STUDIES ON

3-HYDROXYANTHRANILIC ACID

OXIDASE

by

M.C.L. COX.

Thesis submitted for the degree of Master of Science
at the University of the Witwatersrand,
Johannesburg, South Africa.

DECLARATION

This is to certify that the work presented in this thesis is my own, and has not been presented previously for examination.

Signed .....

Date 19-6-67

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CONTENTS

Declaration	(i)
Acknowledgements	(ii)
Contents	(iii) - (iv)
List of Figures	(v) - (vi)
List of Tables	(vii)
INTRODUCTION	1 - 7
DISCUSSION	
(A) Synthesis of Substrates for 3-Hydroxyanthranilic acid Oxidase:	8 - 22
(i) 3-Hydroxyanthranilic acid-3-^{14}C	8 - 12
(ii) 4-Methyl-3-hydroxyanthranilic acid-3-^{14}C	13 - 22
(B) Observations on the Primary Oxidation Product of 3-Hydroxyanthranilic acid	23 - 31
(C) Studies on 3-Hydroxyanthranilic acid Oxidase	32 - 63
(i) Partial Purification of 3-Hydroxyanthranilic acid Oxidase	32 - 54
(a) Buffers	32 - 34
(b) Solubility and Stability of 3-Hydroxyanthranilic acid	34 - 37
(c) Enzyme Assay Systems	37 - 43
(d) Purification of 3-Hydroxyanthranilic acid Oxidase	44 - 54
(ii) Some Properties of 3-Hydroxyanthranilic acid Oxidase	55 - 63
EXPERIMENTAL PART	64 - 103
Instruments	64
Analyses	65
(I) Synthesis of 3-Hydroxyanthranilic acid-3-^{14}C	66 - 69

- Continued on next page -

CONTENTS (Continued)

(II)	Attempted Synthesis of 4-Methyl-3-hydroxy-anthranilic acid-3-^{14}C	70 - 79
(III)	Attempted Isolation of the 2,4-Dinitrophenyl-hydrazone of the Primary Oxidation Product of 3-Hydroxyanthranilic acid	79 - 81
(IV)	Purification of 3-Hydroxyanthranilic acid Oxidase	82 - 99
(V)	Some Properties of 3-Hydroxyanthranilic acid Oxidase	100 - 103
SUMMARY		104
BIBLIOGRAPHY		105 - 110

LIST OF FIGURES

FIGURE NO.	PAGE NO.	TITLE
1	8	The Tryptophan-Nicotinic acid Pathway
2	12	Synthesis of 3-Hydroxyanthranilic acid-3-^{14}C
3	16	Synthesis of 4-Methyl-3-hydroxyanthranilic acid-3-^{14}C
4	21	Ultra-violet Spectra of Compounds A, B and C
5	22	Infra-red Spectra of Compounds A, B and C
6	26	Acid Degradation of 2-Amino-3-acroleylfumaric acid
7	30	Ultra-violet Spectra of Compound X, 2,4-Dinitrophenylhydrazine, and the 2,4-Dinitrophenylhydrazone of acetone
8	31	Infra-red Spectrum of Compound X
9	35	Stability of $FeSO_4$ Solutions at Different pH
10	36	Absorption Spectra of Tris/Maleate Buffers
11	39	Absorption Spectrum of Cinnabarinic acid
12	40	Standard Curve
13	43	Rate of Formation of Cinnabarinic acid
14	43	Comparison of the Sensitivity of the Two Assay Systems
15	53	Absorption Spectra of the Enzyme Extracts at Different Stages of Purification
16	54	Ammonium Sulphate Fractionation
17	54	Alumina Gel Adsorption

- Continued on next page -

LIST OF FIGURES (Continued)

FIGURE NO.	PAGE NO.	TITLE
18	60	Reaction Spectra with 3-Hydroxyanthranilic acid
19	61	Reaction Spectra with 4-Methyl-3-hydroxy-anthranilic acid
20	62	Ultra-violet Spectra of Pyridine Carboxylic acids
21	63	Lineweaver and Burk Plots
22	85	Effect of the Concentration of $NaIO_3$ on the Formation of Cinnabarinic acid
23	92	Stability of the Acetone Powder

LIST OF TABLES

TABLE NO.	PAGE NO.	TITLE
1	28	Ultra-violet and Visible Spectral Characteristics of the 2,4-Dinitrophenylhydrazone obtained by Wiss and Bettendorf, and Compound X.
2	45 - 47	Published Purifications of 3-Hydroxyanthranilic acid Oxidase
3	59	Michaelis' Constants for 3-Hydroxyanthranilic acid
4	75	Characterization of the Picrate and Hydrochloride of 2-Ethoxy-m-toluidine
5	93	Acetone Fractionation
6	94	Adsorption of Nucleic acids with Charcoal
7	95	The Effect of Heating at Different Temperatures on Enzyme Activity
8	96	The Effect of Duration of Heating on Enzyme Activity
9	99	Purification Procedures Adopted by the Present Author
10	102	Comparison of 3-Hydroxyanthranilic acid and 4-Methyl-3-hydroxyanthranilic acid as substrates for 3-Hydroxyanthranilic acid oxidase

INTRODUCTION

Goldberg and Tanner (1922) reported that under normal dietary conditions the rat does not require nicotinic acid (VII, Fig. 1) to support growth. Krehl et al. (1945) showed that tryptophan (I, Fig. 1) is the factor that substitutes for the nicotinic acid, and it is now well established that tryptophan can be converted into nicotinic acid by a pathway involving numerous intermediates (Fig. 1). 3-Hydroxyanthranilic acid (II,Fig. 1) was first implicated as an intermediate in this pathway in Neurospora crassa by Mitchell and Nyc (1948) and Bonner (1948).

The effect of 3-hydroxyanthranilic acid on the excretion of N'-methylnicotinamide (VIII, Fig. 1) by the rat led Albert et al. (1948) to conclude that nicotinic acid synthesis in mammals also proceeds via 3-hydroxyanthranilic acid. Proof of this relationship was provided by the studies of Hankes and Urivetsky (1954) and Hankes and Henderson (1957) on the metabolism of intraperitoneally administered ^{14}C-carboxyl-labelled 3-hydroxyanthranilic acid in the rat. The latter authors observed that both N'-methylnicotinamide and quinolinic acid (IV, Fig. 1) isolated from the urine were labelled. Henderson and Hirsch (1949) and Henderson (1949) had already demonstrated that an increase in the excretion of quinolinic acid occurs after the feeding of 3-hydroxyanthranilic acid to the rat, while Schweigert (1949), Schweigert and Marquette (1949), Henderson and Ramasarma (1949), Bokman and Schweigert (1950), and Moline et al. (1959), reported that 3-hydroxy-anthranilic acid is almost quantitatively converted to quinolinic acid by rat liver slices and homogenates. Neither nicotinic acid nor N'-methylnicotinamide was produced by this

enzyme system in significant amounts and it was concluded that the primary product of the system was quinolinic acid.

A survey of various tissues of the rat, and of beef and pig liver and kidney, for the presence of the enzyme system that converts 3-hydroxyanthranilic acid to quinolinic acid was carried out by Priest et al. (1951), who found that some or all of the 3-hydroxyanthranilic acid was converted to quinolinic acid in rat, beef, and pig liver and kidney, but that rat spleen, muscle, brain, pancreas, and testis did not contain the enzyme system or contained it only in very small quantities. Ginoulhiac et al. (1961) showed that although the serum of normal rats did not appear to contain any of the quinolinic acid-forming enzyme, the latter was clearly demonstrable in severe cases of liver damage.

Crude extracts of this enzyme system (first termed 3-hydroxyanthranilase by Bokman and Schweigert - 1951) were shown to oxidize 3-hydroxyanthranilic acid apparently to an unstable intermediate (III, Fig. 1) (variously called 2-amino-3-acroleylfumaric acid; α-amino-β-carboxymuconic-ε-semialdehyde; 1-amino-4-formyl-butadiene-1,3-dicarboxylic acid) and that spontaneous cyclization of the latter resulted in the formation of quinolinic acid (Bokman and Schweigert - 1951; Long et al. - 1954; Miyake et al. - 1954; Wiss and Bettendorf - 1957). Despite an exhaustive search, Nishizuka and Hayaishi (1963a) were unable to find an enzyme that catalyzed the cyclization of the postulated intermediate to quinolinic acid. In the mammalian liver preparations studied by Mehler (1956) and Mehler and May (1956), the

only enzyme found to react with 2-amino-3-acroleylfumaric acid was picolinic carboxylase which catalyzed the formation of picolinic acid (X, Fig. 1), probably via an unstable intermediate, α-aminomuconic-ε-semialdehyde (IX, Fig. 1) (Gholson et al. - 1962; Nishizuka et al. - 1965; Ichiyama et al. - 1965; Lan and Gholson - 1965). There is no evidence at present for the direct conversion of 2-amino-3-acroleylfumaric acid to nicotinic acid. Instead nicotinic acid ribonucleotide (VI, Fig. 1) is formed from quinolinic acid ribonucleotide (V, Fig. 1) in the presence of phosphoribosylpyrophosphate (PRPP) and a soluble enzyme system derived from mammalian liver or kidney. Free nicotinic acid is apparently not formed in this pathway (Nishizuka and Hayaishi - 1963a).

Mehler and co-workers (1958a,b; 1964) showed that liver extracts from diabetic rats contain normal amounts of 3-hydroxyanthranilic acid oxidase and the enzymes needed to convert quinolinic acid to nicotinic acid ribonucleotide but that greatly increased levels of picolinic carboxylase are present. The decreased capacity of diabetic animals to synthesize nicotinic acid is therefore attributed to competition between picolinic carboxylase and the spontaneous reaction in which quinolinic acid is formed as a precursor of nicotinic acid ribonucleotide. Da Silva et al. (1952) reported that the cat is apparently unable to convert tryptophan to nicotinic acid. However, Nishizuka and Hayaishi (1963a,b) have shown that although the activity leading to nicotinic acid ribonucleotide synthesis in cat liver is only one-fifth that of rat liver, the picolinic carboxylase activity is twenty to fifty times greater in the cat. This evidence is consistent with the

observations of Da Silva et al. (loc. cit.) since the higher levels of picolinic carboxylase would tend to divert the majority of the 2-amino-3-acroleylfumaric acid from quinolinic acid biosynthesis.

It has been claimed that 3,4-dihydroxyanthranilic acid is active as a nicotinic acid precursor in beef, pig, and horse liver slices (Makino et al. - 1951). Experiments by Henderson and by Schweigert (quoted in Bokman and Schweigert - 1951) however, showed that this compound is inactive as a substrate for quinolinic acid formation in rat liver preparations. Attempts made in this laboratory to repeat Makino's work (loc. cit.) have not yet been completed.

Recently it has been shown by Vaidyanathan and his associates that 3-hydroxyanthranilic acid can be converted to cinnabarinic acid (2-amino-phenoxazine-3-one-1,8-dicarboxylic acid) (XI, Fig. 1) by an enzyme present in the nuclear fraction of rat liver (1964, 1965, 1966a) and by the leaves of Tencoma stans (1966b). This latter reaction is an enzyme analoque of the well known in vitro oxidation of o-aminophenols to phenoxazinones (O. Fischer and Jonas - 1894). Cinnabarinic acid has been isolated together with cinnabarin from the fungus Polystictus sanquineus (Gripenberg - 1958). Morgan and Weimorts (1964) have claimed that acetone powder extracts of the livers of several poikilothermic animals (such as fish, turtles, and alligators) convert 3-hydroxyanthranilic acid to cinnabarinic acid and do not form any of the pyridine carboxylic acids, indicating a lack of 3-hydroxyanthranilic acid oxidase. However, the work of Lan and Gholson (1965) indicates that the previous

authors were most probably working with preparations in which 3-hydroxyanthranilic acid oxidase had been destroyed. Cell-free extracts prepared from Streptomyces antibioticus also catalyze an oxidative condensation of 3-hydroxyanthranilic acid and certain other o-aminophenols to form phenoxazinones. With 4-methyl-3-hydroxyanthranilic acid (XII, Fig. 1), the actinomycin chromophore, actinocin (2-amino-4,5-dimethyl-phenoxazine-3-one-1,8-dicarboxylic acid), (XIII, Fig. 1) is synthesized (Weissbach and Katz - 1961; Katz and Weissbach - 1962; Weissbach et al. - 1965).

3-Hydroxyanthranilic acid oxidase has been classified as an oxygen transferase (Mason - 1957a,b) since both atoms of $^{18}O_2$ are incorporated into the product during the enzyme reaction (Hayaishi et al. - 1956). The mechanism of action of the oxygen transferases is largely unknown but most of these enzymes, including 3-hydroxyanthranilic acid oxidase (Stevens and Henderson - 1959), require ferrous ions and sulphydryl groups for activity (Mason - 1957b; Mehler - 1962; Crandall - 1965). 3-Hydroxyanthranilic acid oxidase is unstable in the absence of external reducing agents and it has been suggested that autoxidation of enzyme-bound ferrous ions leads to reaction inactivation (Mitchell et al. - 1963). Activity is regained rapidly by treating the aged or iron-free enzyme with ferrous sulphate at low pH (Stevens and Henderson - 1959; Decker et al. - 1961; Mitchell et al. - 1963; Ogasawara et al. - 1966), and the authors suggest that the low pH might cause conformational changes in the enzyme molecule which allow for rapid binding of the ferrous iron.

