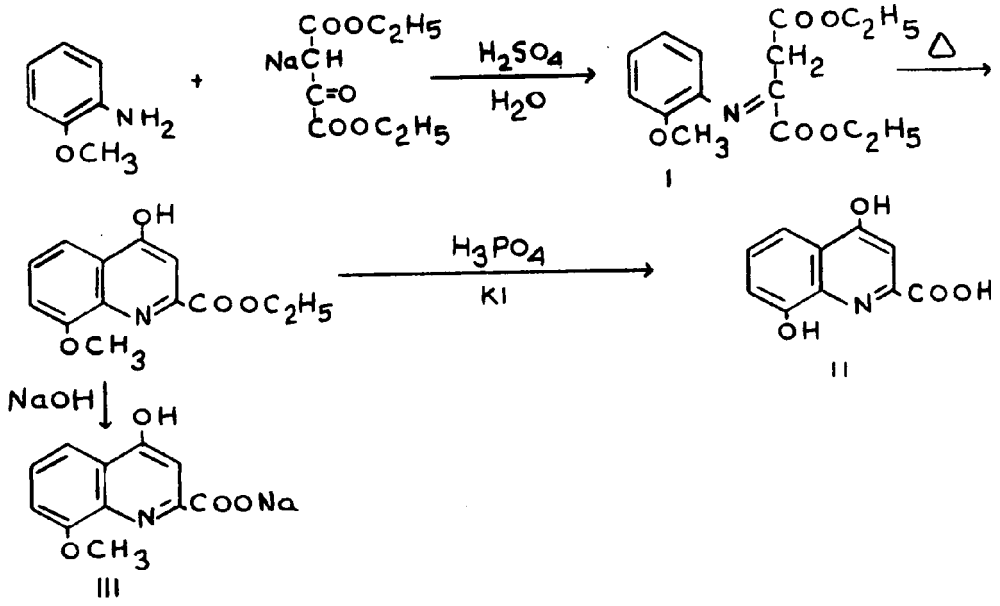
(c) Preparation of 8-methoxyxanthurenic acid, (III): A portion of I was hydrolyzed with alkali to form III.

5. Synthesis of 4, 8-dihydroxyquinoline

The thermal decarboxylation of xanthurenic acid was described by Musajo (92). The procedure used here employs a copper catalyst such as Albert and Magarth used to decarboxylate kynurenic acid (93).

100 Mg. of xanthurenic acid were mixed with 0.1 mg. of extra-fine copper bronze and added to 10 ml. of mechanically stirred paraffin at a temperature of 260-5°. Effervescence occurred as the CO_2 was evolved. The heating and stirring were maintained for about 5 minutes. The solution was cooled and petroleum ether was added before the paraffin solidified. The insoluble xanthurenic acid, 4, 8-dihydroxyquinoline and tar were filtered off. Xanthurenic acid and 4, 8-dihydroxyquinoline were

Figure 68. The synthesis of xanthurenic acid



obtained by washing the precipitate repeatedly with hot water. The solution was clarified with Norite and the 4, 8-dihydroxyquinoline obtained by fractional crystallization. Traces of xanthurenic acid remaining were removed by Dowex-50. This material was positive to Pauly's reagent and melted with decomposition at 264°, reported, above 300°. It gave only one fluorescent spot upon paper chromatography. The R_f values obtained are compared with those reported by Makino and Arai in Table 24 (31). They agree quite well except in the case of butanol-ammonia. This discrepancy may be due to some difference in our solvent composition or method of paper chromatography.

6. Syntheses of m- and p-acetamidobenzoic acids:

These compounds were prepared from the corresponding aminobenzoic acids using acetic anhydride according to the procedure given by Vogel (88). The m-acetamidobenzoic acid melted at 244°, uncorrected, reported 249 - 50° (94). The p-acetamidobenzoic acid melted at 251°, uncorrected, reported 250 - 2° (94).

Table 24

Comparison of R_f values of synthesized 4, 8-dihydroxyquinoline with values reported in the literature

Solvent System	R_f value of 4, 8-dihydroxyquinoline	
	Synthesized	Reported (31)
BAW	0.78	0.79
Butanol saturated with 1% ammonia water	0.23	0.04
Butanol saturated with 0.2% acetic acid	0.70	0.68
MBBW	0.74	0.78
Ethyl acetate: acetic acid: water:: 5: 1: 5	0.53	0.60
2% Acetic acid: isopropanol:: 3: 7	0.73	0.77

APPENDIX II. STUDIES ON THE LUMINESCENT PROPERTIES OF SOME TRYPTOPHAN METABOLITES AND THEIR CHEMICAL ANALOGUES

A. Introduction

Observations made during the isolation and synthesis of certain tryptophan metabolites and during studies using these metabolites in incubations with kidney slices indicated that some of them exhibit a much stronger fluorescence than others differing but slightly in structure. Also, during these studies, spots of certain compounds on paper chromatograms were found to phosphoresce for several seconds if the sheets were laid upon a very cold surface and illuminated with ultraviolet light. Other compounds with slightly different structures would not.

Differences in the luminescent properties of molecules represent differences in their excited electronic states, and it is possible that information concerning such differences might be of some biological application.

In order to obtain more precise information concerning these differences in luminescent properties, a group of compounds was studied in a more quantitative manner. This group consists of tryptophan intermediates (especially those in the Quinoline Pathway) or close structural analogues of these intermediates.

Fluorescent and phosphorescent compounds reveal by the fact that they emit light that they are capable of existing in a fairly stable, excited state, holding energy which in the case of luminescence is eventually lost as light energy (79). Many of these molecules have π electrons which are not connected to an individual atomic nucleus but to the molecule as a whole (e.g. aromatic compounds). These electrons

are easily excited to higher molecular orbits. Fluorescence and phosphorescence occur when such excited electrons drop back into lower energy levels. If the electrons in an excited state are spinning in the same direction a so-called singlet state is obtained. Fluorescent light is emitted when such electrons fall back to their normal levels. On the other hand, if the electrons in an excited state are spinning in opposite directions a triplet state is obtained. Under ordinary conditions such a state occurs very rarely and the triplet state is termed a "forbidden" state. Light emitted when electrons drop back to their normal levels from a triplet level is termed phosphorescent (95).

Electrons in the triplet state cannot readily return to their normal levels and this results in a longer period during which they reside in the excited orbit before dropping back with the emission of photons to their normal levels. When viewing a great number of such molecules, the result of this difference in the stability of the triplet and singlet states is manifested by the duration of light emission after cessation of the exciting illumination, unless interatomic collisions prevent such prolonged light emission by permitting the electrons to lose their energy and return to their normal levels through a loss of heat energy. Originally, it was this duration of afterglow which was considered to distinguish phosphorescence from fluorescence, rather than the different spectral (as implied by the terms "triplet" and "singlet") nature of the light emitted.

Interatomic collisions can be prevented if the motion of the molecules is limited. Such rigidity can be obtained by freezing the solution into a solid-solution or by absorption in gels (79, 95).

The probability of excited electrons going into a triplet state

can be increased through the influence of neighboring molecules. Water can greatly change such probabilities, making the triplet state probable in some cases rather than improbable (79).

Under certain conditions, many natural products exhibit luminescent properties. Some examples are the porphoryins, certain of the proteins, ribonucleic acid, desoxyribonucleic acid, cholesterol, thiamin, riboflavin, carotene, pyridoxine, epinephrine, morphine, polycylic-hydrocarbon carcinogens[1], and extracts of plants[2], skin, tissues, teeth, fats and many more (95, 96). Szent-Györgyi has proposed that their biological significance and their ability to luminesce are not unrelated, but that, in the protoplasm, long-lived, electronic excitations, improbable in ordinary solution, can occur and that living matter forms such an activated system of water and organic matter (79).

B. Experimental

1. Origin of the compounds used

In addition to the compounds which were synthesized and whose properties were discussed in Appendix I and those compounds described in Part I, several other compounds were studied. Quinoline was an Eastman Kodak Co. product. Quinine, N. F., was a product of Merck and Co., Inc. 8-Methoxyquinaldic acid was a gift of Dr. J.M. Price, University of

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1. From coal tar, "...Hieger (1930) first discovered that those [hydrocarbons] causing cancer showed a beautiful, ghostly whitish fluorescence in ultraviolet light.", and by this means isolated the first, chemically pure carcinogenic hydrocarbon (97).

2 The first known report of fluorescence in the literature is by Nicolas Monardes, 1565, concerning the blue color of aqueous extracts of lignum nephriticum (98).

Wisconsin. Anthranilic acid and m-aminobenzoic acid were products of Eastman Kodak Company. The p-aminobenzoic acid was a product having a melting point of 187°, reported 187° (94). The o-acetamidobenzoic acid was a product having a melting point of 177°, reported 185° (94).

2. Fluorescence spectra and fluorescence intensities

The instrument used was an Aminco-Bowman spectrophotofluorimeter. Solutions of the chemicals studied were prepared by dissolving either (1) ten mg. in one liter, or (2) one mg. in 100 ml. of distilled water. The solutions, warmed if necessary, were stirred until the material had dissolved. From these solutions, a ten-fold dilution was made. The two solutions containing ten gamma per ml. and one gamma per ml. were stored at approximately -15° until their spectra were taken. In certain other cases, the solutions were made up in 0.01 N NaOH, 0.01 N HCl and M/15 phosphate buffer, pH 7.4.

3. Phosphorescence of certain compounds which are spotted on paper and their duration of afterglow

Twenty lambda of a solution near saturation (20°) with the compound to be studied were spotted on Whatman #1 paper and allowed to dry. Tests were sometimes made on the spot after chromatographic development. Next, a block of dry-ice was laid flat on the table and covered with a sheet of aluminum foil. The spotted paper was placed flat on the foil and covered with a sheet of thin pliofilm. This prevents water condensed from the air from dampening the paper. The paper was firmly held against the cold surface and an ultraviolet lamp (Mineralite) was placed face down over the position of the compound on the paper. The spot was illuminated for ten seconds and then the lamp was turned off. At the same time, a stop-watch was started and the duration of the

phosphorescent afterglow was roughly timed. As the intensity of the phosphorescence fades away, it is difficult to observe a definite end-point, but, with practice, fairly consistent results can be obtained.

C. Results and Discussion

Figures 69 to 90 show the fluorescence spectra of a number of compounds. Two curves were obtained. The activation curve (drawn with a solid line) represents the intensity of fluorescent light (having the wavelength of the emission maximum) obtained as the wavelength of the incident light is varied. The emission curve (drawn with a dotted line) represents the intensity of fluorescent light at various wavelengths, the incident light having the wavelength of the activation maximum. Light intensities are in arbitrary units, being galvanometer readings. The peak obtained in the activation spectrum which coincides with the peak in the emission spectrum is an artifact of the instrument. Readings were only taken at close enough intervals to obtain a general shape of the curve and in some cases values of maxima may be shifted a small amount from the true values. Also these curves were obtained in unbuffered water solutions. The results shown in these graphs are summarized for better comparison in Tables 25 and 26. In these tables, the values obtained on one gamma per milliliter solutions have been multiplied by ten so that all are equivalent to 10 gamma per milliliter solutions, except that the solutions of higher concentration may be more subject to self-quenching.

The quantitative results confirm in a more precise way what is readily seen in most cases by visual inspection of the developed chromatograms under ultraviolet light, namely, that certain changes in