A mechanism of action for the oxygen transferases

in which the function of the enzyme-bound ferrous iron is to bind and activate molecular oxygen rather than to chelate the substrate has been proposed (Mason - 1957b; Hayaishi - 1963), and electron spin resonance studies on metapyrocatechase have been tentatively interpreted as showing the presence of the perferryl ion in this oxygen transferase (Hayaishi - 1965). It has also been suggested that sulphydryl groups in homogentisic acid oxidase (another oxygen transferase) play a part in substrate binding (Tokuyama - 1959), but this has not been substantiated by the work of Flamm and Crandall (1962). Orgel (1956) has shown that the preferred ligands for the ferrous ion are the amino and the sulphydryl groups, so that it is possible that the latter could play a part in the binding of the ferrous ion and not the substrate. Norman and Lindsay-Smith (1965) have outlined a chemical analogy for the oxidation of phenols by molecular oxygen based on established chemical reactions.

The present author's interest has centred on reactions (II) to (IV) (Fig. 1). The importance of these reactions in the biosynthesis of the nicotine adenine nucleotides and in the elucidation of the mechanism of the conversion of benzenoid structures to pyridine-containing compounds is obvious. The intermediate (III, Fig. 1) has never been isolated in pure form, although Wiss and Bettendorf (1957) obtained what appeared to be the 2,4-dinitrophenylhydrazone of (III). There have been several reports (Stevens and Henderson - 1959; Decker *et al.* - 1961; Iaccarino *et al.* - 1961; di Prisco *et al.* - 1961; Vescia and di Prisco - 1962; Ichiyama *et al.* - 1965; Ogasawara *et al.* - 1966) of the purification of 3-hydroxyanthranilic acid oxidase but relatively little work has

been done on the physical and chemical properties of the enzyme.

The work to be described in this thesis may be outlined as follows:-

(A) The preparation of substrates for 3-hydroxyanthranilic acid oxidase:

(i) 3-hydroxyanthranilic acid,

(ii) 4-methyl-3-hydroxyanthranilic acid.

(B) Observations on the primary oxidation product (III,Fig.1) of 3-hydroxyanthranilic acid.

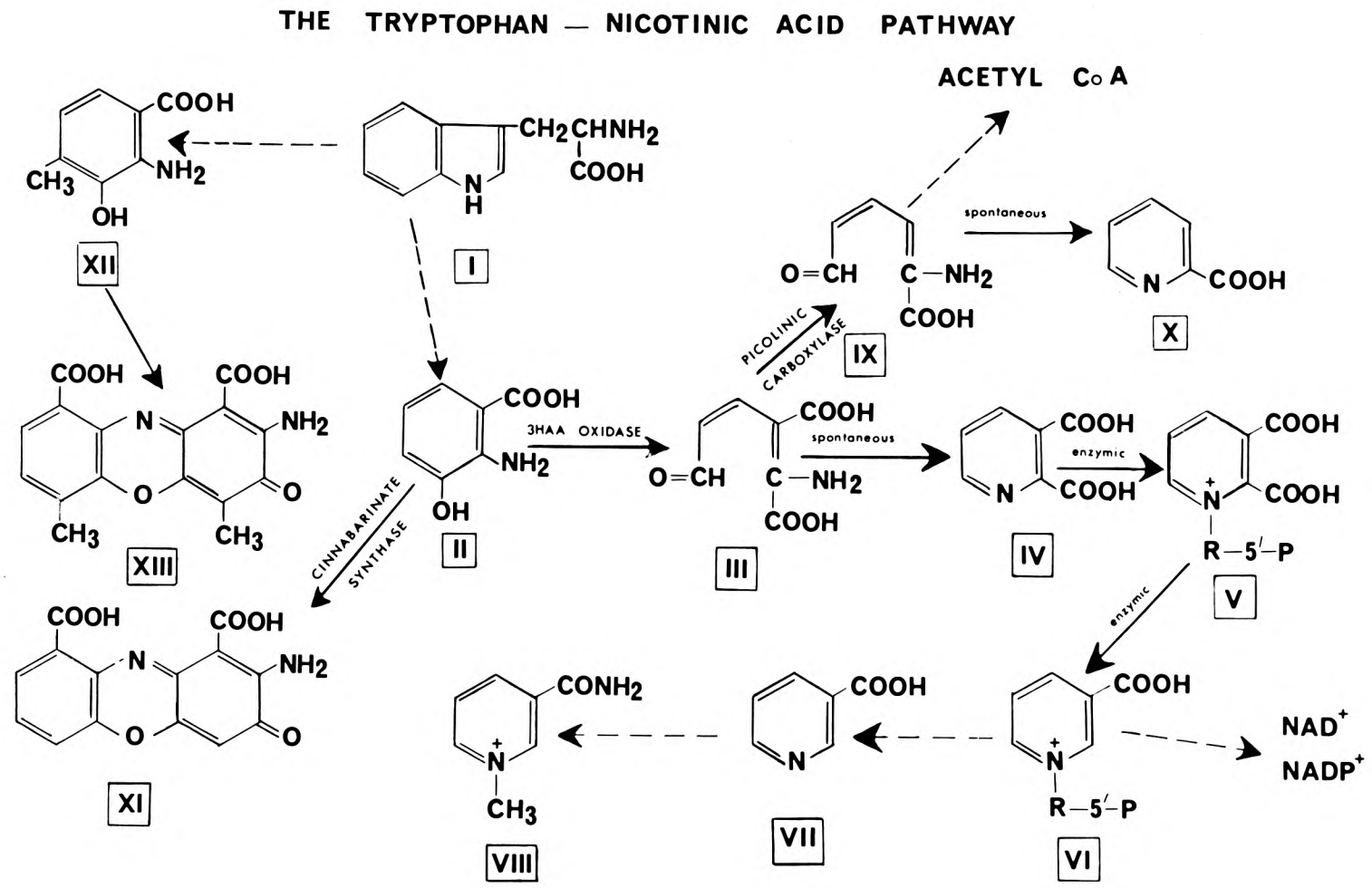
(C) Studies on 3-hydroxyanthranilic acid oxidase:

(i) Partial purification,

(ii) Determination of the enzyme reaction spectra with different substrates,

(iii) Determination of Michaelis' and Inhibitor constants.

FIGURE 1


THE TRYPTOPHAN — NICOTINIC ACID PATHWAY
ACETYL CoA
COOH
CH3
NH2
OH
XII
CH2CHNH2
COOH
N
H
I
O=CH
C—NH2
COOH
spontaneous
N
COOH
X
PICOLINIC CARBOXYLASE
IX
COOH
NH2
OH
II
3HAA OXIDASE
O=CH
C—NH2
COOH
III
spontaneous
COOH
N
COOH
IV
enzymic
COOH
N
COOH
R—5′—P
V
enzymic
COOH
N
R—5′—P
VI
NAD+
NADP+
COOH
N
VII
CONH2
N
CH3
VIII
CINNABARINATE SYNTHASE
COOH
COOH
N
NH2
O
O
CH3
CH3
XIII
COOH
COOH
N
NH2
O
O
XI

D I S C U S S I O N

(A) THE SYNTHESIS OF SUBSTRATES FOR 3-HYDROXYANTHRANILIC ACID OXIDASE

(i) 3-HYDROXYANTHRANILIC ACID

3-Hydroxyanthranilic acid was first synthesized by Nyc and Mitchell (1948). Radioactive 3-hydroxyanthranilic acid has been synthesized by a number of workers:

(i) Ciereszko and Hankes (1954) synthesized carboxyl-labelled 3-hydroxyanthranilic acid and this was utilised in metabolic studies by Hankes and Urivetsky (1954) and Hankes and Henderson (1957) which confirmed that 3-hydroxyanthranilic acid is an intermediate in nicotinic acid biosynthesis in mammals.

(ii) Mehler and May (1956) also synthesized carboxyl-labelled 3-hydroxyanthranilic acid, but by a different method and in better yield than Ciereszko and Hankes (*loc. cit.*). The labelled compound was used in the characterization of picolinic carboxylase, discovered by Mehler (1956).

(iii) May *et al.* (1962) synthesized 3-hydroxyanthranilic acid-1,2-di-^{14}C from 1,3 butadiene and ^{14}C-labelled maleic anhydride, and Mehler *et al.* (1964) utilized the same basic scheme to synthesize 3-hydroxyanthranilic acid-3,6-di-^{14}C which was utilized in metabolic studies to be described (*vide infra*).

(iv) 3-Hydroxyanthranilic acid-3-^{14}C has been synthesized by Moline *et al.* (1959) in 0.03% yield and by Diamond and Levy (1965) in 12.2% yield, in both cases from phenol-1-^{14}C.

The synthesis of 3-hydroxyanthranilic acid-3-^{14}C

(Fig. 2) described in this work is based on the method of Diamond and Levy (loc. cit.); only two modifications were introduced. First, dilute sulphuric acid was added to the reduction product of o-nitrophenetole (III, Fig. 2), and the o-phenetidine isolated as the sulphate (Stage 3, p. 67). Secondly, the Skraup synthesis was modified in the hope of obtaining a better yield: iodine was replaced by sodium-m-nitrobenzene sulphonate as the oxidizing agent in this reaction (Long and Schofield - 1953). However, the present author only obtained a yield of ca. 30% as against 45% (Stage 4, p. 68).

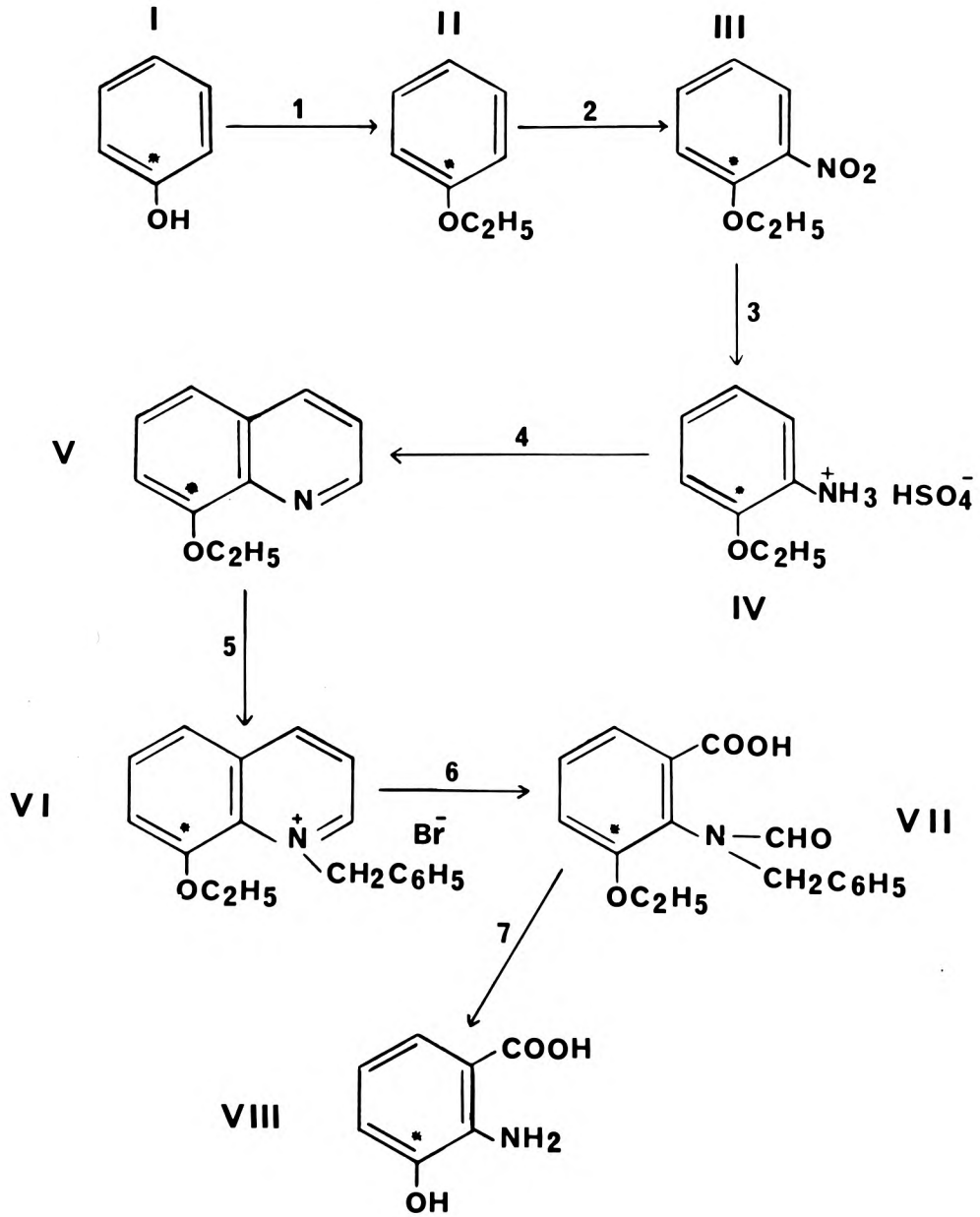
Ethylation of phenol-1-^{14}C (I, Fig. 2) proceeded smoothly and the subsequent nitration using Francis' (1906) method gave o-nitrophenetole-1-^{14}C exclusively (III, Fig. 2). Catalytic reduction (platinum oxide catalyst) gave o-phenetidine-1-^{14}C, which was isolated as the sulphate (IV, Fig. 2). Up to this point all the yields were good, of the order of 70% or above, but in the Skraup synthesis of 8-ethoxyquinoline-8-^{14}C (V, Fig. 2) the yield was only of the order of 30%. By refluxing with benzyl bromide the quinoline was smoothly converted into N-benzyl-8-ethoxyquinoline-8-^{14}C bromide (VI, Fig. 2) and the latter was oxidized with calcium permanganate to the corresponding anthranilic acid (VII, Fig. 2). Both these reactions gave good yields. Finally N-benzyl-3-ethoxy-N-formyl-anthranilic acid-3-^{14}C (VII, Fig. 2) could be simultaneously hydrolyzed and dealkylated by phosphorus and hydriodic acid to give 3-hydroxyanthranilic acid-3-^{14}C (VIII, Fig. 2) in over 90% yield.