Figure 69, Fluorescence spectra of quinine

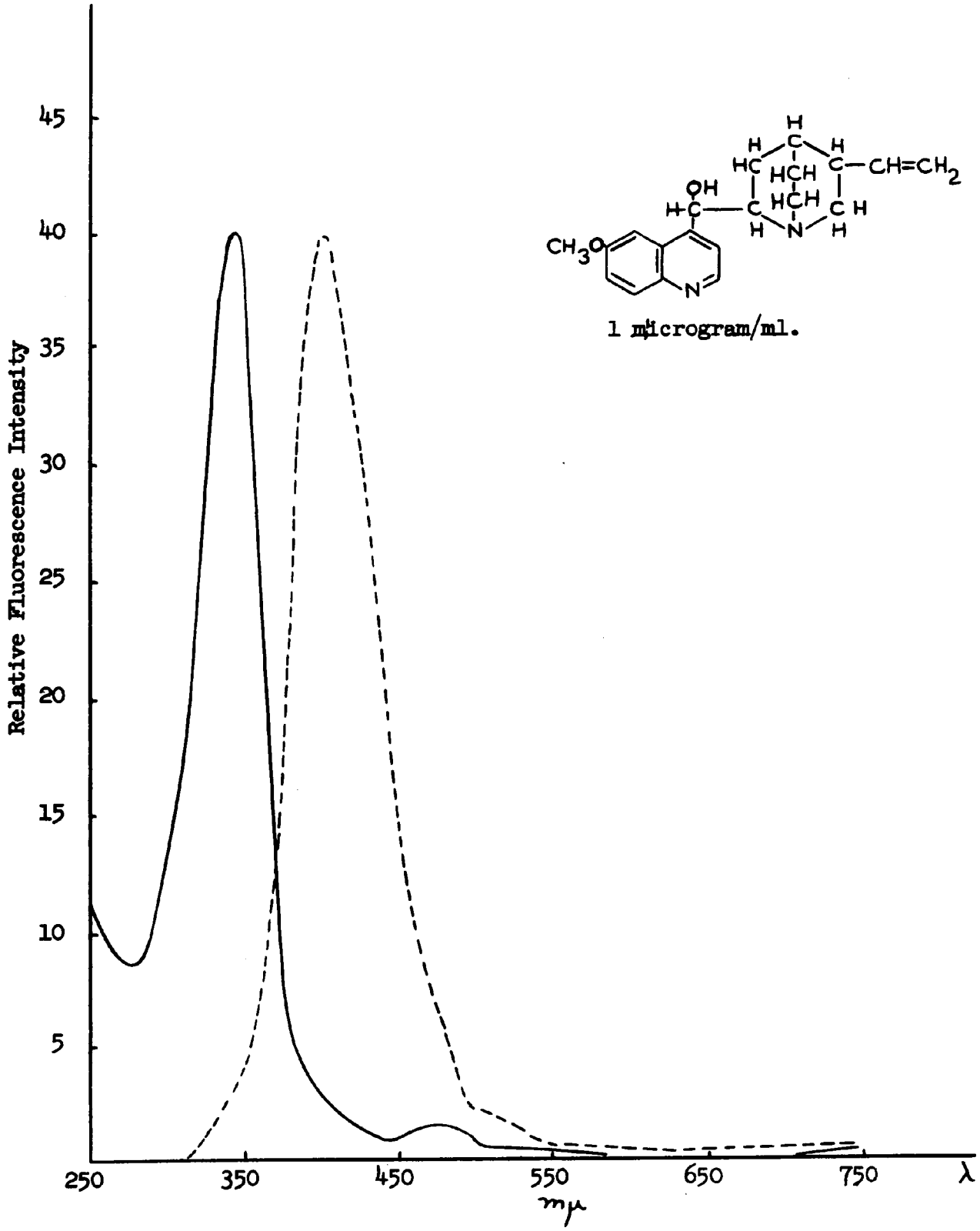


Figure 70. Fluorescence spectra of kynurenine

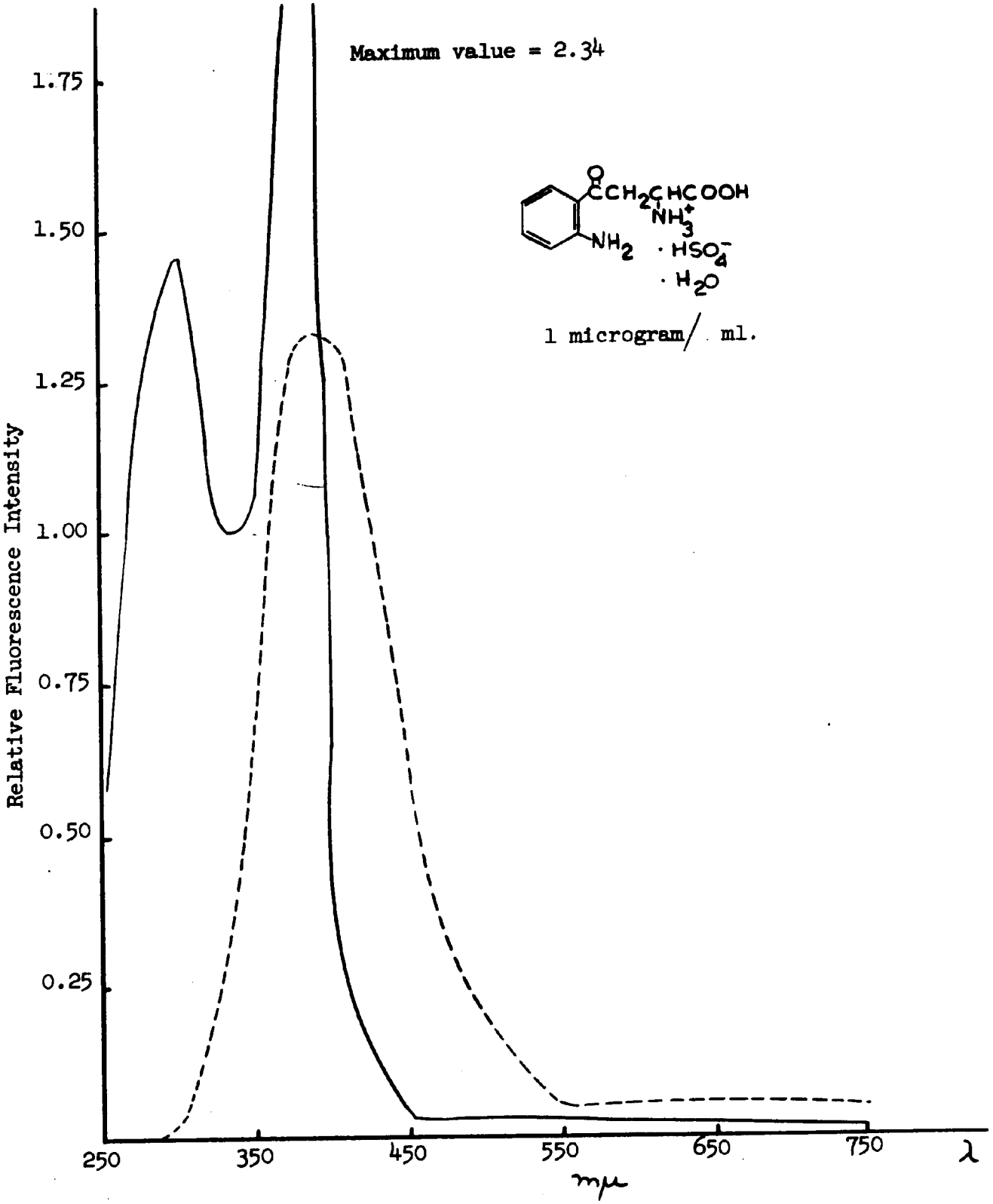


Figure 71. Fluorescence spectra of 3-hydroxykynurenine

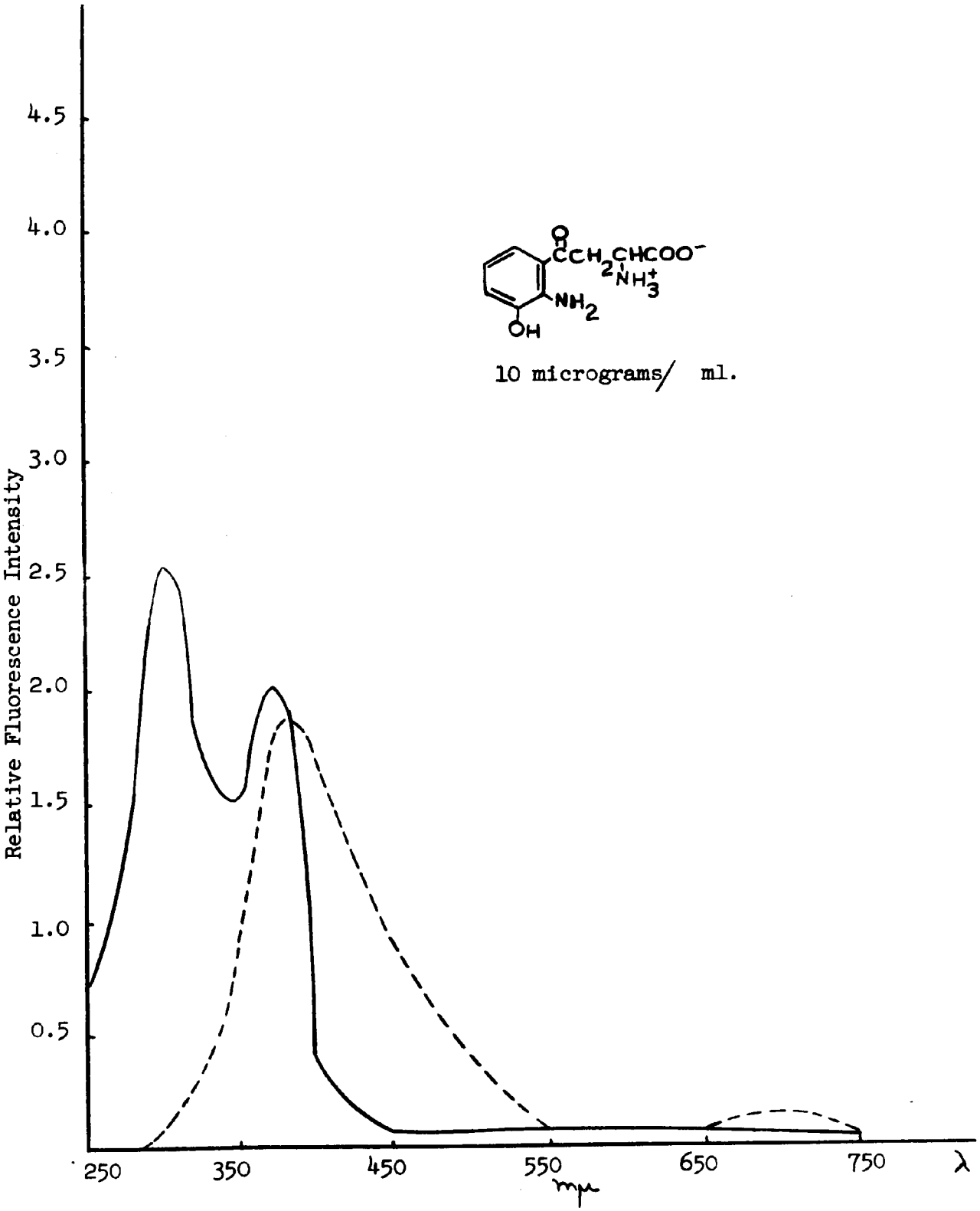


Figure 72. Fluorescence spectra of 3-methoxykynurenine

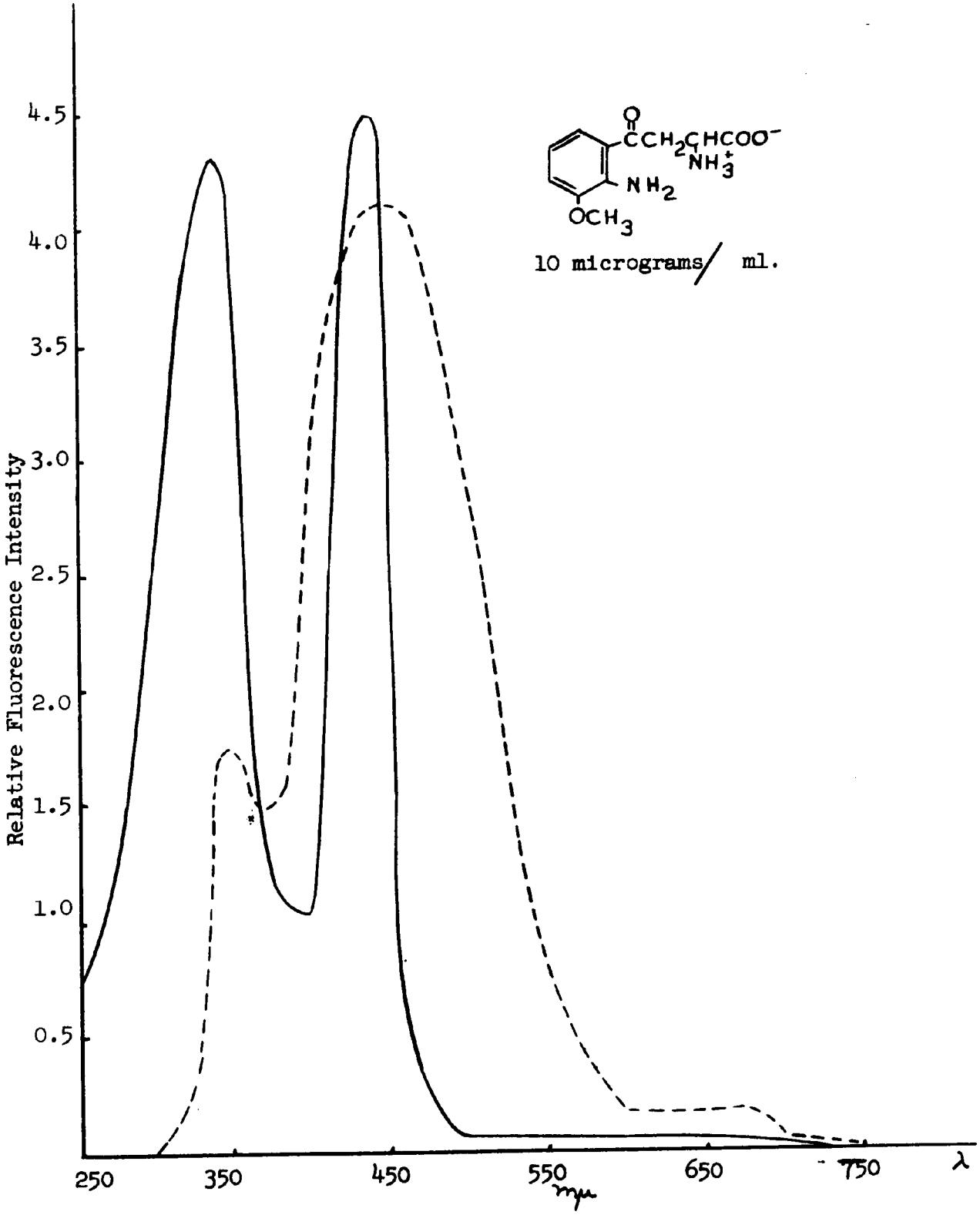


Figure 73. Fluorescence spectra of quinoline

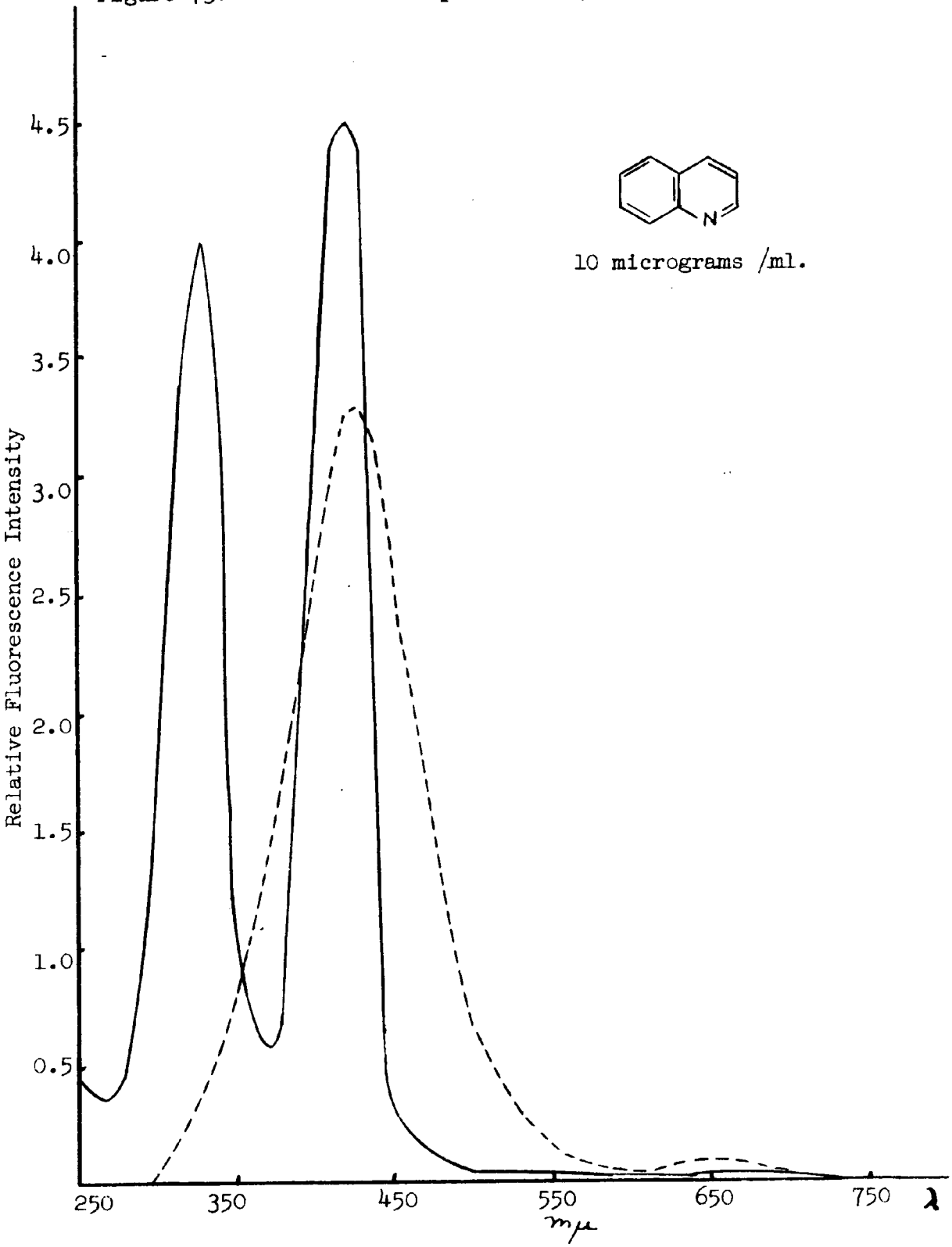


Figure 74. Fluorescence spectra of 8-hydroxyquinoline

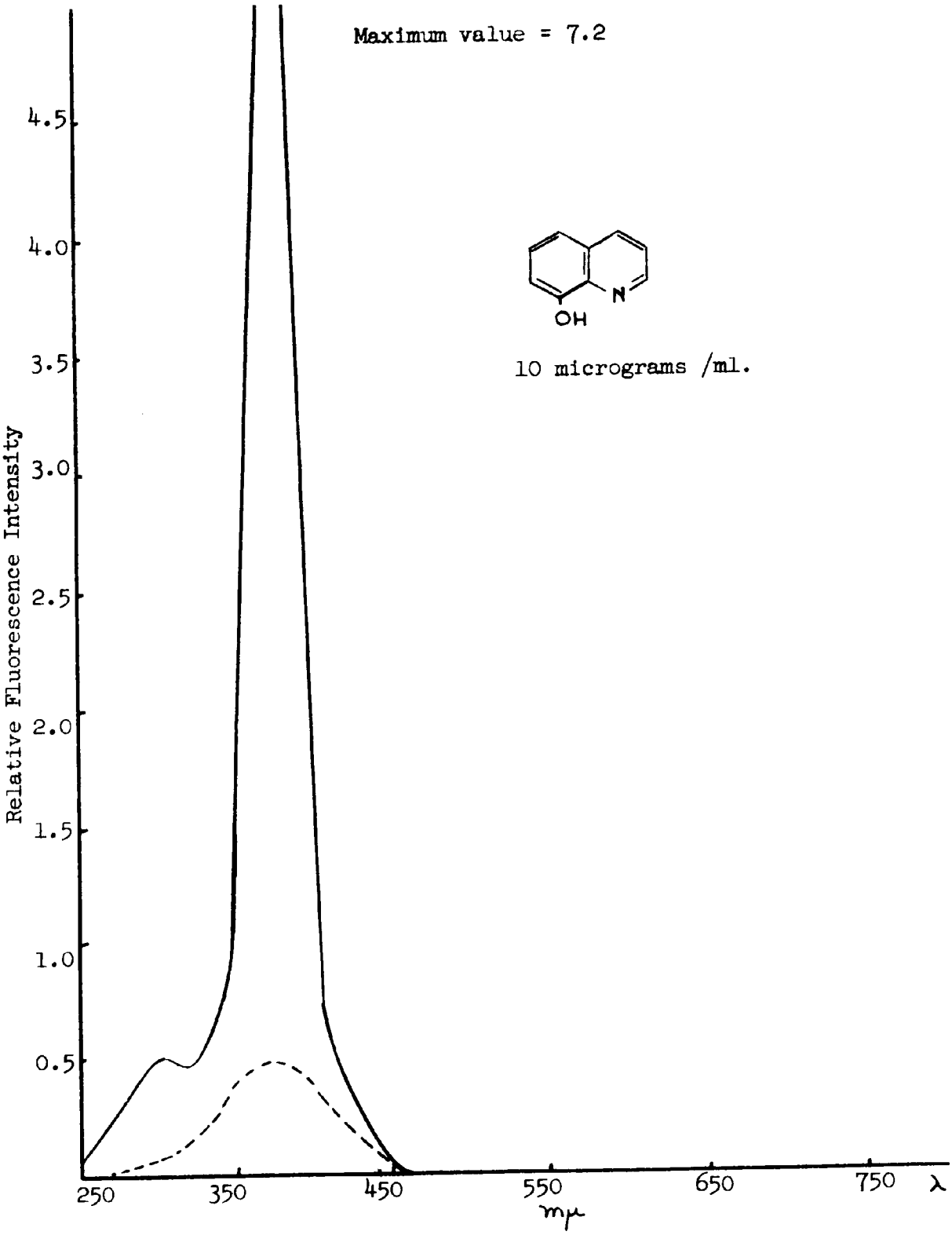


Figure 75. Fluorescence spectra of 8-hydroxyquinoline glucuronide

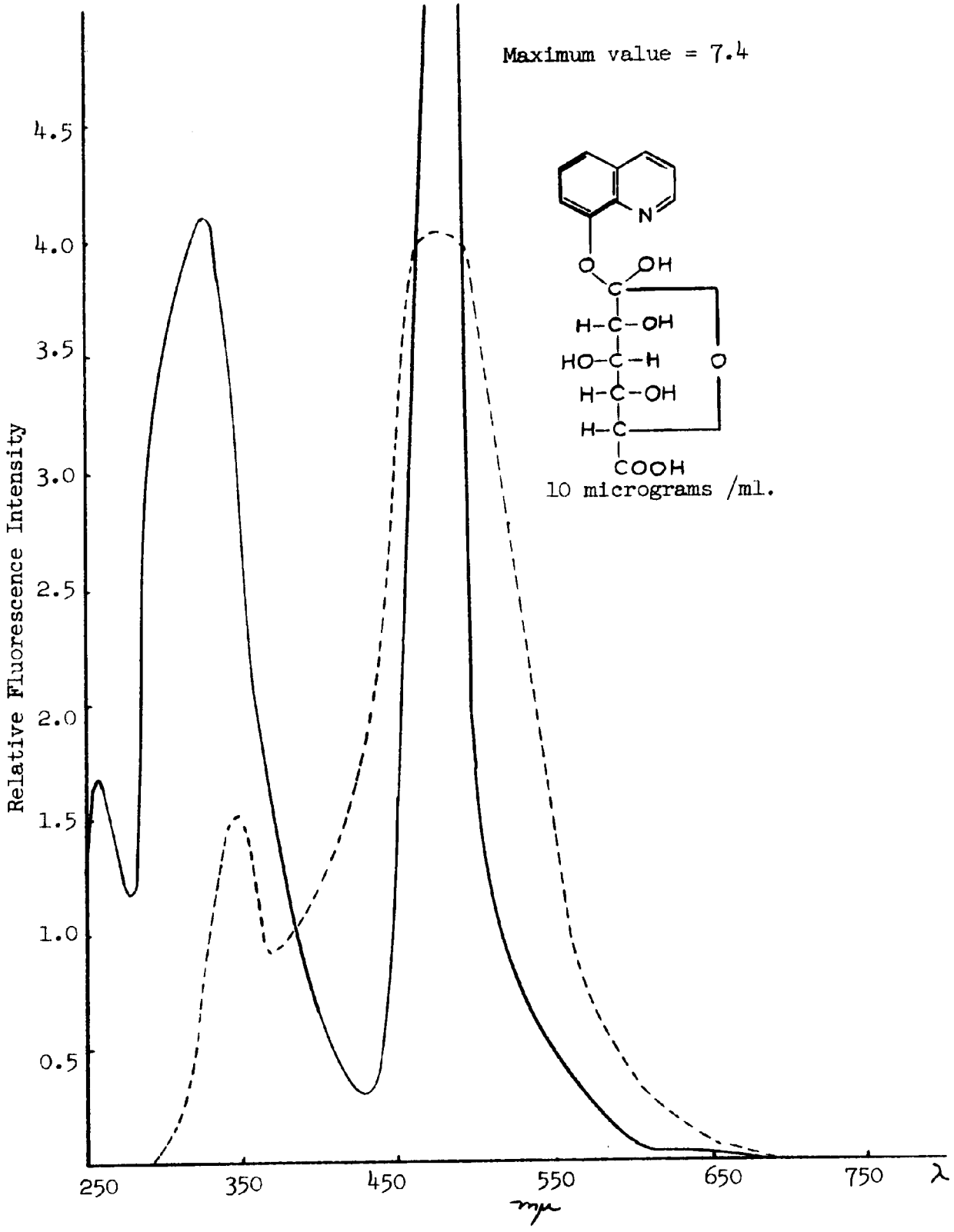


Figure 76. Fluorescence spectra of 8-hydroxyquinaldic acid

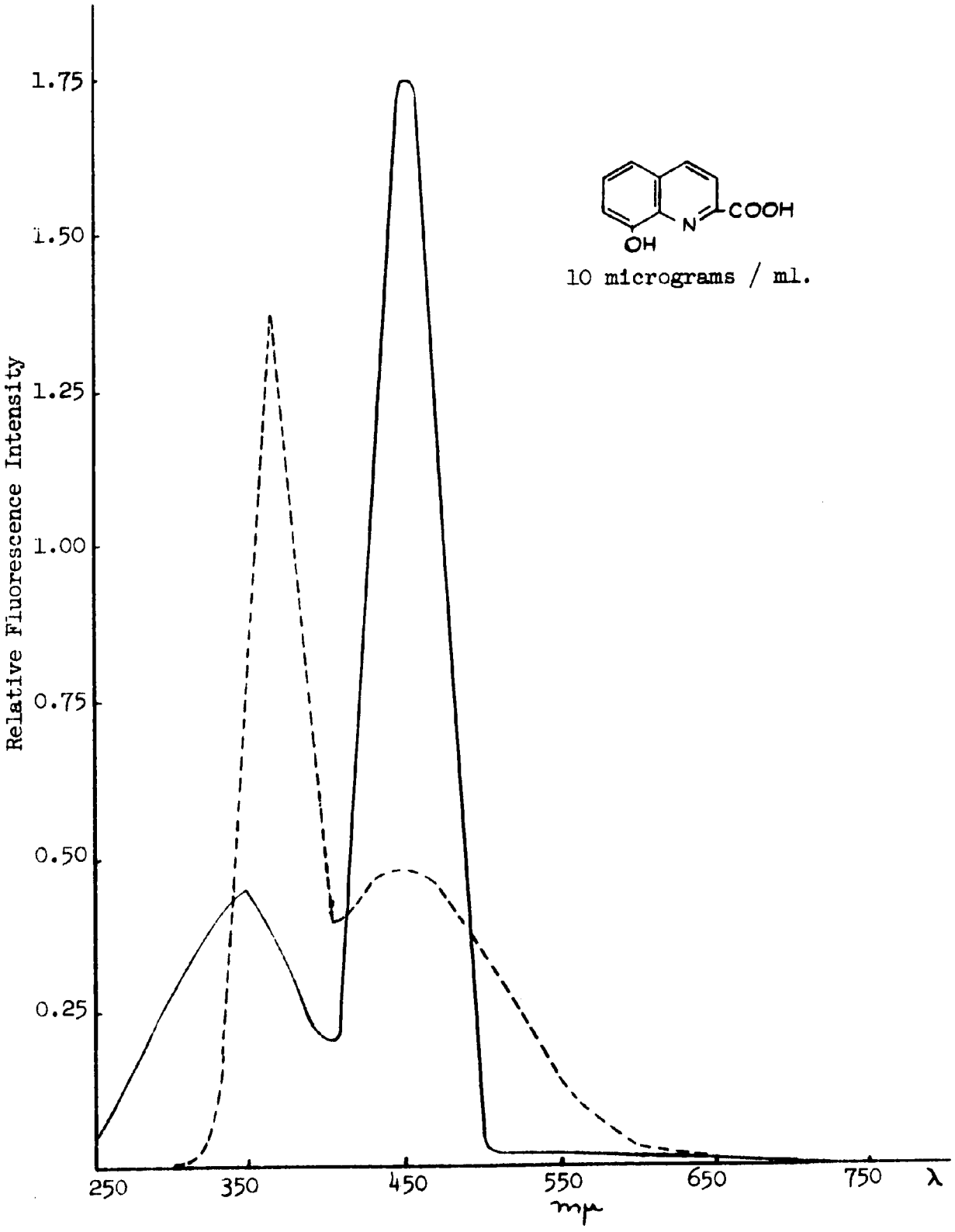


Figure 77. Fluorescence spectra of 8-methoxyquinaldic acid

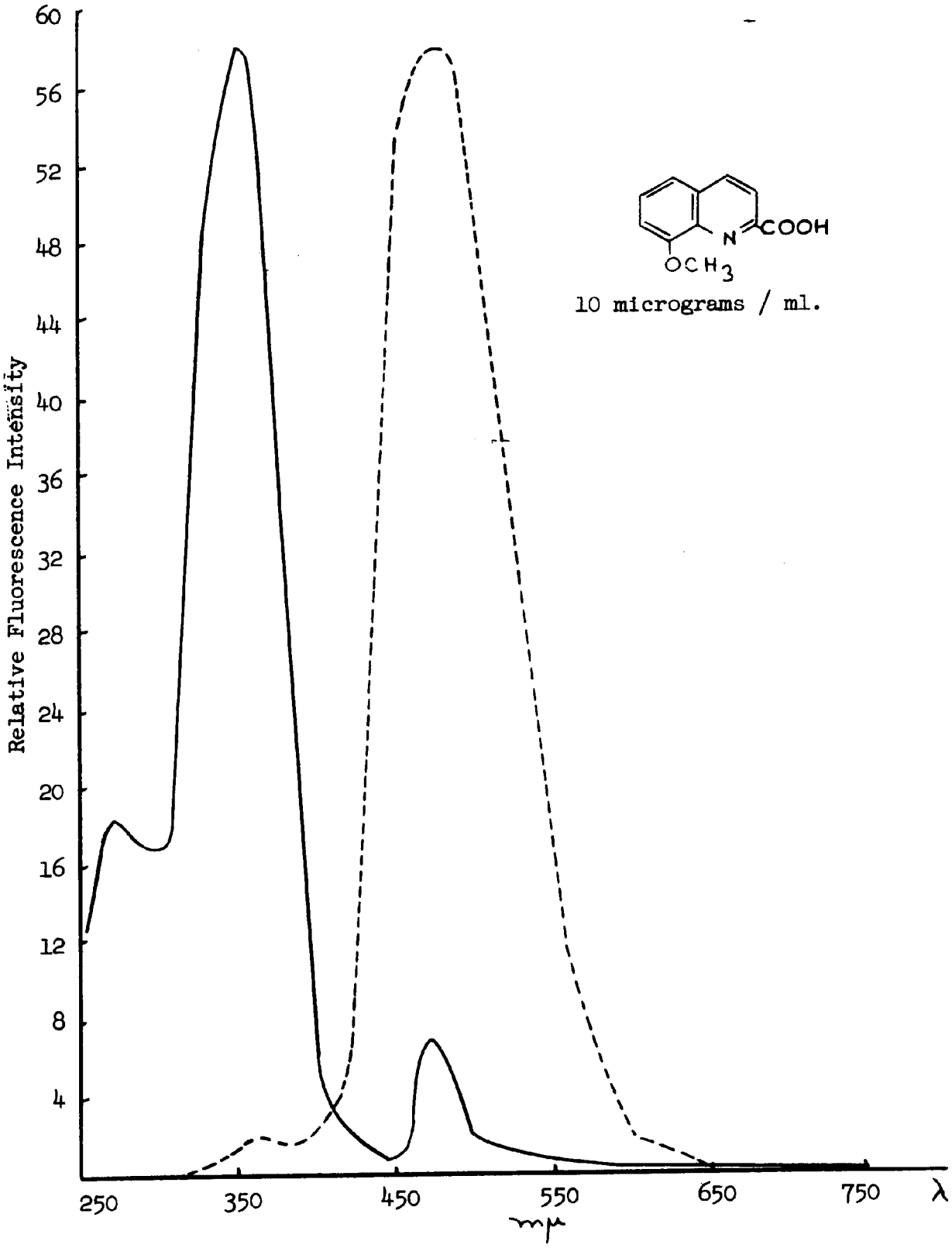


Figure 78. Fluorescence spectra of 8-methoxyxanthurenic acid ethyl ester

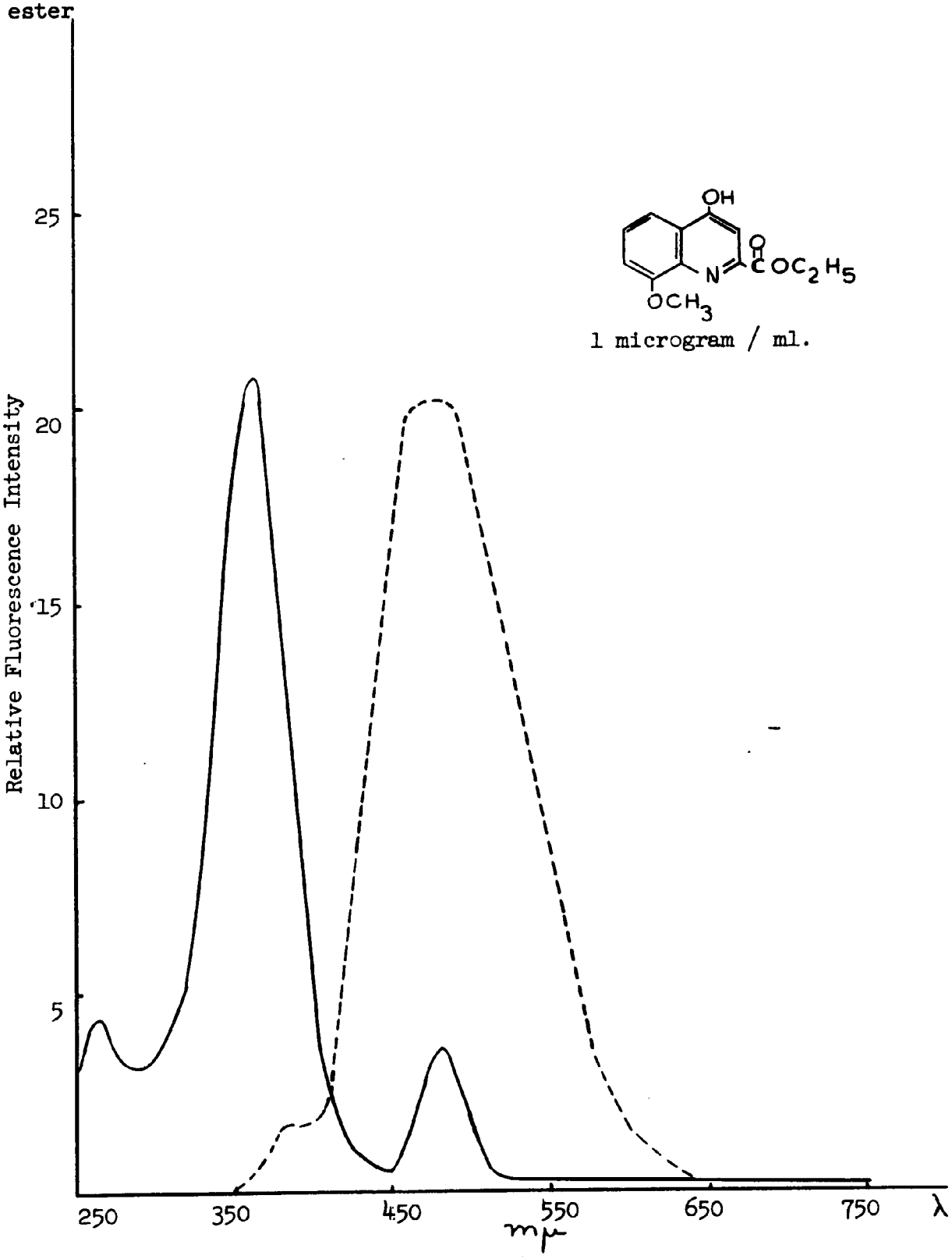


Figure 79. Fluorescence spectra of 8-methoxyxanthurenate

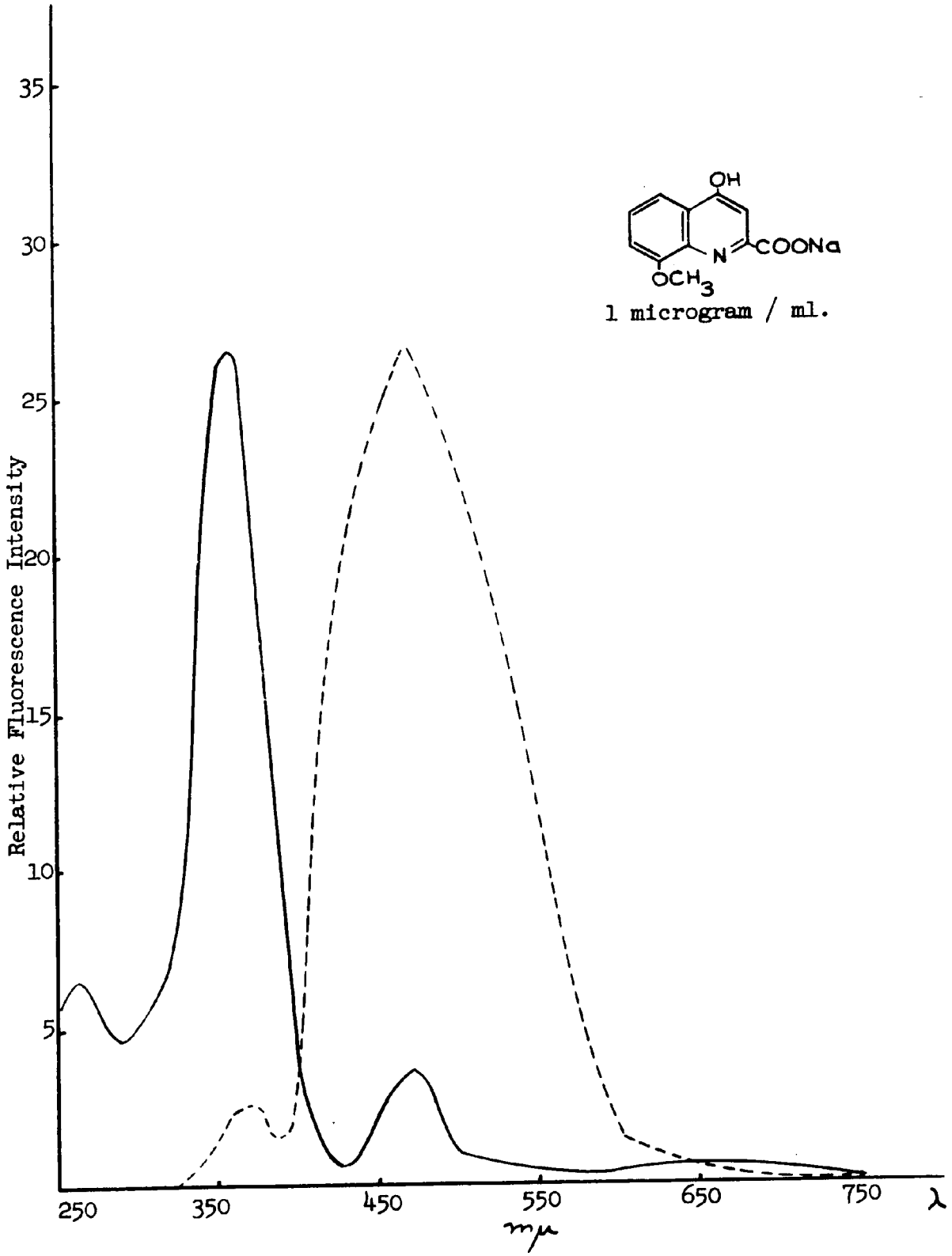


Figure 80. Fluorescence spectra of xanthurenic acid

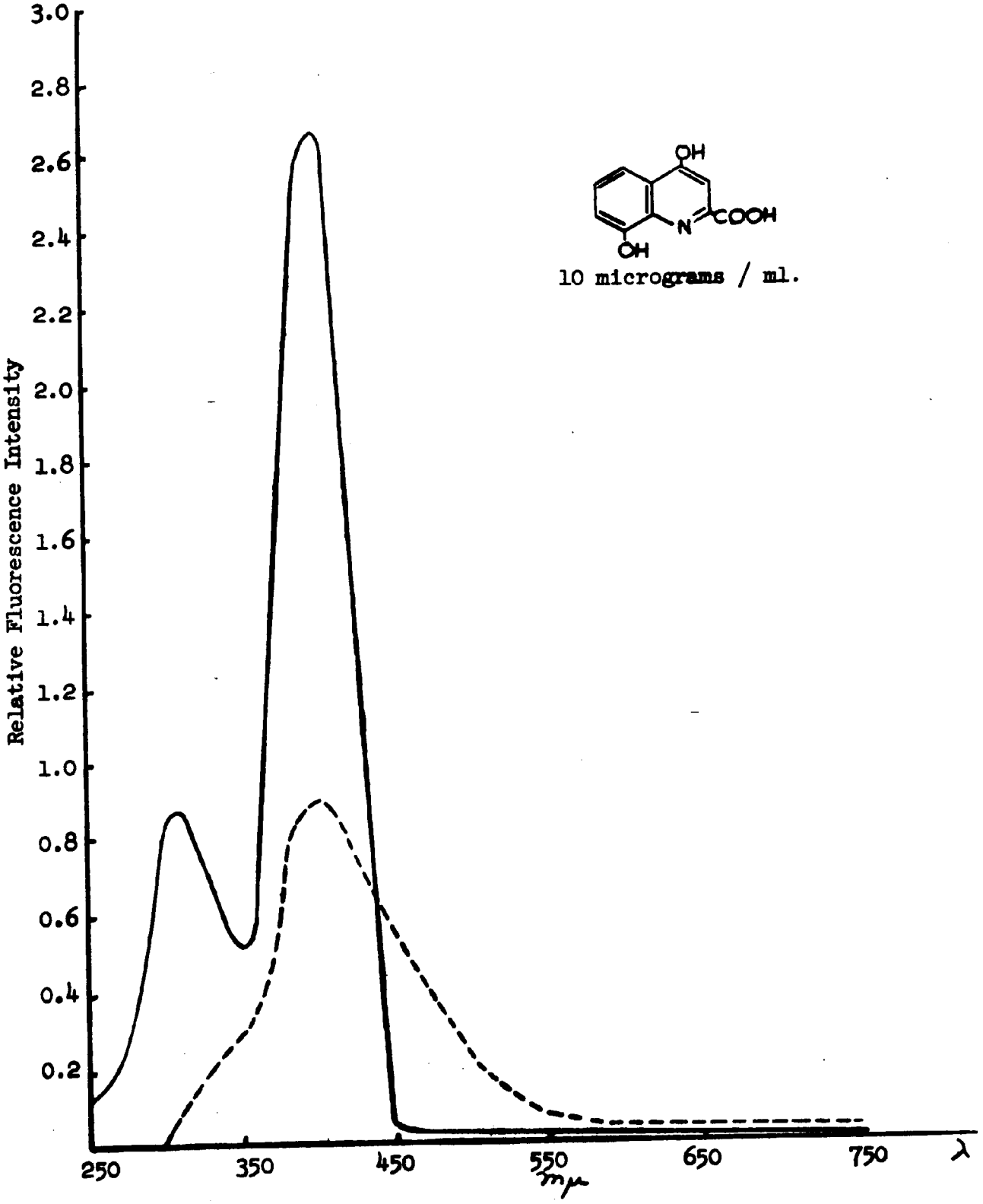


Figure 81. Fluorescence spectra of 4, 8-dihydroxyquinoline

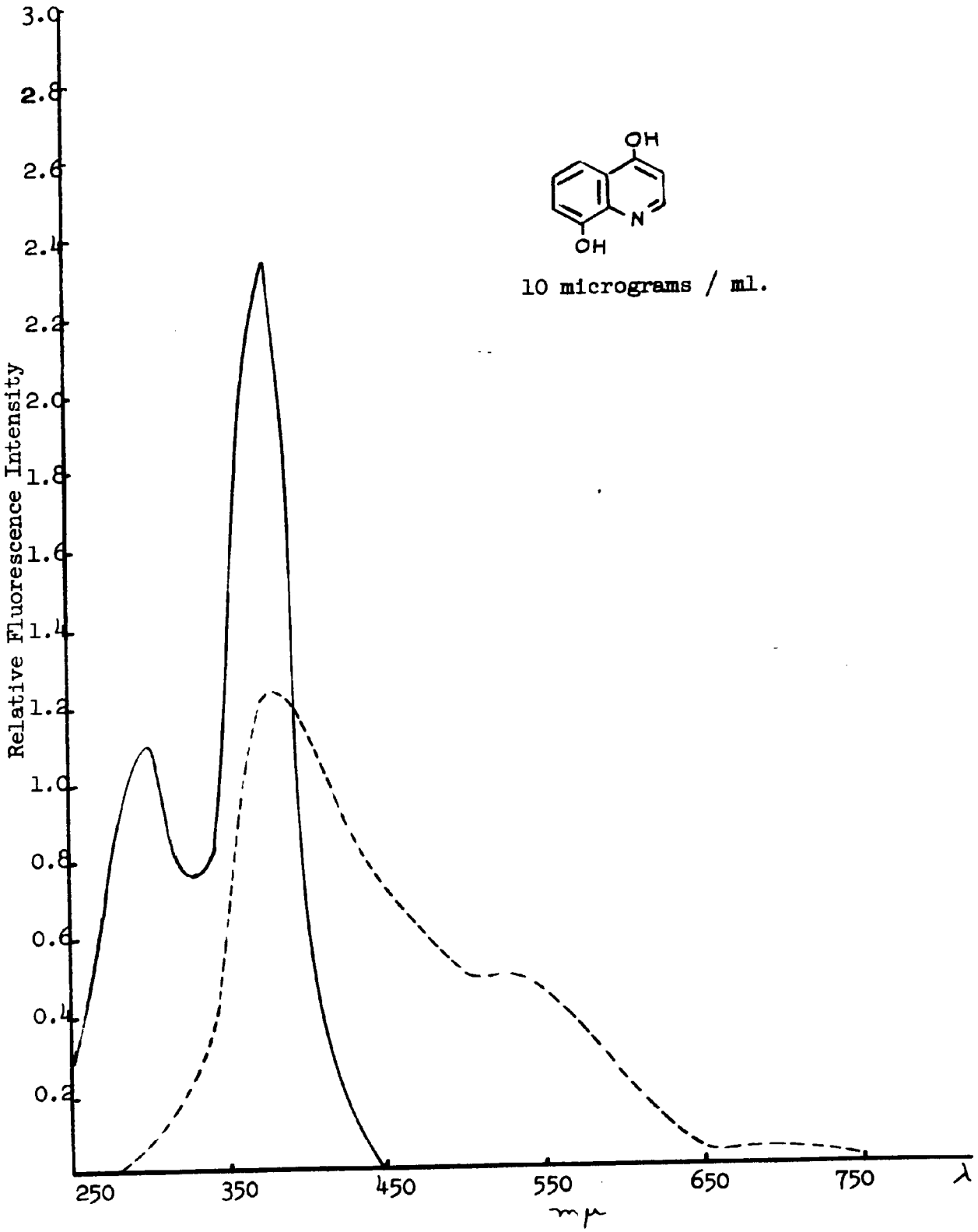


Figure 82. Fluorescence spectra of kynurenic acid

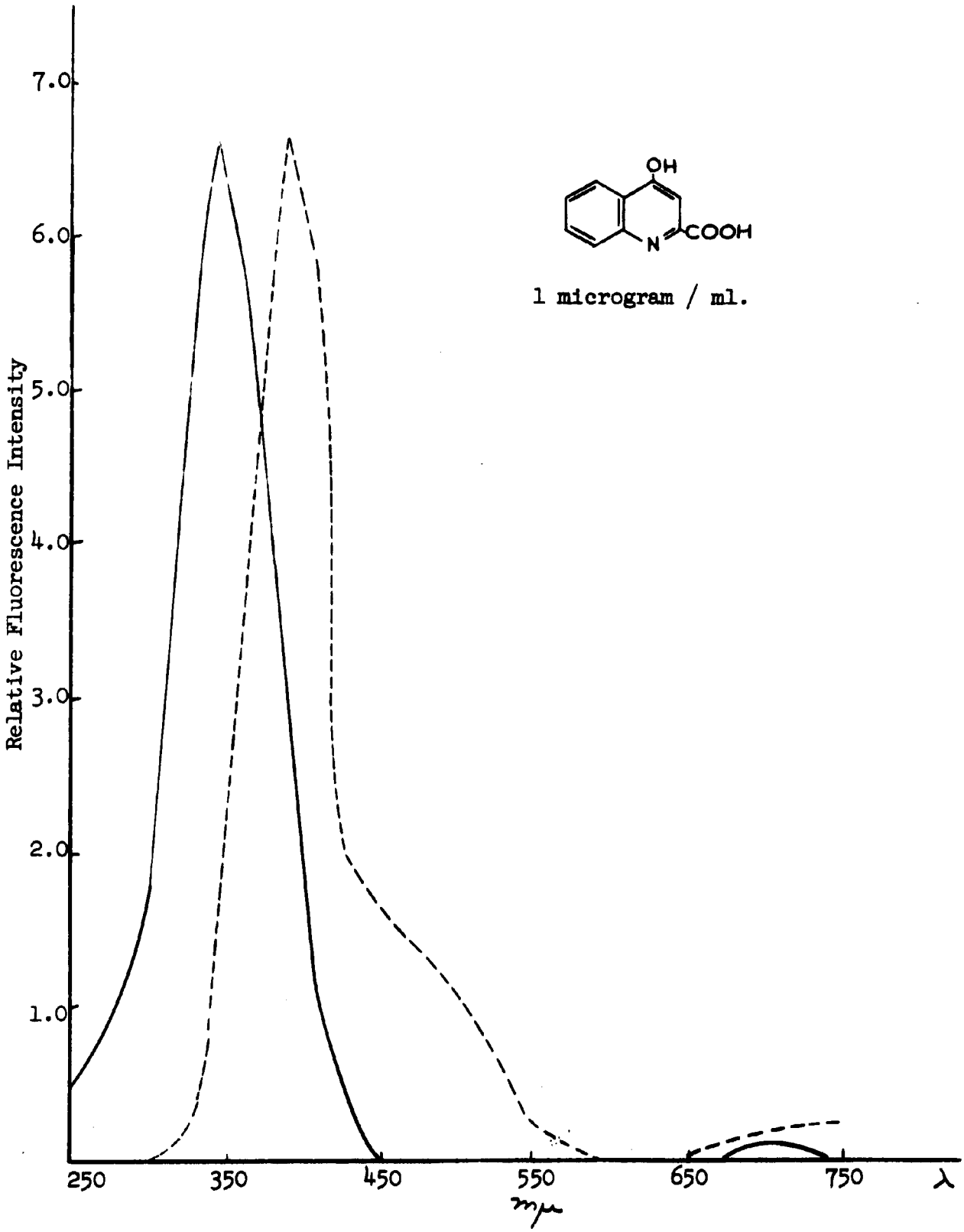


Figure 83. Fluorescence spectra of quinaldic acid

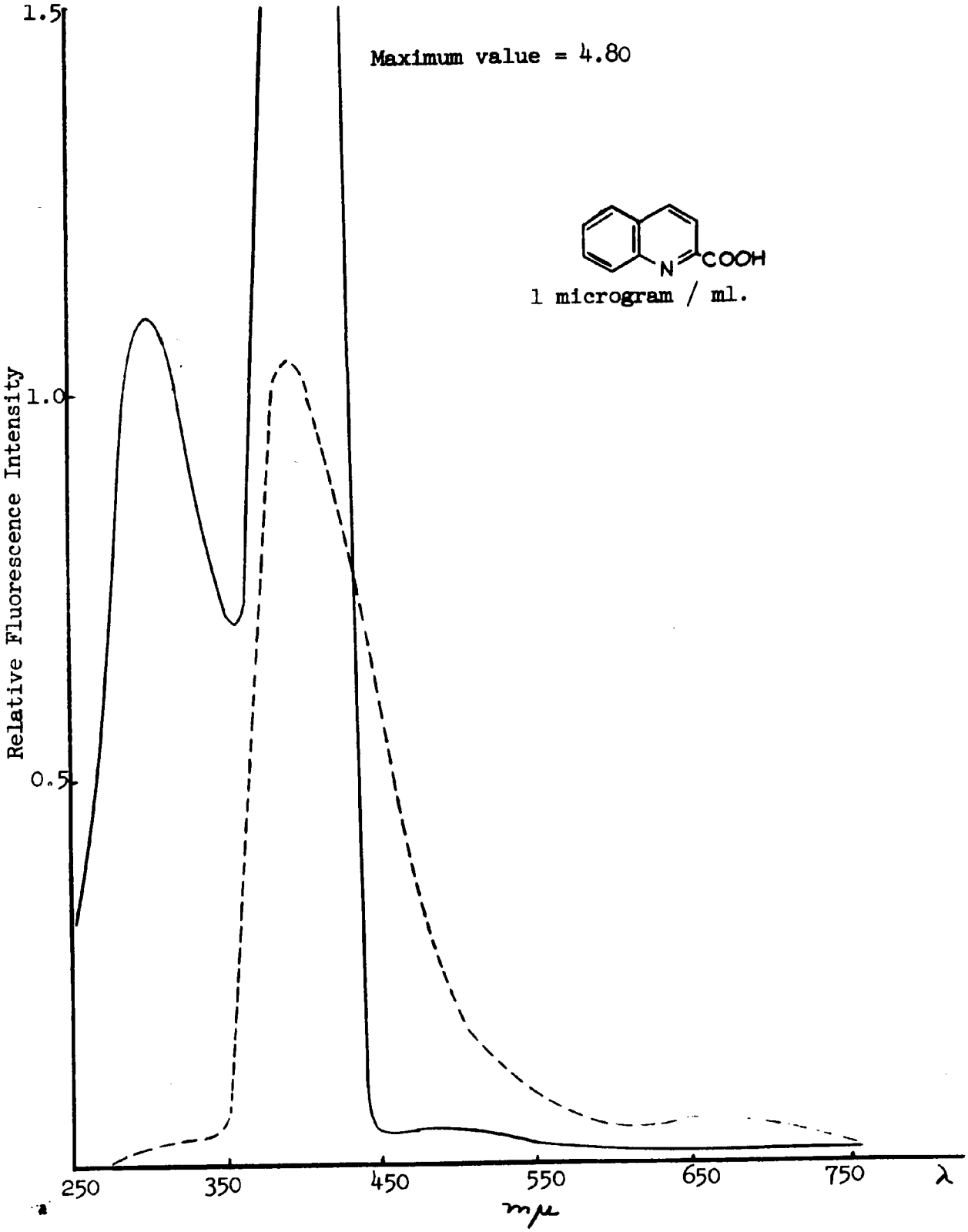


Figure 84. Fluorescence spectra of kynurine

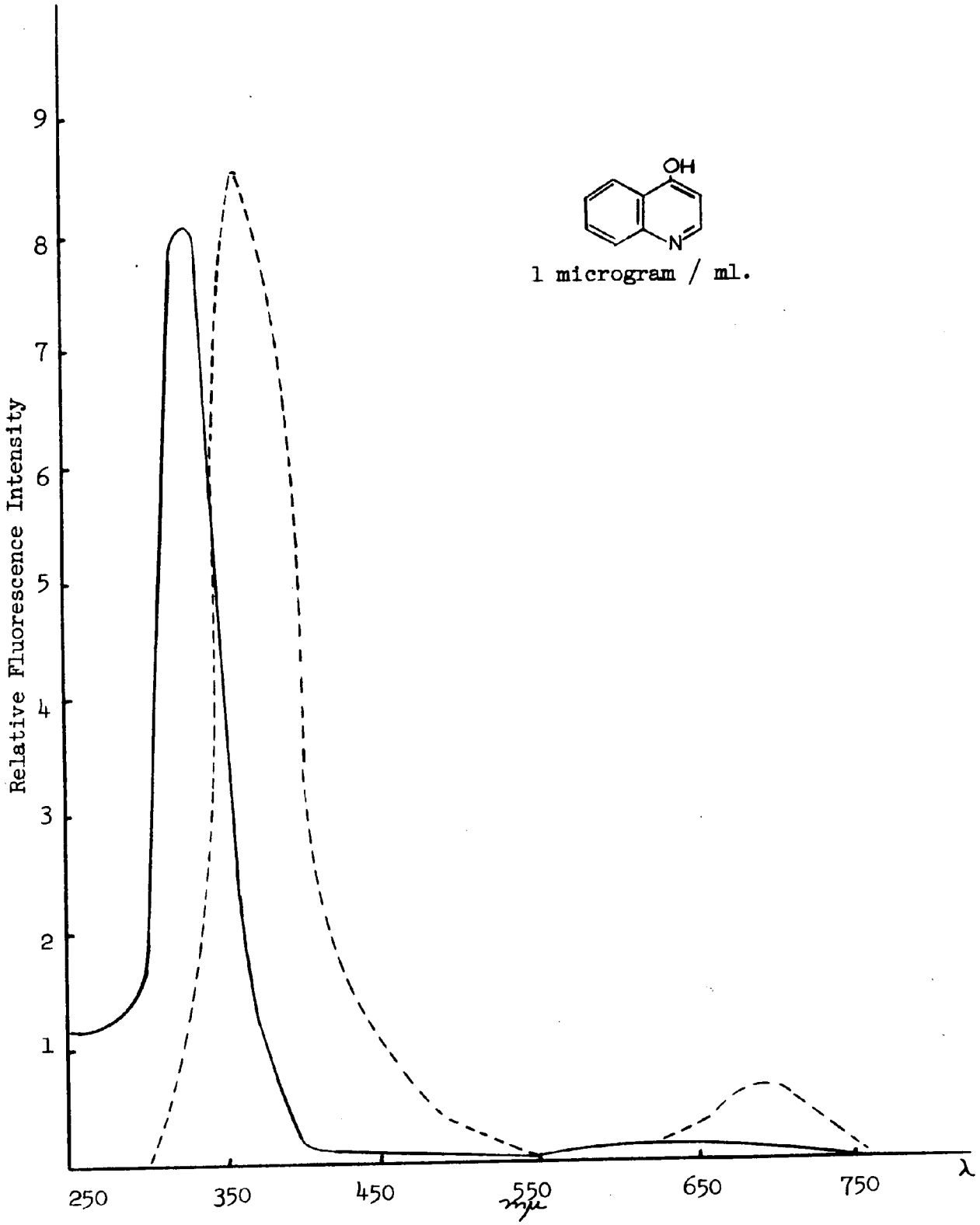


Figure 85. Fluorescence spectra of anthranilic acid

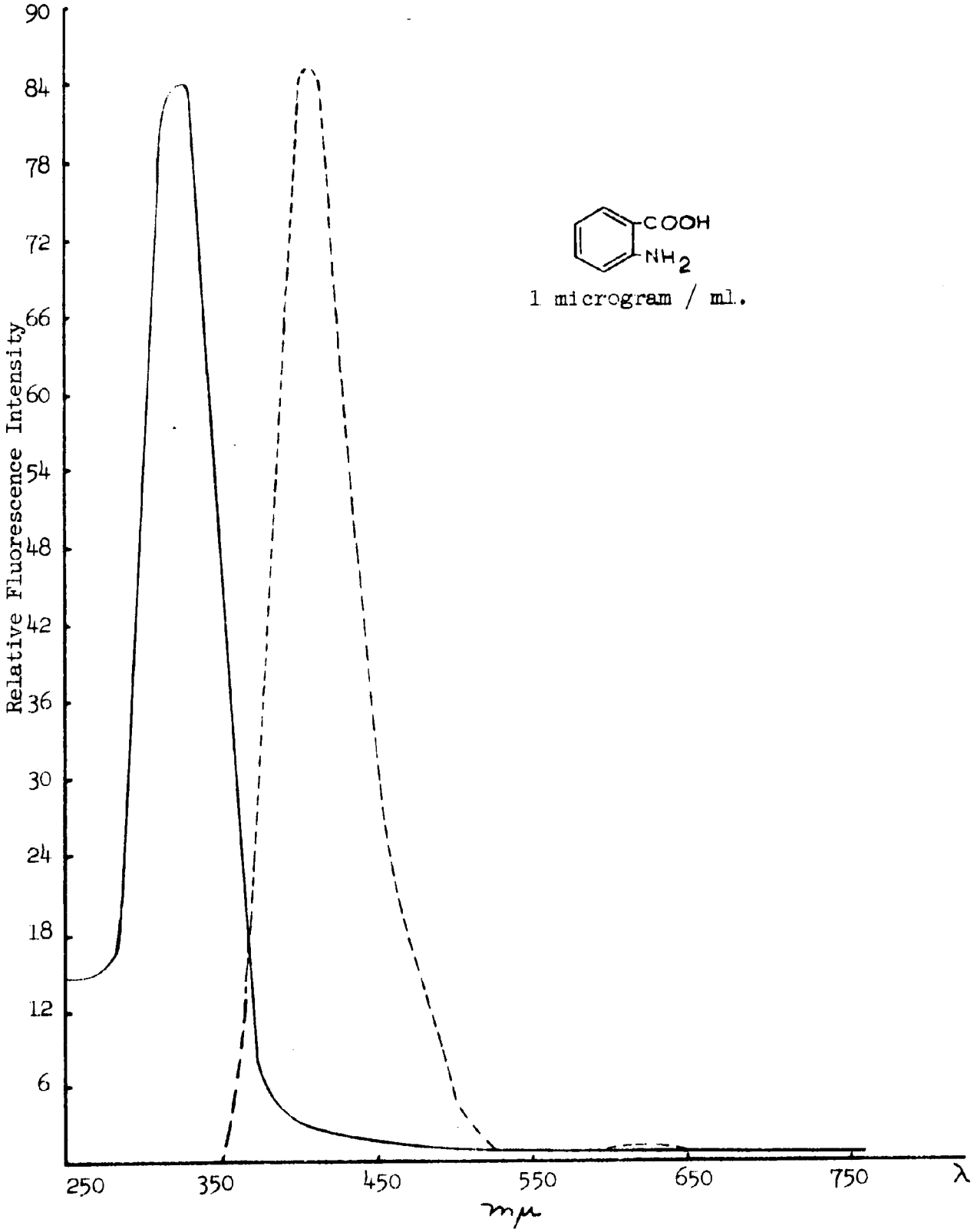


Figure 86. Fluorescence spectra of o-acetamidobenzoic acid

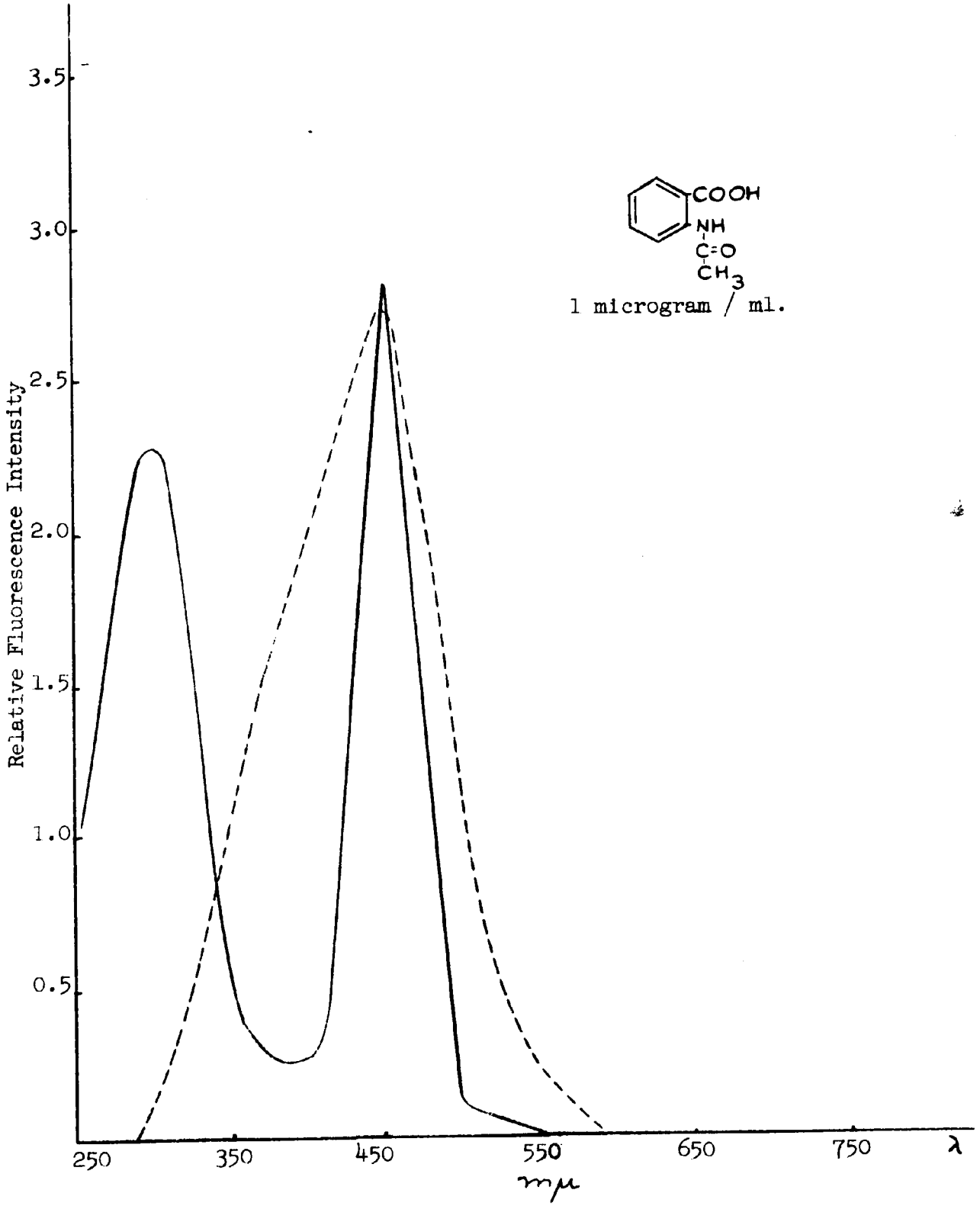


Figure 87. Fluorescence spectra of m-aminobenzoic acid

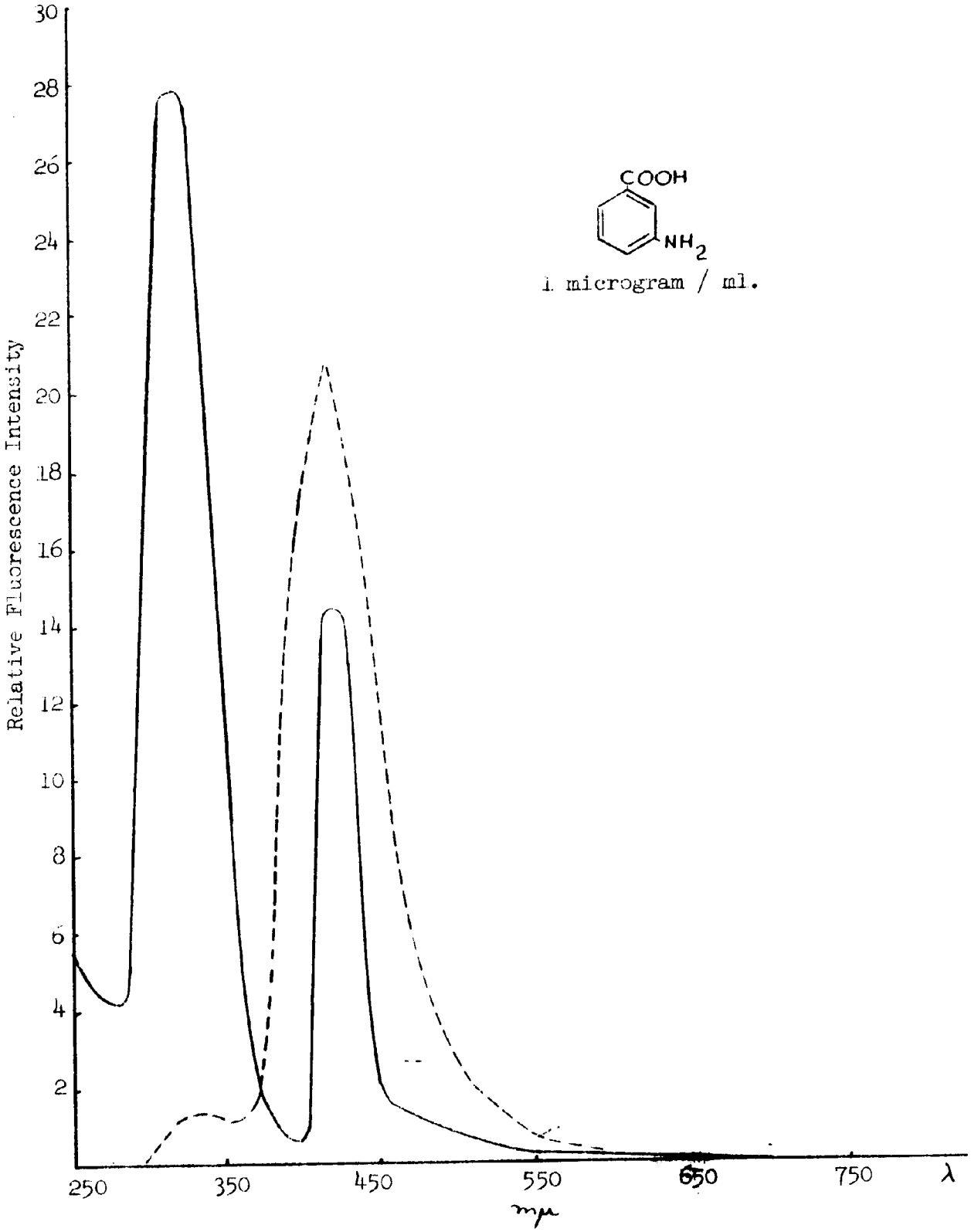


Figure 88. Fluorescence spectra of m-acetamidobenzoic acid