This synthesis of 3-hydroxyanthranilic acid-

3-^{14}C was devised in order to study the exact position of cleavage of the molecule in the conversion of 3-hydroxyanthranilic acid to quinolinic acid by determining the position change of carbon-3 in the benzene nucleus of 3-hydroxyanthranilic acid. If, as postulated, the conversion of 3-hydroxyanthranilic acid via 2-amino-3-acroleylfumaric acid to quinolinic acid occurs by cleavage between carbon atoms 3 and 4 of 3-hydroxyanthranilic acid, then carbon-3 would form the α-carbon atom of quinolinic acid (Fig. 1). Utilising an enzyme preparation which did not contain any picolinic carboxylase or phosphoribosylpyrophosphate (PRPP), labelled quinolinic acid would accumulate. Eventually, essentially all the 3-hydroxyanthranilic acid-3-^{14}C would be converted into labelled quinolinic acid. Then in the presence of the soluble liver enzyme system described by Nishizuka and Hayaishi (1963), and on the addition of PRPP, the quinolinic acid would be converted into nicotinic acid mononucleotide with the liberation of radioactive carbon dioxide which could be collected and analysed. Mehler (1964) has shown that this is indeed what occurs and it is now generally accepted that 3-hydroxyanthranilic acid oxidase cleaves 3-hydroxyanthranilic acid between carbon atoms 3 and 4.

FIGURE 2

SYNTHESIS OF 3-HYDROXYANTHRANILIC ACID-3-^{14}C



(ii) ATTEMPTED SYNTHESIS OF 4-METHYL-3-HYDROXYANTHRANILIC ACID-3-^{14}C.

Non-radioactive 4-methyl-3-hydroxyanthranilic acid has been synthesized by Brockman and Muxfeldt (1958) and utilized by Weissbach and co-workers (1961, 1962, 1965) in their studies of the synthesis of actinocin. However, Brockman and Muxfeldt's scheme is completely unsuitable for a radioactive synthesis and consequently the present author has attempted to synthesize 4-methyl-3-hydroxyanthranilic acid-3-^{14}C (X, Fig. 3) from phenol-1-^{14}C (I, Fig. 3) according to the scheme shown in Fig. 3. Preliminary experiments were carried out using non-radioactive phenol.

(a) Synthesis of Salicylaldehyde (II, Fig. 3) from Phenol.

The classical Reimer-Tiemann (1876) synthesis - as described by Gattermann (1943) - and several modifications of this technique were carried out. Gattermann (loc. cit.) claims that a yield of 30% can be obtained provided that no sodium phenoxide crystallizes out of solution during the addition of the chloroform. It is possible to prevent the crystallization by maintaining the temperature at 65 - 70° throughout the addition of the chloroform and by gently swirling the reaction mixture at the beginning of the reaction. Even relatively gentle shaking during the early stages of the reaction invariably results in sodium phenoxide crystallizing out of solution. However, even when no crystallization occurred, the best yield obtained by the present author was ca. 18%. Several other authors (cf. Armstrong and Richardson - 1933; Brady and Jakabovits - 1950) have found that yields of the order of 10%

are characteristic of the Reimer-Tiemann reaction rather than 30%. In fact Brady and Jakabovits (loc. cit.) state: "The yields of aldehyde in the Reimer-Tiemann reaction were of the usual low order and the results would not be credible were they not so remarkable and repeatable within reasonable limits."

A possible cause for the low yields obtained in the Reimer-Tiemann reaction is the non-homogeneity of the reaction mixture. Armstrong and Richardson (loc. cit.) substituted trichloroacetic acid for chloroform but found no improvement in yield. When the present author repeated this method the very small amount of crude product obtained decomposed during distillation. Sten and Ray (1932) claimed that the yield in the Reimer-Tiemann reaction was improved by mixing the chloroform with absolute alcohol (1:3) and adding a little pyridine to the reaction mixture. Using this technique the authors obtained yields of the order of 30%. However, the present author only obtained a yield of ca. 15% by this method, and a large amount of resinous material appeared to be formed. Sten and Ray (loc. cit.) also claimed that aqueous pyridine could entirely replace the alkali hydroxide normally used in the Reimer-Tiemann reaction, the yield in this case being of of the order of 10%. In addition, p-hydroxybenzaldehyde was not produced by the latter method.

Reimer and Tiemann (1876) state that in their synthesis of hydroxyaldehydes the proportion of ortho- and para- derivatives is unaffected by changing the alkali from sodium to potassium hydroxide. The present author, however, found that the yield of salicylaldehyde obtained using potassium

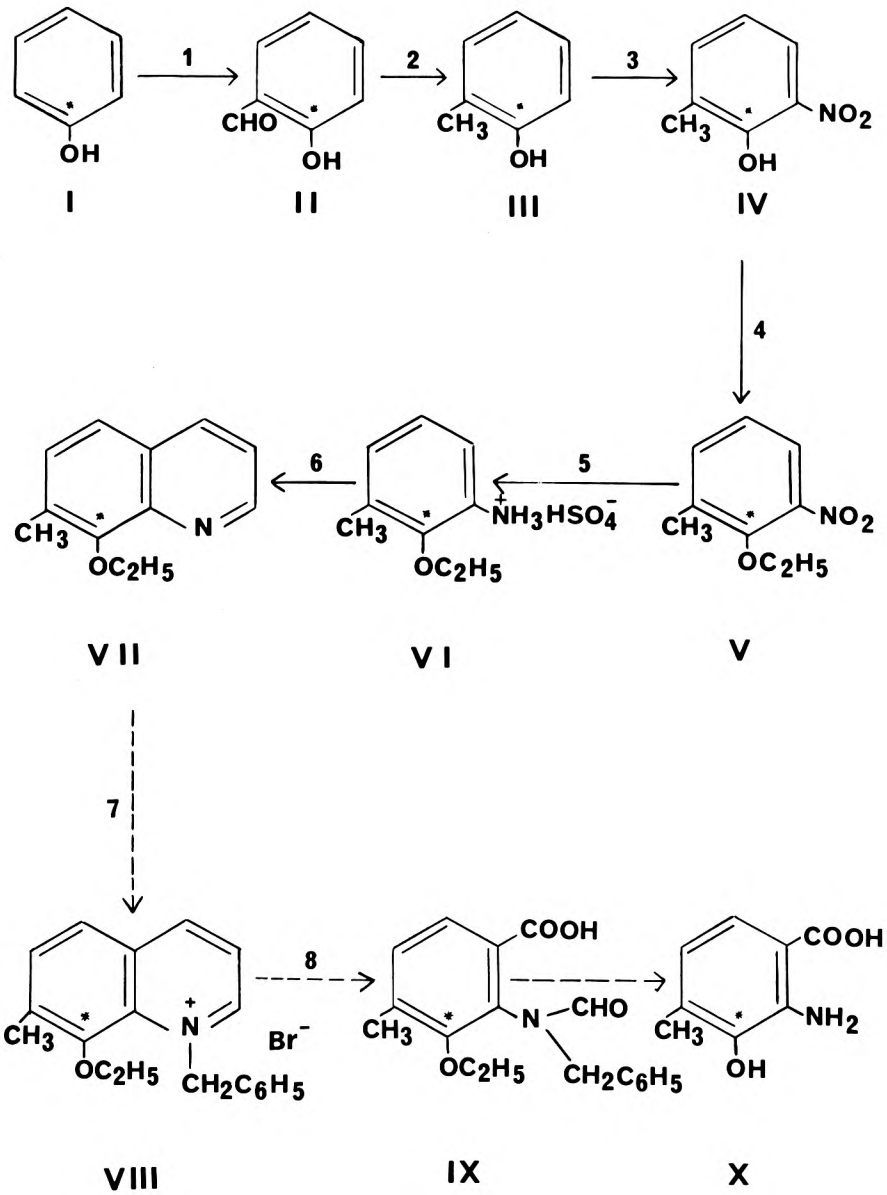
hydroxide was only 6.8% whereas using sodium hydroxide a yield of 17.9% was obtained. Brady and Jakabovits (1950) have re-investigated the effect of change of alkali in the Reimer-Tiemann reaction. In 15-N alkali the ratio of o-hydroxybenzaldehyde (salicylaldehyde) to p-hydroxybenzaldehyde was NaOH (2.08), KOH (1.24), CsOH (0.98), and methyltriethyl ammonium hydroxide (0.52), while in 2-N alkali the differences virtually disappeared, the ratio being about 0.90. These observations provide an explanation of the decreased yield using the method of Gattermann (25-N alkali) when KOH (6.8%) is substituted for NaOH (17.9%), especially when one considers that in 25-N alkali the ortho- to para- ratios may be even greater than in 15-N alkali. It appears that LiOH, at a concentration of 25-N, may well be the ideal alkali to use in the Reimer-Tiemann reaction. Unfortunately, however, lithium phenoxide is very insoluble and persistently crystallizes out of solution.

(b) Synthesis of o-Cresol (III, Fig. 3) from Salicylaldehyde

Clemmensen reduction of salicylaldehyde by the Martin (1942) procedure was tried several times, but the best yield obtained was 11.3%. The Wolff-Kishner reduction provided an alternative pathway for the synthesis of o-cresol from salicylaldehyde and the Huang-Minlon (1946) modification of this reaction was found to be suitable. Using NaOH and 90% hydrazine hydrate in diethylene glycol, o-cresol was obtained in 60% yield.

FIGURE 3

SYNTHESIS OF 4-METHYL-3-HYDROXYANTHRANILIC ACID-3-^{14}C



(c) Synthesis of 2-Ethoxy-*m*-toluidine sulphate (VI, Fig. 3) from *o*-Cresol.

The method of nitration described by Gibson (1925) produces a mixture of 4-, 4,6- and 6-nitro-*o*-cresol. The 4,6- and 6- isomers can be separated by fractional steam distillation; 4-nitro-*o*-cresol is not steam volatile. Using ethyl-*p*-toluene sulphonate the ethylation of 6-nitro-*o*-cresol (IV, Fig. 3) proceeds readily and with *ca.* 67% yield. In addition, the unchanged 6-nitro-*o*-cresol can be recovered, and the yield of 2-methyl-6-nitrophenetole (V, Fig. 3) taking this recovery into account is 88%. Catalytic reduction of 2-methyl-6-nitrophenetole (platinum oxide catalyst) gave 2-ethoxy-*m*-toluidine, which was isolated as the sulphate (VI, Fig. 3) in over 90% yield. Thus the synthesis up to this stage had been successful and the starting material used in the Skraup synthesis of 7-methyl-8-ethoxyquinoline (VII, Fig. 3) was definitely 2-ethoxy-*m*-toluidine sulphate.

(d) Synthesis of 7-Methyl-8-ethoxyquinoline (VII, Fig. 3)

Using iodine as the oxidizing agent in the Skraup synthesis 7-methyl-8-ethoxyquinoline was obtained from 2-ethoxy-*m*-toluidine sulphate, but only in poor yield (*ca.* 30%). The reaction solution should have been diazotized to remove any unchanged amine but this step was omitted because it proved impossible to steam distil the diazotized solution (due to persistent foaming) or even to extract it with ether (due to the formation of a stable emulsion). On reflection, the best method of working up the solution after the Skraup reaction

would probably be as follows:

(i) render it alkaline, and steam distil the alkaline solution,

(ii) acidify the steam distillate and diazotize with $NaNo_2$,

(iii) render the diazotized solution alkaline and steam distil once again.

This procedure, though laborious, would remove any unchanged amine and would probably prevent foaming because of the removal of any by-products of the Skraup synthesis before diazotization is carried out.

A picrate prepared from the product of the Skraup reaction had a melting point of 152 - 156°. The picrate of an authentic sample of 7-methyl-8-ethoxyquinoline (characterized by elemental analysis) prepared by using sodium-m-nitrobenzene sulphonate as the oxidizing agent in the Skraup synthesis had a melting point of 157 - 158.5° (Diamond - unpublished results). It thus seems probable that the present author did, in fact, prepare 7-methyl-8-ethoxyquinoline, and that the latter was free from unchanged 2-ethoxy-m-toluidine. The poor yields in the Skraup reaction obtained by the present author were explained when a compound was isolated from the residue left behind after steam distillation (p. 76) which elemental analysis, and comparison with an authentic sample prepared by a different route (Levy - unpublished results), showed to be 7-methyl-8-hydroxyquinoline. De-ethylation in the Skraup reaction had obviously occurred. It is interesting to note that while 7-methyl-8-ethoxyquinoline and quinoline itself are steam volatile, 8-ethoxyquinoline is not.

(e) Attempted synthesis of N-Benzyl-4-methyl-8-ethoxyquinoline bromide (VIII, Fig. 3)

By analogy with the synthesis of N-benzyl-8-ethoxyquinoline bromide it was thought that by treating 7-methyl-8-ethoxyquinoline, dissolved in dry benzene, with benzyl bromide the corresponding benzobromide would be easily formed. This proved not to be the case and Diamond (unpublished results), using this method, obtained a series of compounds all with relatively sharp, but different, melting points. Consequently the present author decided to modify the procedure by omitting the benzene and simply adding the benzyl bromide directly to the quinoline. Three such syntheses were carried out and once again a series of compounds was obtained, each with a separate melting point (p. 77). None of the compounds obtained by the present author corresponded with those obtained by Diamond. The three compounds all had the same R_F (=0.73) when run in a mixture of butanol:dioxan:water (6:3:1) (p. 77). Ultra-violet spectrophotometry once again showed the compounds to be very similar; each has an absorption maximum in alcohol at 314 mu (Fig. 4). The infra-red spectra of the three compounds (Fig. 5) are also very similar, although not identical. Elemental analysis of one of the compounds (Compound A; p. 78) showed that this compound, at least, is not N-benzyl-4-methyl-8-ethoxyquinoline bromide.

Attempts to oxidize the three compounds to N-benzyl-3-ethoxy-N-formyl-4-methylanthranilic acid (IX, Fig. 3) were completely unsuccessful. At the present time these three compounds, and also those prepared by Diamond (loc. cit.) remain an enigma. To add to the confusion Levy and Diamond

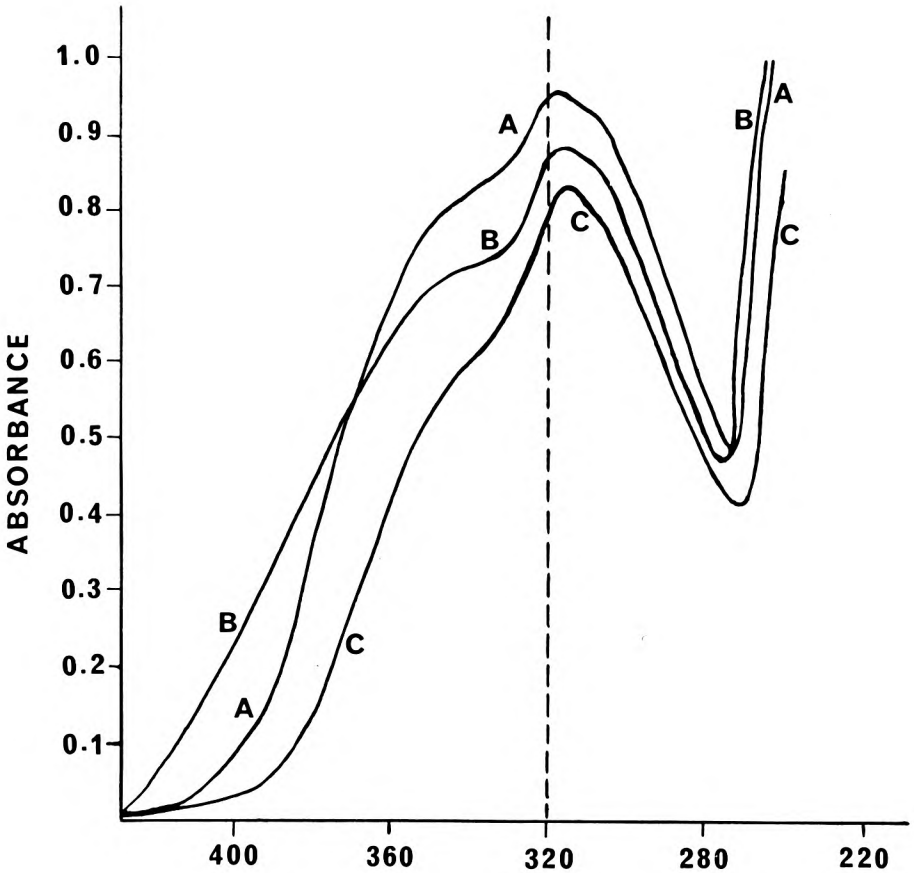
(unpublished results) have managed to synthesize N-benzyl-4-methyl-8-ethoxyquinoline bromide from 7-methyl-8-ethoxyquinoline using conditions which appeared to be identical with those used by the present author. Oxidation of this sample of benzobromide with calcium permanganate gave the corresponding anthranilic acid (IX, Fig. 3) and simultaneous hydrolysis and dealkylation of the latter with phosphorus and hydriodic acid gave 4-methyl-3-hydroxyanthranilic acid (X, Fig. 3). A further anomaly is that the 4-methyl-3-hydroxyanthranilic acid prepared by the method of Brockman and Muxfeldt (1958) (repeated by P.R. Levy) has a different melting point to that prepared via the quinoline (vide supra). However the ultra-violet and infra-red spectra of the two samples are identical in every respect.

It should be noted that the 4-methyl-3-hydroxy-anthranilic acid used in the kinetic studies of 3-hydroxy-anthranilic acid oxidase was prepared by E.M. Diamond by essentially the same pathway as shown in Fig. 3, i.e., via the quinoline.

FIGURE 4

ULTRA-VIOLET SPECTRA OF COMPOUNDS

A, B AND C

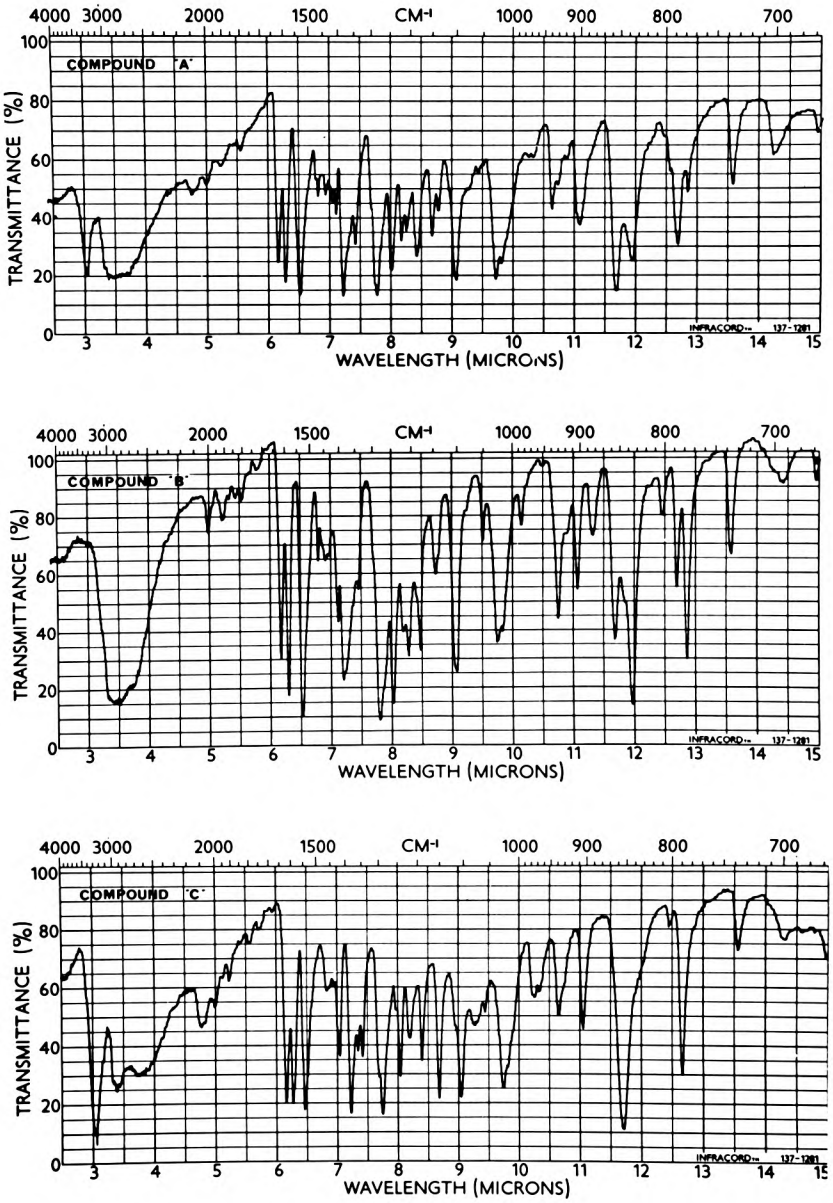


WAVELENGTH IN MILLIMICRONS

FIGURE 5

INFRA-RED SPECTRA OF COMPOUNDS

A, B AND C



(B) OBSERVATIONS ON THE PRIMARY OXIDATION PRODUCT OF 3-HYDROXYANTHRANILIC ACID

In the presence of 3-hydroxyanthranilic acid oxidase, 3-hydroxyanthranilic acid is converted to an unstable intermediate characterized by an intense absorption band at 360 mμ (Bokman and Schweigert - 1951). Miyake *et al.* (1954) proposed a quinonoid-type structure for the intermediate, but Wiss and Bettendorf (1957) and Wiss and Weber (1960) have presented evidence that it is an aliphatic compound, 2-amino-3-acroleylfumaric acid (III, Fig. 1). The ultraviolet spectrum of the intermediate contains two peaks, one at 275 mμ (molar absorption coefficient about 6,000) and one at 360 mμ (molar absorption coefficient about 47,000) (Wiss and Weber - *loc. cit.*).

Even if a quantitative conversion of 3-hydroxyanthranilic acid to the intermediate is assumed, it is difficult to understand how an accurate estimation of the molar absorption coefficients can be made because:

(i) the intermediate is being spontaneously converted into quinolinic acid, although Wiss and co-workers (*loc. cit.*) have tried to prevent this by working at 0°;

(ii) If any picolinic carboxylase is present in the crude enzyme extracts used to convert the 3-hydroxyanthranilic acid into the intermediate, both α-aminomuconic-ϵ-semialdehyde (IX, Fig. 1) and picolinic acid (X, Fig. 1) will almost certainly be formed;

(iii) The structure of the intermediate is by no means definitely established.

These points are emphasized by the fact that Mehler (1955)

independently estimated the molar absorption coefficient at 360 mμ to be ca. 30,000.

Although the primary oxidation product of 3-hydroxyanthranilic acid has not been isolated, there are several lines of evidence which indicate that it is 2-amino-3-acroleylfumaric acid. Two atoms of oxygen are consumed for every molecule of 3-hydroxyanthranilic acid oxidized (Hayaishi et al. - 1956) and 2-amino-3-acroleylfumaric acid ($C_7H_7O_5N$) is two oxygen atoms richer than 3-hydroxyanthranilic acid ($C_7H_7O_3N$). Cis-trans isomerization of 2-amino-3-acroleylfumaric acid would bring the amino group close enough to the aldehyde group to form the stable pyridine ring of quinolinic acid ($C_7H_5O_4N$) in a Schiff-type condensation. The fact that quinolinic acid is formed from the oxidation product is evidence that the latter retains all the carbon and nitrogen atoms of 3-hydroxyanthranilic acid.

A phenolytic oxygenation at carbon atoms 3 and 4 of 3-hydroxyanthranilic acid would be expected to yield a compound similar to 2-amino-3-acroleylfumaric acid. Quinolinic and picolinic acids formed from 3-hydroxyanthranilic acid in the presence of $^{18}O_2$ contain one atom of oxygen derived from molecular oxygen and none derived from water (Hayaishi et al. - 1956). These findings are consistent with the view that both atoms of $^{18}O_2$ are incorporated into the primary oxidation product, one of these being in the aldehyde group, and the other in the carboxyl group attached to carbon 1. The former is eliminated during the formation of the pyridine ring.

The high molar absorption coefficient at 360 mμ is in agreement with an unsaturated conjugated compound such as 2-amino-3-acroleylfumaric acid (Wiss and Weber - 1960).

If a solution of the primary oxidation product is acidified, the 360 mμ absorption band disappears and a peak is seen at 317 mμ (Long et al. - 1954; Mehler - 1960). Neutralization of the acid solution does not restore the band at 360 mu, but shifts the 317 mu peak to 373 mu. This latter shift is reversible. Miyake et al. (1954) concluded that the primary oxidation product was degraded in acid solution, with the loss of nitrogen, to a secondary product which still contained the aldehyde group. The latter fact was proved by the formation of a 2,4-dinitrophenylhydrazone of the acid degradation product. Mehler (loc. cit.) confirmed the loss of nitrogen during acid degradation and also found that the carboxyl group initially derived from 3-hydroxyanthranilic acid was also lost. Mehler proposes the sequence of reaction shown in Fig. 6 to explain the acid degradation.

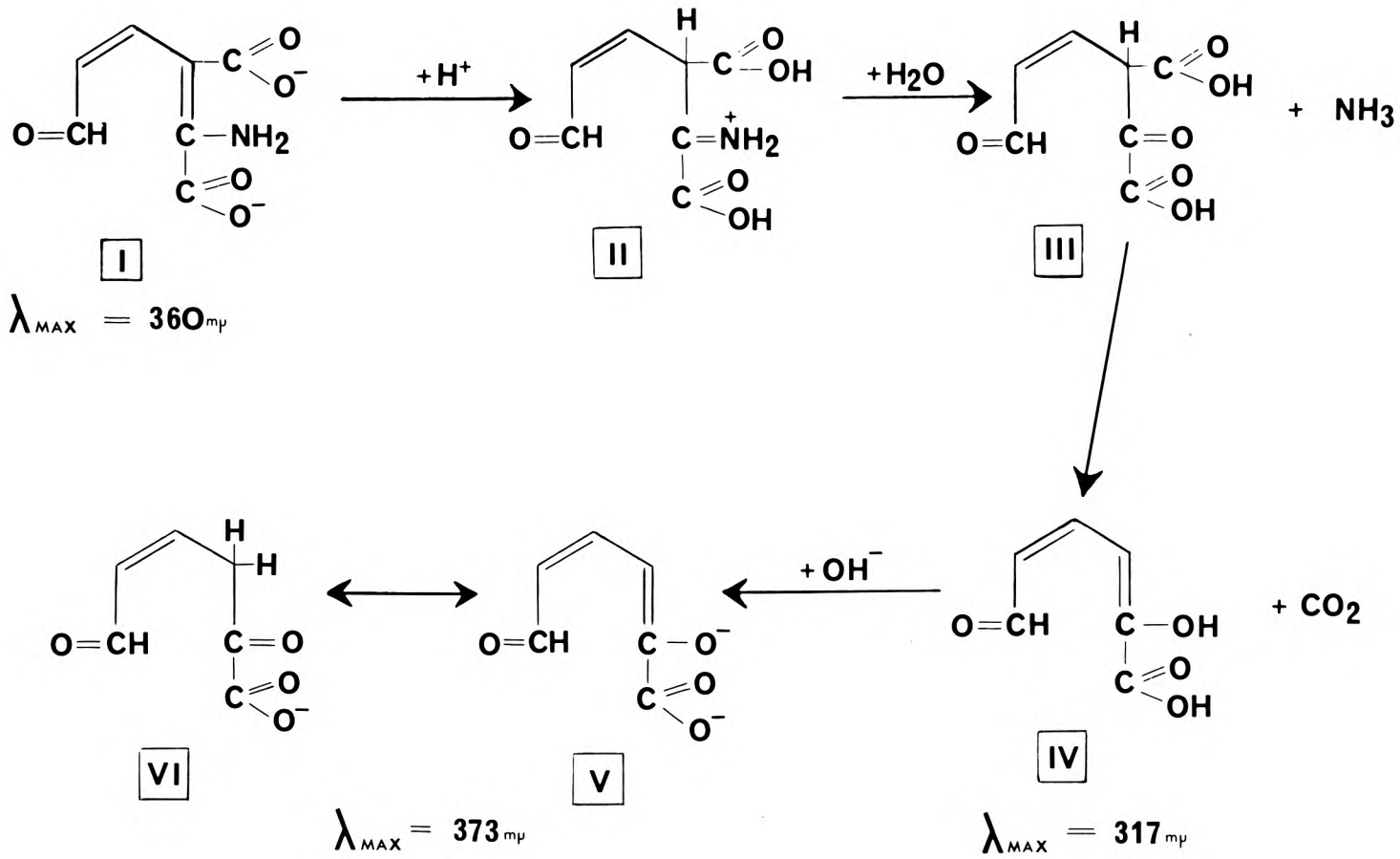
Wiss and Weber (1960) however, claim that during acid degradation quinolinic acid and particularly picolinic acid are formed at 0°, and that picolinic acid is produced exclusively at 100°. In the absence of acid, quinolinic acid is formed exclusively. It is difficult to reconcile these opposing views, but two facts seem to support Mehler's interpretation:

(i) Both Miyake et al. (1954) and Mehler (1960) report that nitrogen is lost during acid degradation. If, as Wiss claims, picolinic and quinolinic acids are formed during acid degradation, no nitrogen could have been released. This unusual high stability of an amino group related to a double bond is attributed by Wiss to the stabilizing effect of the conjugated system of 2-amino-3-acroleylfumaric acid.