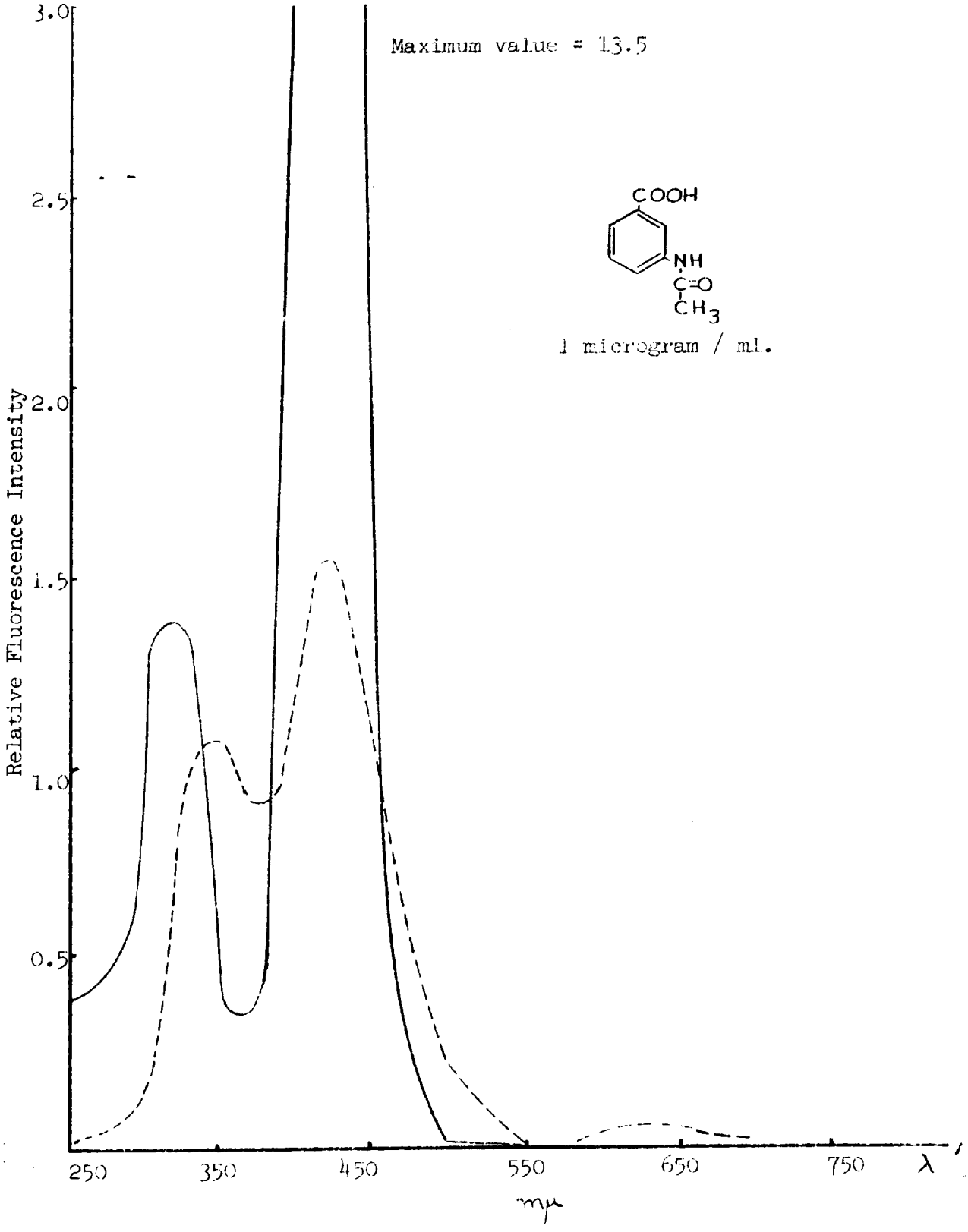


Figure 89. Fluorescence spectra of p-aminobenzoic acid

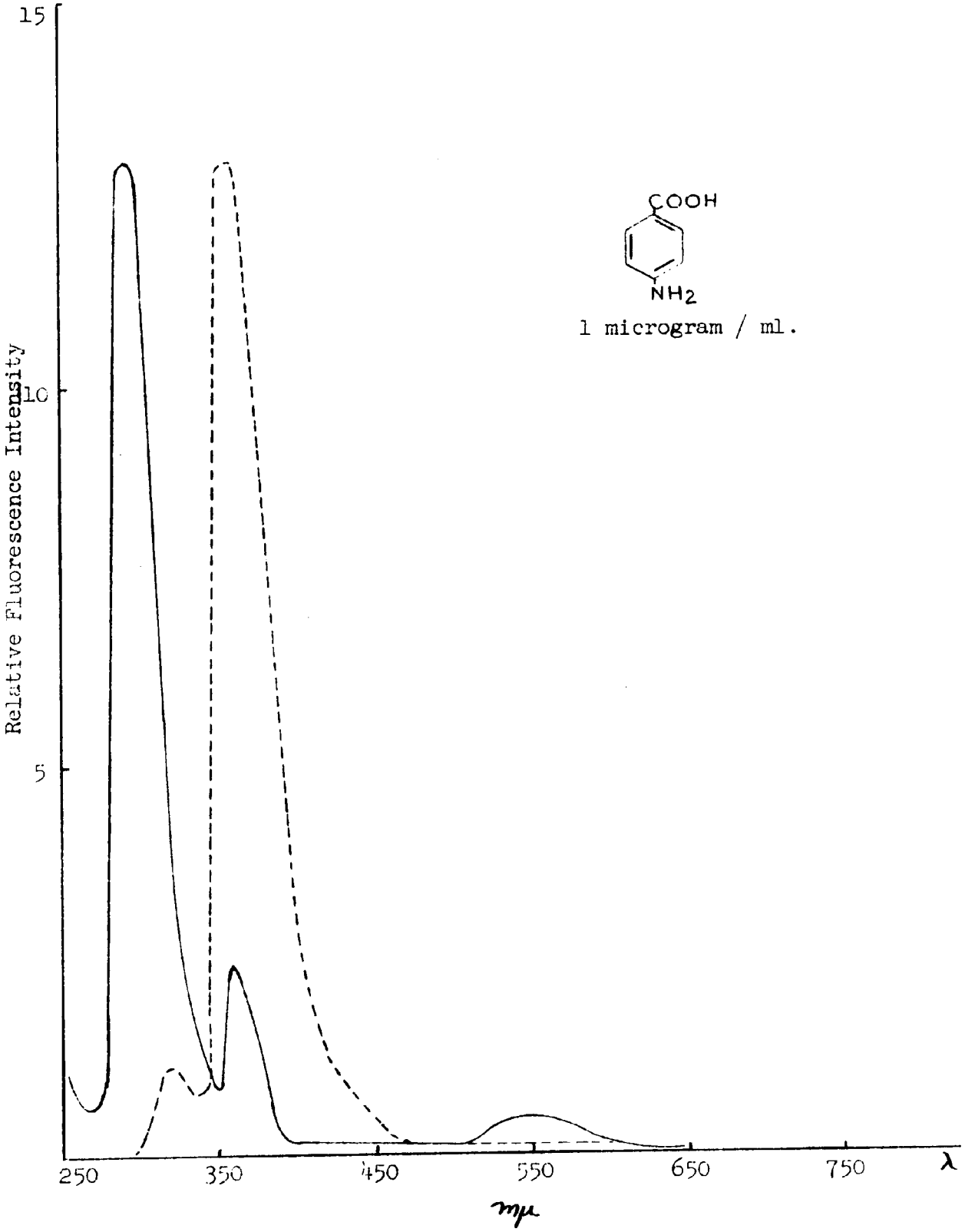


Figure 90. Fluorescence spectra of p-acetamidobenzoic acid

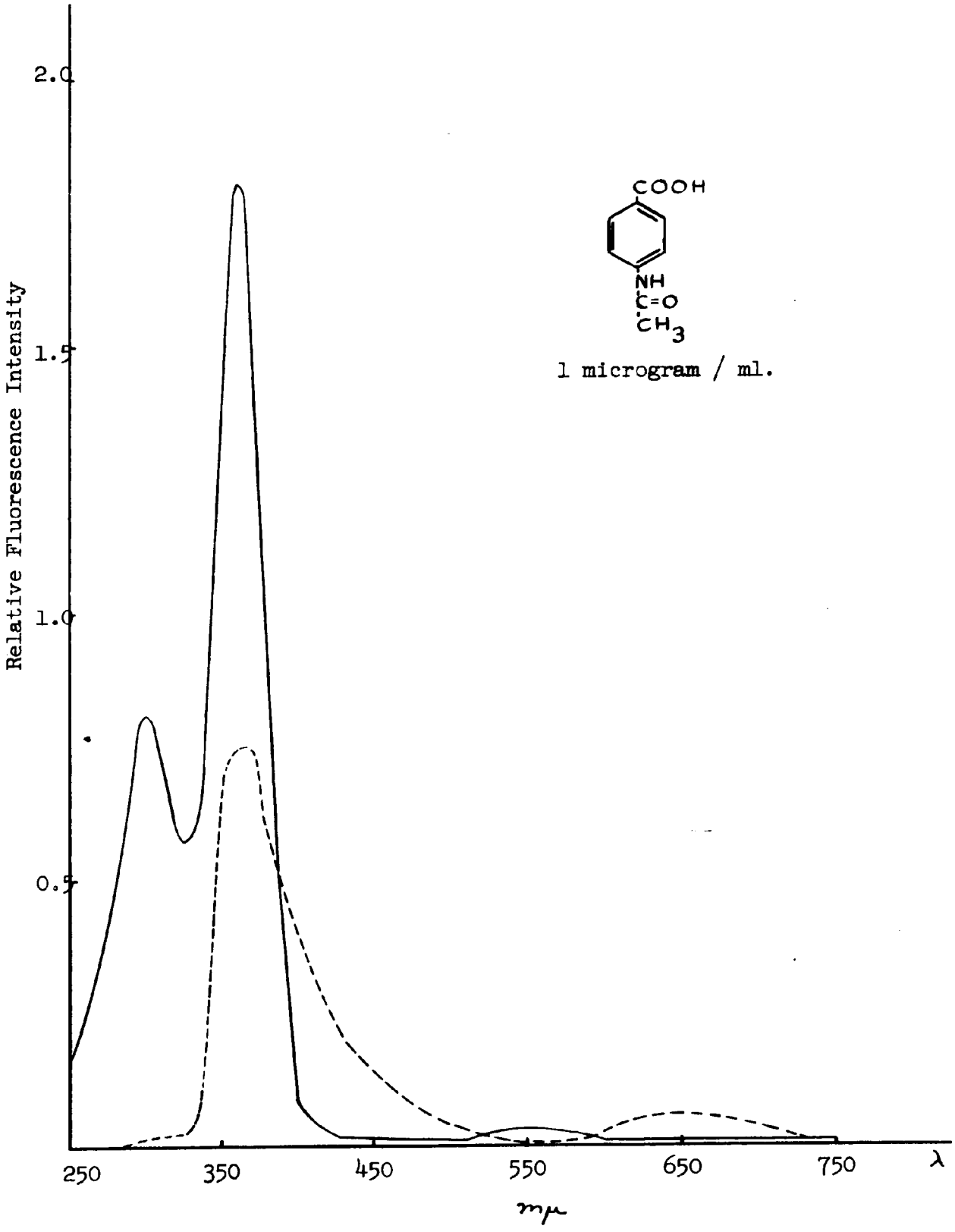
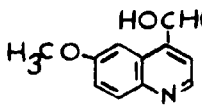
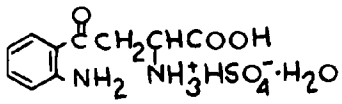
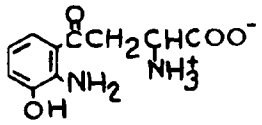
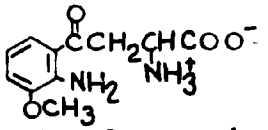
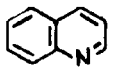
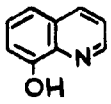
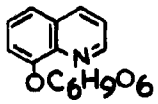
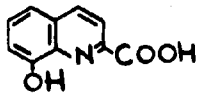


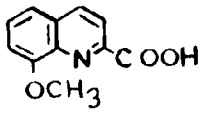
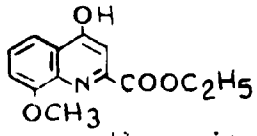
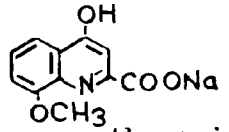
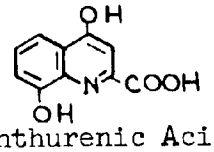
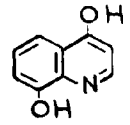
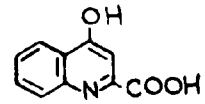
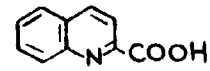
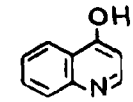
Table 25