(ii) It is difficult to see how picolinic acid could account for the shift in absorption maximum observed during neutralization of

FIGURE 6

ACID DEGRADATION OF 2-AMINO-3-ACROLEYLFUMARIC ACID



the acid solution.

Wiss and Bettendorf (1957) have prepared a 2,4-dinitrophenylhydrazone of what was presumably the primary oxidation product of 3-hydroxyanthranilic acid. The derivative was formed at 0° which may have prevented secondary changes but the fact remains that trichloroacetic acid was used to deproteinize the solution and the 2,4-dinitrophenylhydrazine was dissolved in 2-N HCl, and the presence of acid may well have caused an alteration prior to the formation of the derivative. This problem is emphasized by the fact that the present author also prepared a 2,4-dinitrophenylhydrazone using what appeared to be the identical conditions used by Wiss and Bettendorf (1957). This 2,4-dinitrophenylhydrazone (Compound X) is not the same as that described by Wiss and Bettendorf. It is difficult to decide which of the two derivatives, if either, represents the 2,4-dinitrophenylhydrazone of the primary oxidation product, unaffected by acid degradation. The problem may well be settled by using a non-acid deptroteinizing agent and forming the derivative in neutral solution. The possibility that Compound X is either unchanged 2,4-dinitrophenylhydrazine or the 2,4-dinitrophenylhydrazone of acetone (an acetone powder was used as the source of enzyme) is discounted by the ultra-violet spectra of these compounds (Fig. 7) and by thin-layer chromatography (p. 81).

The ultra-violet and visible absorption maxima of Wiss and Bettendorf's compound and Compound X are shown in Table 1.

TABLE 1: ULTRA-VIOLET AND VISIBLE SPECTRAL CHARACTERISTICS OF THE 2,4-DINITROPHENYLHYDRAZONE OBTAINED BY WISS AND BETTENDORF AND COMPOUND X

Compound	Absorption Maxima (mμ) (in alcohol)	
Wiss and Bettendorf	325	405
Compound X	215	372

The peak at 405 mμ agrees very well with a 2,4-dinitrophenylhydrazone of a conjugated diethenoid compound such as 2-amino-3-acroleylfumaric acid. The corresponding peak for compound X (372 mμ) appears to be at too short a wavelength for such a compound, but agrees excellently with the peak found in 2,4-dinitrophenylhydrazones of mono-ethenoid aldehydes such as crotonaldehyde (absorption maximum in alcohol = 373 mμ) (Johnson - 1953). It is thus possible that the present author has isolated the 2,4-dinitrophenylhydrazone of one of the initial acid degradation products of 2-amino-3-acroleylfumaric acid (II or III, Fig. 6) which are mono-ethenoid compounds. (III) is far more likely than (II) because of the extreme lability of the imino group in the presence of water.

The infra-red spectrum of the compound obtained by Wiss and Bettendorf contains all the peaks one would expect for the 2,4-dinitrophenylhydrazone of 2-amino-3-acroleylfumaric acid, with one conspicuous exception - viz. the carbonyl stretching vibration round about 1700 cm^{-1}. However, if the amino group exists in the charged state (as claimed by Wiss and Bettendorf) it is possible that the carbonyl absorption would be reduced or obscured

by the strong absorptions of the $-NH_3^+$ group round about 1600 cm^{-1}, as in the case of, say, aspartic acid.

The infra-red spectrum of Compound X (Fig. 8) also contains all the peaks one would expect for the 2,4-dinitrophenylhydrazone of 2-amino-3-acroleylfumaric acid, including the carbonyl absorption at 1700^{-1}. The latter peak is small but this could be accounted for by the presence of the $-NH_3^+$ absorption. It is thus seen that the infra-red and ultra-violet evidence concerning the structure of Compound X is conflicting. If one assumes that Compound X is the 2,4-dinitrophenylhydrazone of one of the acid degradation products of 2-amino-3-acroleylfumaric acid (presumably III, Fig. 6) (as indicated by the ultra-violet spectrum) one is left with the difficulty of explaining away the ridiculously weak carbonyl absorption at 1700^{-1} cm. It should also be noted that compound III (Fig. 6) contains two keto groups and consequently a mixture of the mono- and di-2,4-dinitrophenylhydrazones may well be formed. This would serve to further confuse a situation which, because of the presence of acid, contains far too many uncertainties as it is.

Wiss and Bettendorf (loc. cit.) quote elemental analysis figures for their compound which appear to fit the 2,4-dinitrophenylhydrazone of 2-amino-3-acroleylfumaric acid quite well. Unfortunately the elemental analysis of Compound X was valueless, due to the presence of a non-combustible residue (p. 81).

FIGURE 7

ULTRA-VIOLET SPECTRA

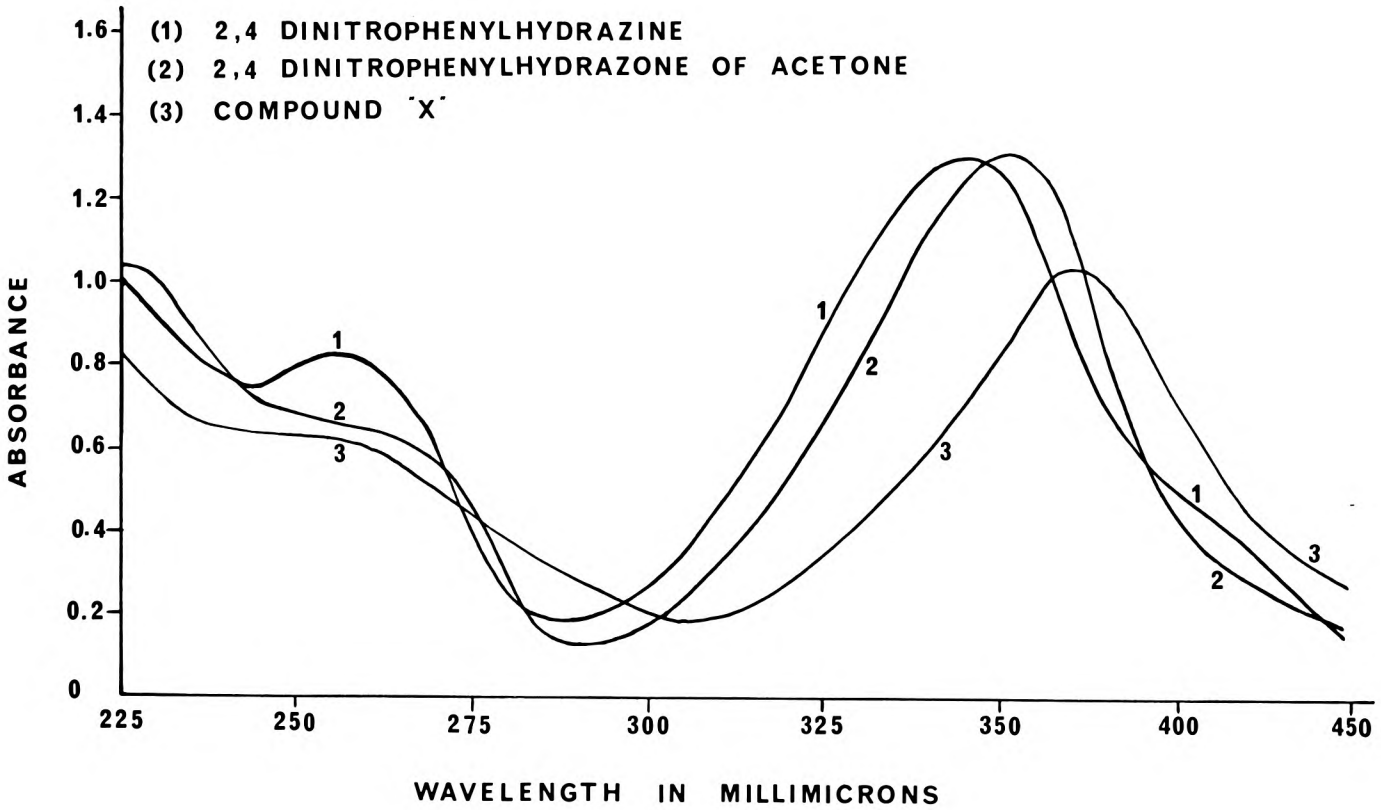
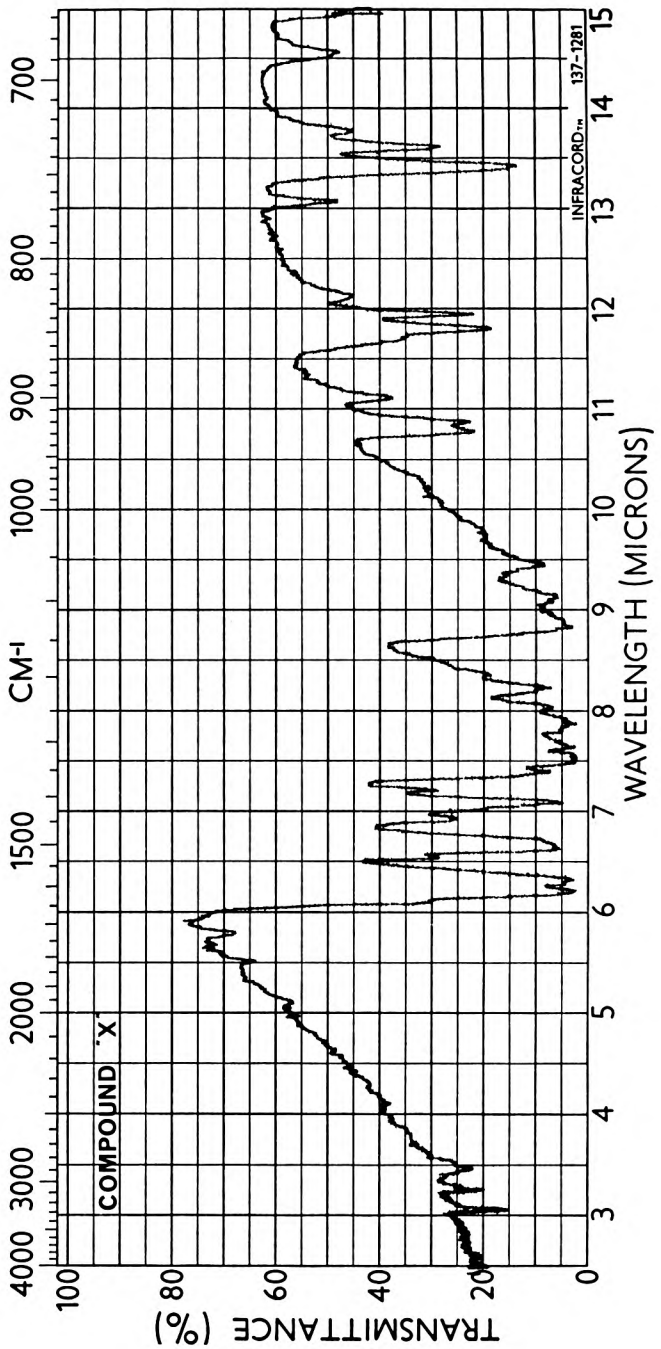


FIGURE 8

INFRA-RED SPECTRUM OF COMPOUND "X" (FIG.)



(C) STUDIES ON 3-HYDROXYANTHRANILIC ACID OXIDASE

(i) PARTIAL PURIFICATION OF 3-HYDROXYANTHRANILIC ACID OXIDASE

(a) Buffers

3-Hydroxyanthranilic acid oxidase is unstable in the absence of external ferrous ions (Stevens and Henderson - 1959), and consequently the present author decided to keep the enzyme in contact with a solution of ferrous sulphate throughout the purification. This decision raised a number of problems. A phosphate buffer could not be used because of the precipitation of ferrous phosphate. Iaccarino et al. (1961) and di Prisco et al. (1961) used a collidine buffer (pH = 6.4) containing ferrous sulphate and another reducing agent, such as ethanethiol or cysteine. The latter were presumably used to try to prevent the autoxidation of ferrous ions to ferric ions and the consequent precipitation of gelatinous ferric hydroxide. However, in later work by the same authors (Vescia and di Prisco - 1962) no reducing agent was added to the ferrous sulphate-containing collidine buffer (pH = 6.5).