Fluorescent and phosphorescent properties of some related quinoline and kynurenine derivatives

Compound 10γ/ml.	Activation maximum λ (mμ)	Emission maximum λ (mμ)	Relative Fluorescence Intensity	Duration of phosphorescence[a] (sec.)
 Quinine	340	400	400	0
 Kynurenine	300	380	13.2	3.5
 3-Hydroxykynurenine	300	380	1.9	1.8
 3-Methoxykynurenine	340	440	4.1	3
 Quinoline	330	420	3.3	0
 8-Hydroxyquinoline	300	380	0.5	0
 8-Hydroxyquinoline Glucuronide	330	480	4.1	2
 8-Hydroxyquinaldic Acid	350	450	0.5	0

[a] See page 156 for experimental procedure.

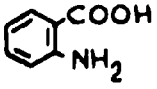
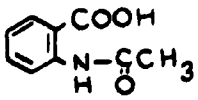
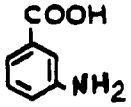
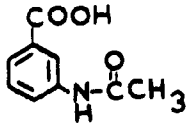
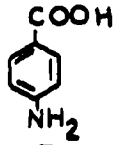
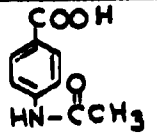
Table 25-Continued

Compound 10γ/ml.	Activation maximum λ (mμ)	Emission maximum λ (mμ)	Relative Fluorescence Intensity	Duration of phosphorescence (sec.)
 8-Methoxyquinaldic Acid	350	480	58	3