The present author has found that reducing agents such as cysteine only retard the autoxidation; they do not prevent it. In the absence of a reducing agent, ferric hydroxide begins to precipitate immediately the ferrous sulphate solution is prepared. It is obvious that ferric hydroxide will not only hamper the reproducibility of the purification procedure but will also render the accuracy of the enzyme assay system used (spectrophotometric measurement of the formation of 2-amino-3-acroleylfumaric acid) more than a little dubious. The production

of ferric ions in the enzyme assay solution also presents another serious complication: ferric ions - of several metallic kations tested - have been shown to be one of the strongest inhibitors of 3-hydroxyanthranilic acid oxidase (Vescia and di Prisco - 1962).

There are two fairly obvious ways of preventing the precipitation of ferric hydroxide from ferrous sulphate solutions. As ferric hydroxide will only form in alkaline or faintly acidic solutions, a buffer of sufficiently low pH could be used (Fig. 9). In this regard, however, one has to be guided by the stability and solubility of the enzyme under consideration at different pH values. Several authors (di Prisco et al. - 1961; Stevens and Henderson - 1959) have shown that the pH optimum of 3-hydroxy-anthranilic acid oxidase is about 6.5 and it is therefore advisable to work at this pH. In particular, the assay of enzyme activity should be carried out at the optimum pH of the enzyme reaction.

Another way in which approximately neutral solutions of ferrous sulphate can be stabilized is to chelate the ferric ions as they are formed. Maleic acid chelates ferric ions and a solution of ferrous sulphate in tri-(hydroxymethyl)-aminomethane (tris)/maleate buffer (pH = 6.4) shows no evidence of a precipitate, even after several days of standing at room temperature. Instead the solution slowly turns yellow presumably due to the formation of the ferric - maleic acid complex. The solution can be further stabilized by adding a reducing agent such as cysteine. Consequently the buffers used in the present work were either:

(i) 0.05M tris/maleate (pH = 6.4) containing ferrous sulphate (3×10^{-3}M), or, more commonly,

(ii) 0.005M tris/maleate (pH = 6.4) containing ferrous sulphate (3×10^{-4}M) and cysteine (6×10^{-4}M).

Kept at 0° buffer (ii) remained completely colourless for about 12 hours and thereafter slowly turned yellow; no precipitation of ferric hydroxide occurred at any time in either of these two buffers. Another important advantage of these buffers is that the chelation of ferric ions by maleic acid should prevent ferric ion inhibition of the enzyme.

One problem encountered with tris/maleate buffers is the intense absorption of maleic acid round about 260 and 280 mμ. If, as in the present work, the protein concentrations of the enzyme solutions are determined by measurement of the absorbance of the solutions at 260 and 280 mμ, the presence of maleic acid can seriously interfere with the accuracy of these measurements. Consequently in the present work the enzyme extracts were diluted considerably with water before measurements were made (Fig. 10) and (p. 89).

(b) Solubility and Stability of 3-Hydroxyanthranilic Acid

3-Hydroxyanthranilic acid is difficult to dissolve in many solvents, especially aqueous ones (p. 83), and this made it impossible to prepare standard solutions of 3-hydroxyanthranilic acid in tris/maleate buffer. However, 3-hydroxyanthranilic acid is more readily soluble in warm absolute alcohol, and consequently stock solutions of the acid in alcohol were prepared and stored at -15° until required. The stock solutions were diluted considerably (ten times or more) with buffer before use in the assay system.

FIGURE 2

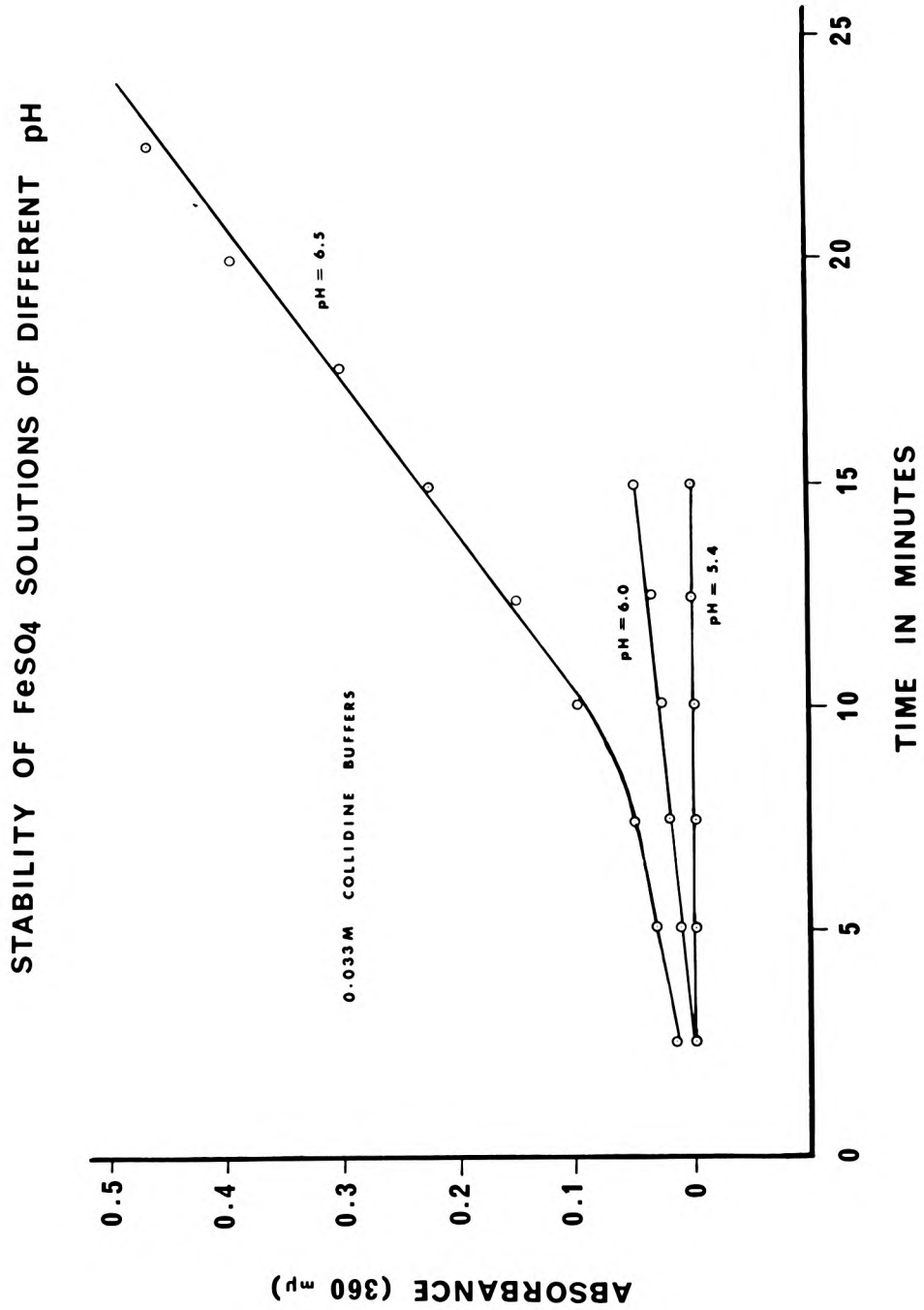
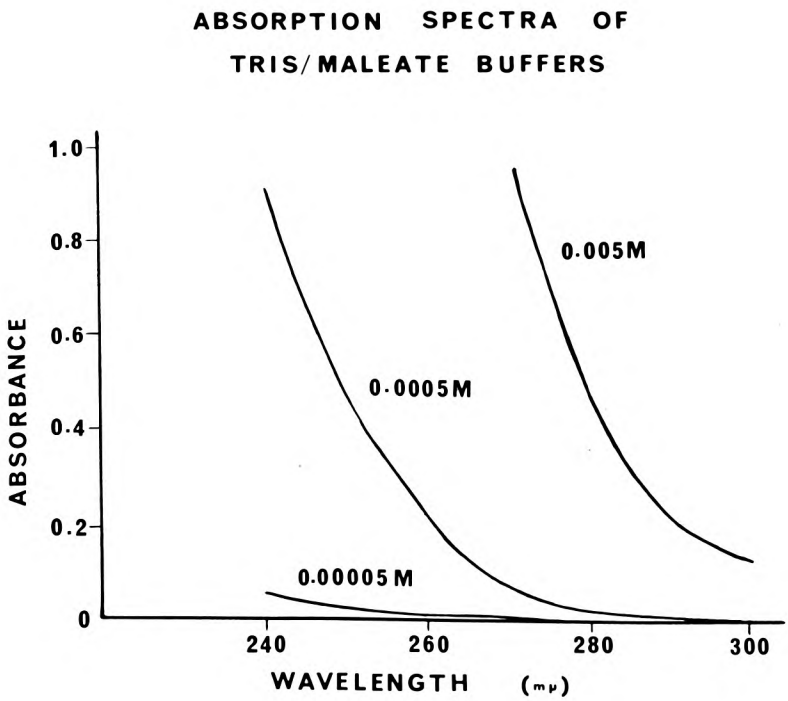

STABILITY OF FeSO4 SOLUTIONS OF DIFFERENT pH
0.033M COLLIDINE BUFFERS
pH = 6.5
pH = 6.0
pH = 5.4
ABSORBANCE (360 mμ)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
TIME IN MINUTES

FIGURE 10


ABSORPTION SPECTRA OF
TRIS/MALEATE BUFFERS
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
0.005M
0.0005M
0.00005M
240
260
280
300
WAVELENGTH (mμ)

Even in alcohol at -15°, 3-hydroxyanthranilic acid tended to dxidize spontaneously and slowly to a yellow-orange compound, presumably cinnabarinic acid. Consequently stock solutions were never kept for more than about two weeks. In aqueous solution the formation of cinnabarinic acid is much more rapid (as judged by the increase in absorbance at 452 mu) and aqueous substrate solutions were always kept at 0° and discarded about two hours after preparation.

(c) Enzyme Assay Systems

Two methods have been used to determine the activity of 3-hydroxyanthranilic acid oxidase by previous workers in the field:

(i) 3-Hydroxyanthranilic acid fluoresces and several workers have used photofluorimetry to determine the concentration of 3-hydroxyanthranilic acid at different intervals during the enzyme reaction (cf. Bokman and Schweigert - 1950).

(ii) Most workers, however, have based their activity unit on the spectrophotometric measurement (at 360 mμ) of the formation of the primary oxidation product of 3-hydroxyanthranilic acid, presumably 2-amino-3-acroleylfumaric acid. This method is simple and rapid and is thus ideally suited for use during the purification of the enzyme. Di Prisco et al. (1961), Iaccarino et al. (1961), Vescia and di Prisco (1962) have used an activity unit defined as mμmoles of primary oxidation product formed per minute, taking the molar absorbancy of this product at 360 mμ as 47,000 (Wiss and Weber - 1960), but the uncertainty regarding the exact nature of the primary oxidation product and the difficulty in obtaining an accurate molar absorbancy co-efficient (see

Section B of Discussion), have led most workers to adopt a simpler, though less rigorous, enzyme activity unit. The latter unit is usually defined as that amount of enzyme which causes an increase in absorbance at 360 mμ of a particular number of absorbance units per minute. For example, the activity unit used by the present author is defined as that amount of enzyme which causes an increase in absorbance at 360 mμ of 0.001 absorbance units per minute. A disadvantage of this assay system is the fact that the absorption band of 3-hydroxyanthranilic acid itself overlaps 360 mμ. Fortunately, however, this overlap is small.

The present author decided to try and develop a new assay system for 3-hydroxyanthranilic acid oxidase based on the well known oxidation of o-aminophenols to phenoxazinones (for method see p. 83 to 87). In acid or neutral solution, sodium iodate oxidizes 3-hydroxyanthranilic acid to an orange-red phenoxazinone, presumably cinnabarinic acid. The latter has an absorption maximum at 452 mμ in 0.05M tris/maleate buffer (pH = 6.4) (Fig. 11). The relationship between the amount of 3-hydroxyanthranilic acid oxidized and the amount of cinnabarinic acid produced is linear over the concentration range tested (Fig. 12).

The enzyme reaction was started by the addition of the enzyme and at various intervals thereafter an aliquot of the incubation mixture was removed and transferred rapidly and quantitatively into a solution of sodium iodate. The 3-hydroxyanthranilic acid present in the aliquot is oxidized by the sodium iodate to cinnabarinic acid. From the absorbance

FIGURE 11

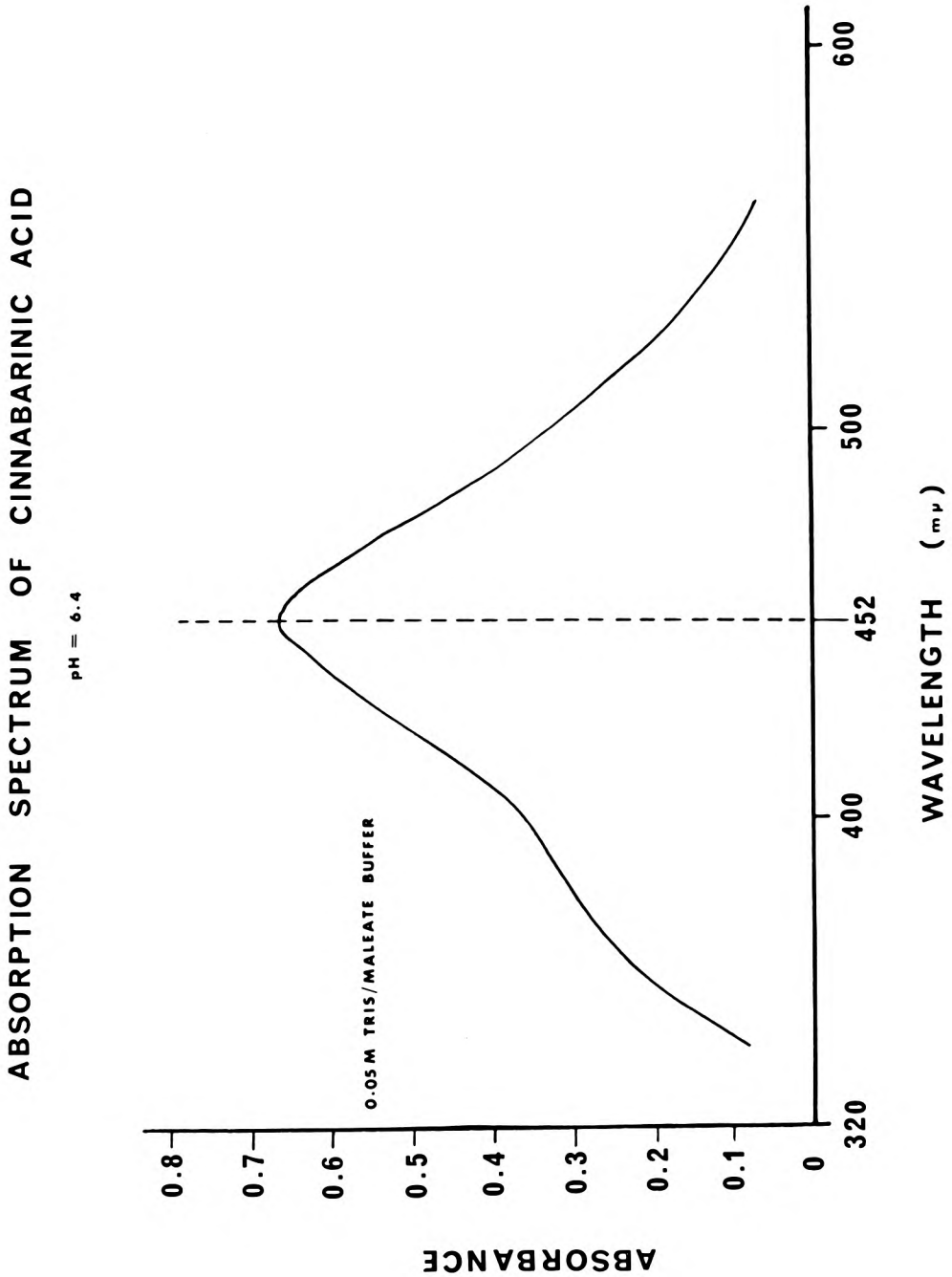
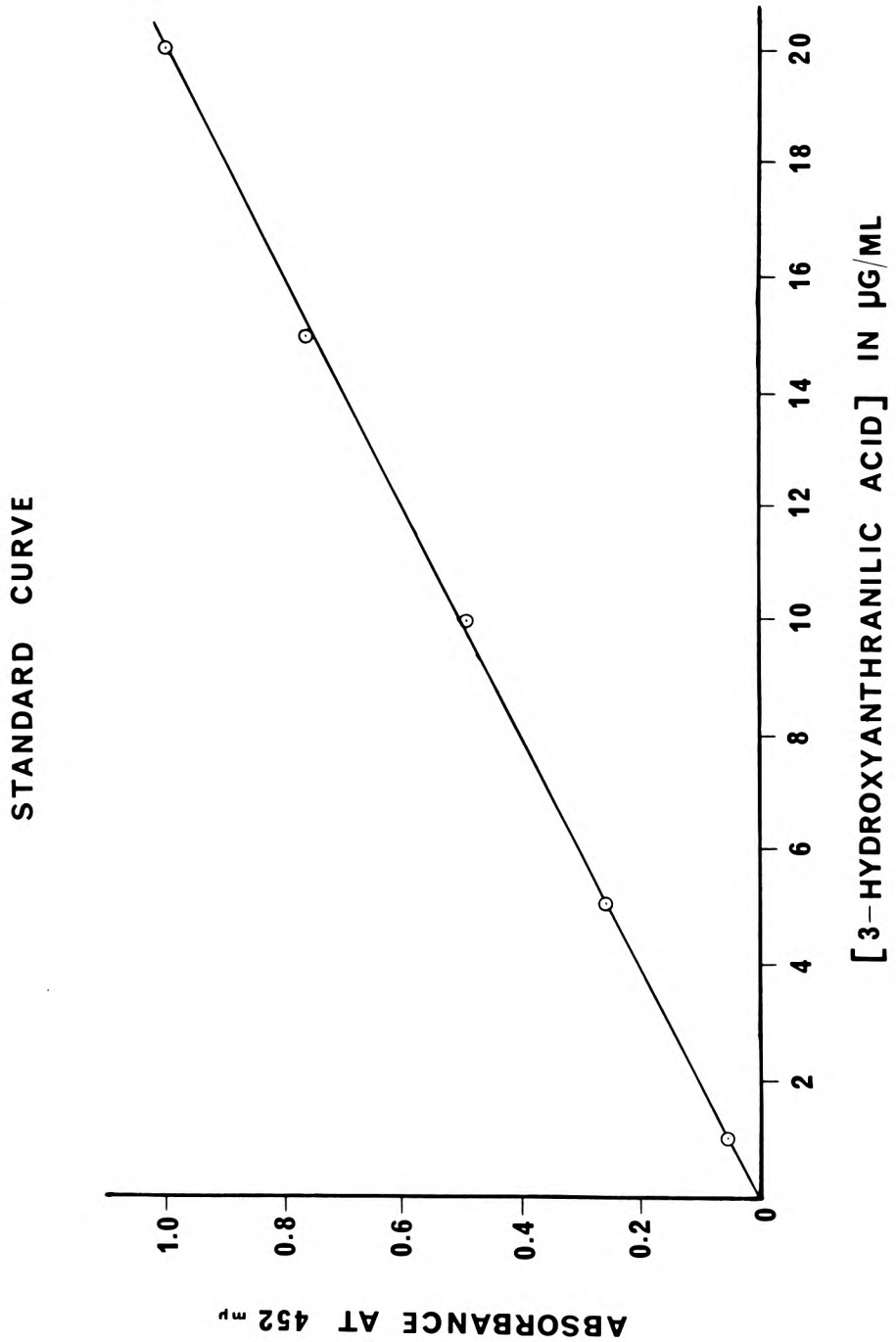

ABSORPTION SPECTRUM OF CINNABARINIC ACID
pH = 6.4
0.05 M TRIS/MALEATE BUFFER
ABSORBANCE
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
320
400
452
500
600
WAVELENGTH (mμ)

FIGURE 12


STANDARD CURVE
ABSORBANCE AT 452 mμ
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
16
18
20
[3-HYDROXYANTHRANILIC ACID] IN μG/ML

of the resulting solution at 452 mμ and by using the standard curve (Fig. 12) the amount of 3-hydroxyanthranilic acid present in the incubation mixture at the time the aliquot was removed can be computed.

At first the aliquots were transferred to a solution of sodium iodate in 1-N sulphuric acid, but the acid caused the precipitation of a large amount of protein. The latter appeared to adsorb a certain amount of the cinnabarinic acid produced, and consequently the assay system was rendered useless. Using sodium iodate in tris/maleate buffer (pH = 6.4) removed the above difficulty, but produced another. At pH = 6.4 considerably more sodium iodate is needed to completely oxidize all the 3-hydroxyanthranilic acid than in very acid solutions. The large amount of sodium iodate required tended to cause protein precipitation. A curve showing the rate of formation of cinnabarinic acid at pH = 6.4 has a plateau between 35 and 90 minutes (Fig. 13), and spectrophotometric measurements of the cinnabarinic acid produced must obviously be made during this period. However, at pH = 6.4 protein starts to precipitate out of solution about 30 to 40 minutes after the addition of the aliquot to the sodium iodate solution. This obviously made accurate spectrophotometric measurements difficult, but by measuring the amount of cinnabarinic acid produced 30 to 35 minutes after the addition of the incubation aliquot to the sodium iodate solution the errors were reduced to a minimum.

Unfortunately, however, it was found that the cinnabarinic acid assay system is considerably less sensitive

than the system based on the formation of 2-amino-3-acroleylfumaric acid (Fig. 14). This point was well illustrated when the present author attempted to determine the Michaelis' constant of the enzyme with 3-hydroxyanthranilic acid as substrate. Using the 2-amino-3-acroleylfumaric acid assay system no difficulty was encountered in obtaining a K_m value, but when the cinnabarinic acid assay system was used a K_m value could not be obtained because the Lineweaver and Burk (1934) reciprocal plot did not yield a straight line.

A further disadvantage of the cinnabarinic acid assay system is that it cannot be used in the presence of cysteine due to the oxidation of the latter to a coloured product. The presence of ferrous sulphate does not appear to affect the measurements possibly because the ferric ions formed by sodium iodate oxidation are chelated by the maleic acid present in the buffer. Consideration of the above, together with the fact that the method is time-consuming, led to the discarding of the cinnabarinic acid assay system as a practical means of assaying 3-hydroxyanthranilic acid oxidase. The one major advantage of this assay system is that, with the use of the standard curve the enzyme activity can be expressed in terms of mμmoles of 3-hydroxyanthranilic acid utilized by the enzyme per minute, in accordance with the recommendations of the International Commission on Enzyme Nomenclature.

FIGURE 13

RATE OF FORMATION OF CINNA-BARINIC ACID

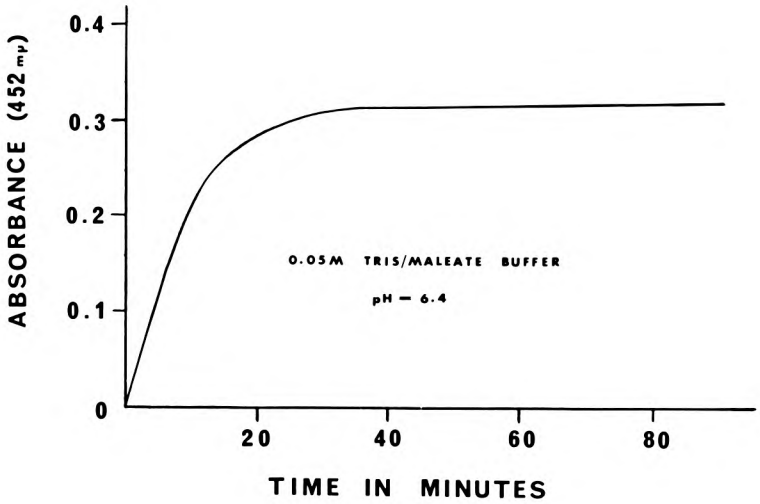
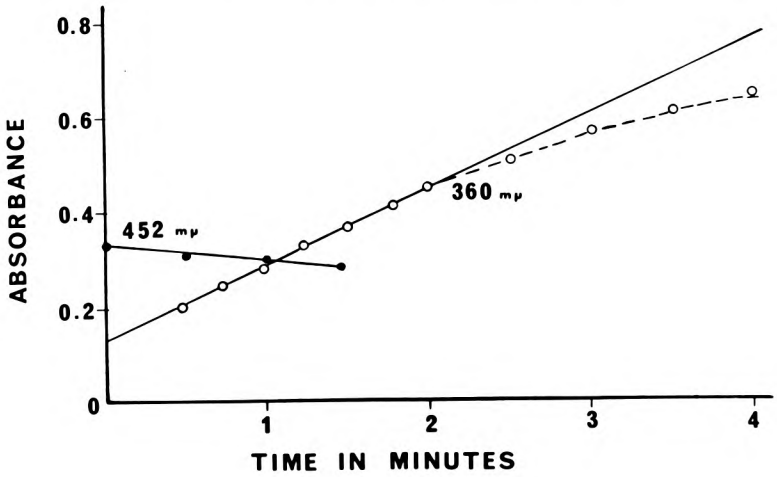


FIGURE 14

COMPARISON OF THE SENSITIVITY OF THE TWO ASSAY SYSTEMS



(d) Purification of 3-Hydroxyanthranilic Acid Oxidase

Long et al. (1954) reported that during attempts to purify 3-hydroxyanthranilic acid oxidase, activity was lost and could not be recovered by the addition of a boiled liver extract or by recombination of protein fractions. This led Mehler (1956) to state that the oxidase was very unstable and could not be purified by conventional protein fractionation methods.

Stevens and Henderson (1959) were the first to partially purify 3-hydroxyanthranilic acid oxidase, and they showed that the enzyme was stable in the presence of ferrous sulphate and reduced glutathione. Since then 3-hydroxyanthranilic acid oxidase has been purified by several different workers in the presence of ferrous sulphate. Most purifications have also been carried out in the presence of a second reducing agent such as reduced glutathione, cysteine, or ethanethiol. Recently Ogasawara et al. (1966) have purified beef kidney 3-hydroxyanthranilic acid oxidase in an inactive form, free of bound ferrous iron. The apo-oxidase was activated in the presence of ferrous sulphate just prior to assay. The work of Ogasawara et al. (1966), Decker et al. (1961), and Mitchell et al. (1963) has shown quite conclusively that the earlier difficulty in purifying 3-hydroxyanthranilic acid oxidase was due to the oxidation and/or loss of bound ferrous iron. Table 2 summarises the purifications of 3-hydroxyanthranilic acid oxidase published to date.