 8-Methoxyxanthurenic Acid Ethyl Ester	365	480	204	0
 8-Methoxyxanthurenic Acid Sodium Salt	360	470	267	3.5
 Xanthurenic Acid	310	400	0.9	1.8
 4, 8-Dihydroxyquinoline	310	380	1.2	4
 Kynurenic Acid	345	390	68	7.5
 Quinaldic Acid	300	390	10.5	7
 Kynurine	330	360	85	16
XI	350	450	520	5[a]

[a]XI was not applied as a saturated solution.

Table 26

Fluorescent and phosphorescent properties of isomeric amino and acetamido benzoic acids

Compound 10γ/ml.	Activation maximum λ (mμ)	Emission maximum λ (mμ)	Relative Fluorescence Intensity	Duration of phosphorescence[a] (sec.)
 Anthranilic Acid	330	415	850	8
 o-Acetamidobenzoic Acid	300	450	27	6
 m-Amino Benzoic Acid	320	420	207	4
 m-Acetamidobenzoic Acid	320	420	15.6	10
 p-Amino Benzoic Acid	295	360	129	16
 p-Acetamidobenzoic Acid	300	360	7.5	11

[a]See page 156 for experimental procedure.

the molecular structure greatly change the intensity of the fluorescence or the duration of the phosphorescence. In Table 27, the relative order of fluorescence intensities is seen to hold over the entire pH range in the cases of kynurenic, xanthurenic and 8-hydroxyquinaldic acids.

In Table 25, the effect of conjugation of the phenolic groups tested is seen to be enhancement of fluorescence, a lengthening of the duration of phosphorescence and a shifting of the emission maxima to longer wavelengths. On the other hand, N-acetylation of aminobenzoic acids (see Table 26) results in diminished fluorescence, but not necessarily in shortened, phosphorescent after-glow.

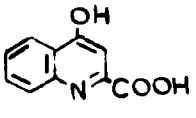
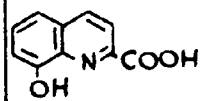
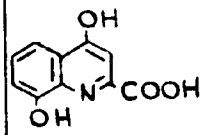
Fluorimetry has proven useful in qualitative and quantitative work and fluorescence data on related compounds may be of use in setting up analytical procedures. Thus the identity of the fluorescence spectra of XI, KI, TA and AXA, described in this thesis, indicates their chemical identity.

Phosphorescence of certain compounds on paper chromatograms can be used in locating or identifying certain spots (99). The paper is necessary for the production of this phosphorescence, i. e., the pure compounds placed on dry-ice and illuminated with ultraviolet light will not phosphoresce. Apparently, the cellulose surface of the paper and its absorbed water layer interact with these compounds to promote the triplet state and the solid-solution formed when the paper is pressed against dry-ice prevents interatomic collisions between the molecules of these compounds in their excited states.

The results obtained in this study are consistent with the idea that when electrons are pulled out of the aromatic ring a decrease in fluorescence will occur. The possible location of electrons in mole-

Table 27

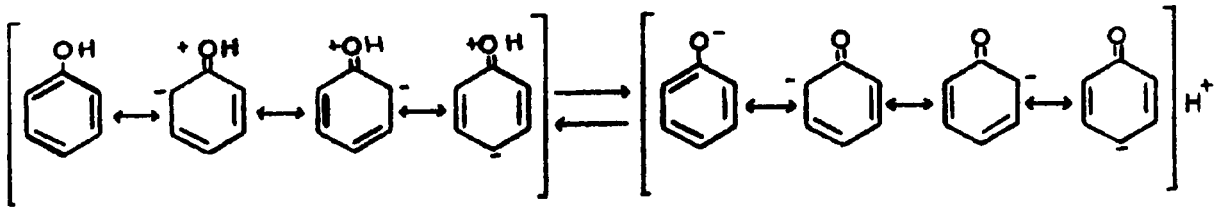
Effects of varying pH values upon the fluorescent properties of solutions of kynurenic, 8-hydroxyquinaldic and xanthurenic acids

Nature of solution, 10γ/ml.	Fluorescent properties			
0.01N Acid	Activation maximum λ (mμ)	340	--[a]	--[a]
	Emission maximum λ (mμ)	410	--[a]	--[a]
	Relative fluorescence intensity	22.2	--[a]	--[a]
Aqueous	Activation maximum λ (mμ)	345	350	310
	Emission maximum λ (mμ)	390	450	400
	Relative fluorescence intensity	68	0.5	0.9
pH = 7.4	Activation maximum λ (mμ)	350	300	300
	Emission maximum λ (mμ)	395	370	380
	Relative fluorescence intensity	51	0.4	0.3
0.01N Alkali	Activation maximum λ (mμ)	345	310	300
	Emission maximum λ (mμ)	405	380	380
	Relative fluorescence intensity	34	0.2[b]	0.2

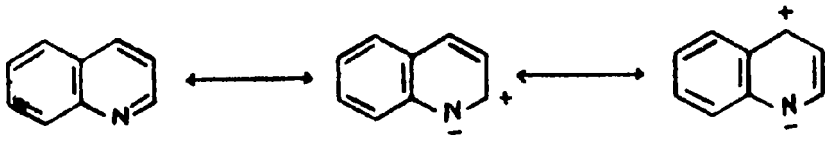
[a]This solution was not fluorescent.

[b]The same value was obtained with a solution containing either ten or one micrograms/ml., possibly because of self-quenching at the higher concentration.

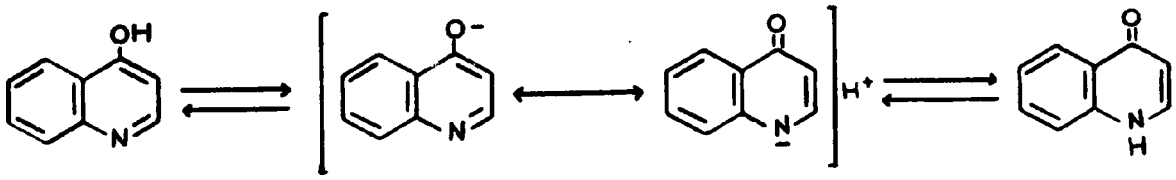
cules may be described more precisely by writing the resonance hybrid structures of the tautomeric isomers. Since fluorescence is generally associated with the availability of π electrons, the assumption can be made that as the contributions of non-aromatic or polarized forms increase the fluorescence intensity of the molecule should tend to decrease. Then it is readily seen that hydroxylation will usually tend to decrease the fluorescence.



Conjugation of a phenolic group would prevent formation of the phenoxide ion, thereby, decreasing the number of nonaromatic forms, reducing the number of atoms with excess electronic charge and diminishing the inductive effect of the oxygen atom. Similarly, the nitrogen atom in the pyridine ring of quinoline pulls electrons out of the ring.



If a group with an attraction for electrons is placed in the 2 or 4 position, the resulting competition between this group and the nitrogen atom for the ring electrons may tend to restore the electron density of the ring.



This net restoration would explain why a hydroxyl or a carboxyl group in the 2 or 4 positions enhances the fluorescent and phosphorescent properties of the derivative relative to those of the unhydroxylated quinoline. The reduction in the fluorescence intensity of the amino-benzoic acids upon acetylation can be attributed to the decreased ability of an acetylated amino group to donate electrons to the ring (100).

The effect of adding certain groups to a molecule has been noted previously in certain cases. For example, the introduction of iron, halides, oxygen, nitrogen, or sulfur atoms into a fluorescent molecule will often result in a molecule with diminished or no luminescent properties (79, 95). These same atoms may affect fluorescent molecules to which they are not chemically attached. Such atoms or compounds which depress the fluorescence of other molecules are called quenchers (95). Quenching may occur by different mechanisms but the result is either a chemical change in some component of the system (i. e., fluorescing molecules, solvent, other solutes, or the quencher itself) or else a loss of energy present in the excited molecules as heat. Some examples of compounds which will powerfully quench nearly all fluorescent solutions are phenol, o-cresol, hydroquinone, resorcinol, pyrogallol, α-naphthol, and the divalent ions of copper, nickel, and

iron. Other substances which are described as quenching certain fluorescent solutions are iodine, thiocyanate, bromide, ethanol, nitrobenzene, sodium stearate and oxygen (95).

The effect of conjugation on enhancing the fluorescent properties of hydroxylated molecules can be considered in connection with in vivo conjugation. Various theories of "detoxication"[1] have been proposed (101).

1. The chemical defense hypothesis, (Baumann, 1876); this theory considers that in vivo changes in foreign molecules result in either less toxicity or greater solubility.

2. The surface tension hypothesis, (Berczeller, 1917); this theory considers that in vivo changes in foreign molecules result in a solution with a surface tension nearer to that of pure water.

3. The increased acidity hypothesis, (Quick, 1932); this theory considers that in vivo changes in foreign molecules form stronger acids, which are more easily eliminated by the organism.

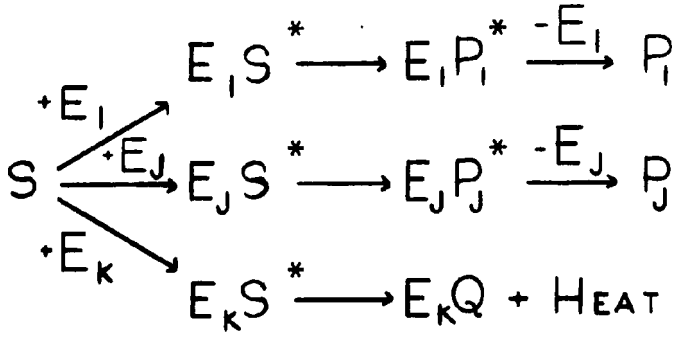
In addition to these proposals, one might consider the following hypothesis. If, as discussed in the Introduction, the existence of excited electronic states is of vital importance in maintaining the integrity of a living system, then a priori, the production of endogenous agents by the living system or the introduction of exogenous

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[1]The word "detoxication" is used to designate that group of chemical reactions which foreign organic compounds undergo in the animal body. These reactions are generally oxidations, reductions, or conjugations. Phenols are conjugated by ethereal sulfate formation, glucuronide formation, or methylation. Inorganic compounds, e.g. hydrogen cyanide, and endogenous compounds are often modified by similar reactions.

agents into the living system which cause enhancement or depression of these excited states would cause a change in the economy of the system, and the conjugation of these agents could diminish their effects upon such excited states.

Suppose (for concreteness) that a compound S is present in a cell and that S can undergo several available reactions (i, j, k, etc.), e.g. oxidation, reduction, conjugation, etc. These reactions will proceed via some enzyme-substrate, activated transition state, ES_i^* . Such an activated, transition state would possibly also involve an excited electronic state (102). If all these pathways were available, compound S would form products, P_i, P_j, etc. If, however, pathway k yielded a quencher, Q, this compound could deactivate the enzyme-substrate transition state as soon as it was formed on the enzyme surface. Pathway k now being blocked, the remainder of S has open to it pathways i and j through which eventually all of S would be shunted.



If S itself were a quencher, it would go by pathways which it did not quench and eventually form products which had poor quenching ability, since other pathways would become blocked as further quenchers were formed. In general, compounds would tend to be formed which do not greatly affect the electronic states essential for the maintenance of

the intact system. Thus the conjugation of some exogenous or endogenous phenols with glucuronic acid could make them poorer quenchers, less likely to distort the electronic patterns in the cell.

APPENDIX III. COMPOSITION OF CHROMATOGRAPHIC SPRAY REAGENTS

(a) Pauly's diazo spray for detecting phenols (103):

(1) Required solutions:

i. Solution #1: 900 mg. (a large excess) of sulfanilic acid are triturated in 9 ml. of concentrated HCl.

ii. Solution #2: 500 mg. of sodium nitrite are dissolved in 10 ml. of water.

iii. 4% Sodium carbonate (w/v).

(2) Procedure:

This reagent is prepared just prior to use with freshly prepared solutions. A small beaker containing $1\frac{1}{2}$ ml. of Solution #1 is placed in an ice-bath. Next $1\frac{1}{2}$ ml. of Solution #2 are added. After 5 minutes, 6 ml. more of Solution #2 are added. After another 5 minute interval, the diazotized sulfanilic acid solution is diluted with 41 ml. of water.

The paper chromatogram is sprayed with 4% sodium carbonate. It is dried and then sprayed with Pauly's diazo reagent and allowed to dry again. A red to pink color will develop at those spots containing a phenolic group.

(b) Ehrlich's spray for detecting aromatic amines (103):

(1) Required solution:

i. 250 Mg. of p-dimethylaminobenzaldehyde are dissolved in 50 ml. of ethanol containing $\frac{1}{2}$ ml. of concentrated HCl.

(2) Procedure:

Ehrlich's reagent is prepared just before use. The chromatogram is sprayed and dried at room temperature. Indole deriva-

tives give a red to blue color, while aromatic amines give yellow to orange colors.

(c) Ammoniacal silver nitrate spray for detecting reducing materials (104):

(1) Required solutions:

i. Solution #1: 850 mg. of silver nitrate are dissolved in 50 ml. of water.

ii. Solution #2: 17 ml. of ammonia water (a solution of 28-30% NH_3 in water) and 2 ml. of 2.5 N NaOH are mixed with 23 ml. of water.

(2) Procedure:

Equal amounts of Solutions #1 and #2 are mixed just prior to use. The chromatogram is sprayed and dried at 110° for exactly 3 minutes. A brown or black spot develops in the presence of reducing substances.

(d) Ninhydrin spray for detecting aliphatic amines (103):

(1) Required solution:

i. 250 Mg. of ninhydrin are dissolved in 100 ml. of acetone.

(2) Procedure:

The chromatogram is sprayed and dried at 60° for 30 minutes. A pink to purple color forms in the presence of aliphatic amines.

(e) Folin's spray for detecting phenols (105):

(1) Required solutions:

i. Folin's reagent (106): Transfer to a flask (capacity about 1 liter):

(a) 375 ml. of water,
(b) 50 g. of sodium tungstate,
(c) 10 g. of phosphomolybdic acid,
(d) 25 ml. of phosphoric acid (85%),
(e) 50 ml. of concentrated HCl.

Insert a funnel in the flask and partly close the opening with a watch-glass. Boil gently for two hours. A deep straw-yellow solution should be obtained. Dilute to 500 ml. and store in a ground-glass stoppered bottle, protected from dust and light.

ii. Sodium carbonate solution: Dissolve 53 g. of sodium carbonate in 500 ml. of water.

(2) Procedure:

Spray the chromatogram with Folin's reagent and allow the paper to dry at room temperature. Spray the dried chromatogram again with the sodium carbonate solution and allow the paper to dry at room temperature. A blue spot is formed by a phenolic compound.

APPENDIX IV. EVALUATION OF PROBABLE ERROR

Advantages of using the expression for probable error (designated "r") when presenting the results of an experimental analysis are that the data can be more easily summarized, and that, if the probable error of a method is known, it can be applied to individual analyses (where there may not be a group of values all measuring a particular fixed quantity). The formula which was used for calculating the probable error of a single analysis was the following (known as Bessel's formula) (107):

$$r = \pm A \sqrt{\frac{\sum d_i^2}{n - k}} \quad .$$

The symbol "d_i" represents the difference between the known value, x_s, (perhaps in grams per milliliter) in a standard solution analyzed, and the x_i determined, following analysis, from the best-fitting standard curve, $f_i(x,y)$, at the experimentally obtained value of y_i (perhaps in optical density units). That is, $d_i = x_s - x_i$. A series of standards analyzed simultaneously with x_i was used to obtain d_i, and the d_i from all such series was used in the summation $\sum d_i^2$.

The symbol "n" represents the number of determinations, i. e., the number of experimental values y_i obtained (including duplicates on the same aliquot where these were obtained).

The symbol "k" represents the number of constants (B, C...) necessary to define the standard curve fitting the plotted experimental points. A straight line was assumed in all our experiments, i. e., $x_i = By_i + C$, and the best-fitting straight line was drawn by visual

inspection. The value of k was always 2 in our calculations.

"A" is a constant, proposed by H. Jeffreys. This constant has a different value depending upon the number of determinations, n. The values used are given in the following table:

n	Jeffrey's Constant
2	1.000
3	0.816
4	0.766
5	0.740
6	0.728
7	0.718
10	0.703
values above 10	0.674.

The probable error of the sum of two independent determinations equals the square root of the sum of the squares of the probable errors for each determination (r_1, r_2), i. e.,

$$r = \pm \sqrt{r_1^2 + r_2^2} \quad .$$

This formula was used to calculate the probable errors of those determinations in which the analysis required that a correction, obtained using another analytical method, be introduced.

The interpretation applied to the probable error is that if the analysis is repeated under the same conditions with the same care, half of the results will fall within a range of $\pm r$, and that there is only one chance in a hundred of obtaining a random error exceeding $\pm 4r$. The probable error is not a definition of the limits of the "true" value. This value can be estimated by a comparison of values obtained using independent methods for which the magnitude of random errors is known.

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