TABLE 2: PUBLISHED PURIFICATIONS OF 3-HYDROXYANTHRANILIC ACID OXIDASE

Starting Material	Overall Purification	Purification Procedure Used	Reference
Beef liver acetone powder	4 X	(i) Heat acetone powder extract at 51° for 5 minutes, (ii) Protein precipitation with lead subacetate.	Stevens & Henderson (1959)
Fresh rat and ox liver	40 X	(i) Cytoplasmic fraction obtained by differential centrifugation, (ii) Acidification to pH = 5.0, (iii) Heat at 55° for 5 minutes, (iv) Acetone precipitation (fraction between 45 - 60% saturation collected), (v) Ammonium sulphate fractionation (fraction between 35 - 60% saturation collected).	Iaccarino, Boeri & Scardi (1961)
Fresh calf liver	600 X	(i) - (iv) Same as Iaccarino *et al.* (1961), (v) Ammonium sulphate fractionation (fraction between 35 - 52% saturation collected), (vi) Ethanol fractionation (fraction between 25 - 45% v/v collected),	di Prisco, Vescia & Boeri (1961)

- Continued on next page -

TABLE 2: PUBLISHED PURIFICATIONS OF 3-HYDROXYANTHRANILIC ACID OXIDASE (Continued)

Starting Material	Overall Purification	Purification Procedure Used	Reference
Fresh calf liver	600 X	(vii) Gt-alumina gel adsorption, (viii) Carboxymethylcellulose chromatography.	di Prisco, Vescia & Boeri (1961)
Fresh calf liver	3450 X	(i) Cytoplasmic fraction obtained by differential centrifugation, (ii) Acetone fractionation (fraction between 45 - 58% v/v collected), (iii) Heat at 55° for 5 minutes, (iv) Ammonium sulphate fractionation (fraction between 35 - 52% saturation collected), (v) Carboxymethylcellulose chromatography.	Vescia & di Prisco (1962)
Fresh beef liver	1840 X	(i) Heat homogenate supernatant at 55° for 5 minutes, (ii) Acidification to pH = 3.5, (iii) Calcium phosphate gel adsorption, (iv) Ammonium sulphate fractionation (fraction between 50 - 60% saturation collected).	Decker, Kang, Leach & Henderson (1961)

- Continued on next page -

TABLE 2: PUBLISHED PURIFICATIONS OF 3-HYDROXYANTHRANILIC ACID OXIDASE (Continued)

Starting Material	Overall Purification	Purification Procedure Used	Reference
Fresh beef kidney	270 X	(i) Acidification of homogenate supernatant to pH = 4.0, (ii) Ammonium sulphate fractionation (fraction between 40 - 65% saturation collected), (iii) Acetone fractionation (fraction between 45 - 55% saturation collected), (iv) Ammonium sulphate fractionation (fraction between 40 - 65% saturation collected), (v) Sephadex G-100 gel filtration.	Ogasawara, Gander & Henderson (1966)
Rat liver acetone powder	5 X	(i) Acetone powder extract treated with protamine sulphate, (ii) Ammonium sulphate fractionation, (iii) Calcium phosphate gel adsorption.	Ichyama et al. (1965)

The present author has achieved a ca. thirty times purification of 3-hydroxyanthranilic acid oxidase starting with a rat liver acetone powder (Table 9) (Fig. 15). If this is compared to the purifications outlined in Table 2 it will be seen that those authors who started with an acetone powder achieved relatively low orders of purification while purification of homogenates of fresh material yielded much higher values. It thus appears that the ideal starting material for the purification of 3-hydroxyanthranilic acid oxidase is the cytoplasmic fraction of liver or kidney.

In the present work the rat liver acetone powder was extracted with buffer (pH = 6.4), centrifuged, the supernatant liquid acidified to pH = 3.5, left at this pH for 30 minutes, and then brought to pH = 6.65. After standing for 15 minutes the solution was centrifuged and the precipitate discarded. Nucleic acids were removed from the supernatant solution by adsorption onto charcoal (Table 6) (Dixon and Kodama - 1926; Morton - 1953, 1954). Ammonium sulphate fractionation - the fraction precipitating between 50 and 70% saturation was collected (Fig. 16) - completed the purification. The latter step resulted in a large activation of the enzyme. Ammonium sulphate activation of 3-hydroxyanthranilic acid oxidase has been reported by Decker et al. (1961).

The purification outlined above was used for general purposes. In addition it was found that by heating the charcoal-treated enzyme solution at 55° for 5 minutes a two to three times purification could be achieved. Heating

for longer periods or at higher temperatures resulted in a marked decrease in enzyme activity (Tables 7 and 8). The heating step was not used in the general purification procedure outlined above because it was not consistently successful. On some occasions a 3-fold increase in specific activity occurred but on others the purification was only of the order of $1\frac{1}{2}$ times or no purification was obtained at all. It should be pointed out, however, that the specific activity of the enzyme after heating was never lower than it had been before the heating.

It is possible that by heating in the presence of substrate (Bach et al. - 1946) consistent results might have been obtained. Stevens and Henderson (1959), Iaccarino et al. (1961), di Prisco et al. (1961), and Vescia and di Prisco (1962) have obtained purification of 3-hydroxyanthranilic acid oxidase by heating in the presence of substrate. Vescia and di Prisco (loc. cit.) obtained a ca. 10 times purification with an 830% yield by this procedure. This large activation is presumably due to conformational changes in the enzyme molecule caused by heat. Heat activation of 3-hydroxyanthranilic acid oxidase was also observed by Decker et al. (1961) who heated the enzyme in the absence of substrate and obtained a 3-fold purification.

Several other purification techniques were applied to 3-hydroxyanthranilic acid oxidase by the present author - none with success. Acetone fractionation of the supernatant solution obtained after acidification resulted consistently in a tremendous decrease in the specific activity

of the various fractions as compared with the unfractionated solution (Table 5). The reasons for this are not apparent, especially as several other authors have successfully purified 3-hydroxyanthranilic acid oxidase by acetone fractionation, although under somewhat different conditions (Iaccarino et al. - 1961; di Prisco et al. - 1961; Vescia and di Prisco - 1962; Ogasawara et al. - 1966).

Protamine sulphate has been used by several workers to remove nucleic acid from enzyme extracts (cf. Korkes et al. - 1950; Seaman - 1963). Ichyama et al. (1965) have used protamine sulphate in their purification of 3-hydroxyanthranilic acid oxidase but it is not immediately apparent whether it was used to remove nucleic acids or to precipitate inactive protein. Both Korkes et al. and Seaman used 2% solutions of protamine sulphate at pH = 5.0 for nucleic acid precipitation, and the latter author prepared the solution at 0°. However, the present author found it impossible to prepare a 2% solution of protamine sulphate in 0.005M tris/maleate buffer (pH = 5.0)containing ferrous sulphate (83 µg/ml) and cysteine (73 µg/ml) at 0°. At room temperature the protamine sulphate dissolved with difficulty but on cooling to 0° precipitation occurred. The highest concentration of protamine sulphate which could be prepared in the above buffer at 0° was 10 mg/ml (1%). Treatment of the enzyme extract - purified by acidification - with a 1% solution of protamine sulphate (pH = 5.0) had no result: neither nucleic acids (as judged by the ratio of the absorbance at 280 mµ to the absorbance at 260 mµ) nor inactive protein (as judged by the specific activity) were

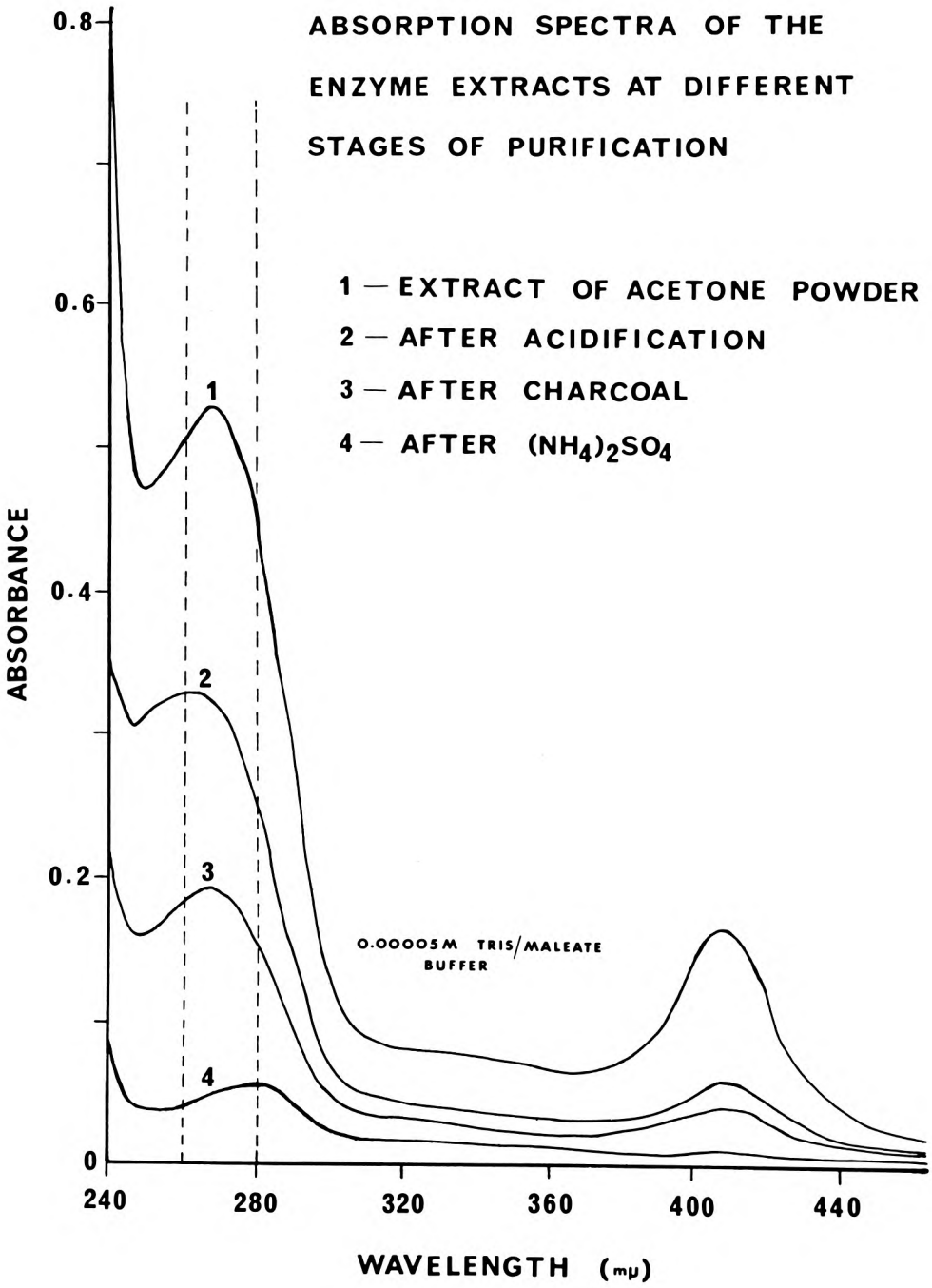
removed.

Purification of 3-hydroxyanthranilic acid oxidase by adsorption of the enzyme onto Cγ-alumina gel has been obtained by di Prisco et al. (1961), but Ogasawara et al. (1966) report that their attempts to purify the enzyme by this technique were not successful because of failure to recover more than a small percentage of the enzyme activity. Similarly, the present author has found that Cγ-alumina gel adsorption is not a useful purification technique for 3-hydroxyanthranilic acid oxidase. Fig. 17 shows that the ammonium sulphate fractionated enzyme is well adsorbed between Gel/Protein ratios (p. 97) of 0.47 and 0.71. Removal of the enzyme from the gel surface proved difficult however. Several solvents were tried (p. 97) but the only one which met with even partial success was an 0.005M tris/maleate buffer (pH = 7.6) containing 10% ammonium sulphate (cf. Bach et al. - 1946). Unfortunately even the latter solvent appeared to remove only a small portion of the adsorbed enzyme, and consequently the procedure was abandoned.

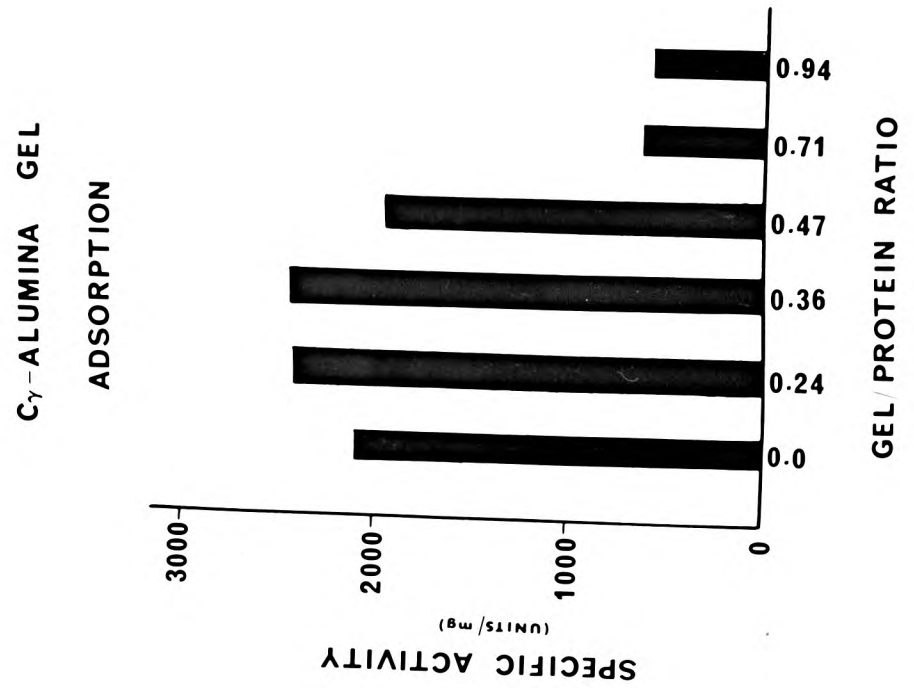
An attempt to purify the ammonium sulphate fractionated enzyme by gel filtration (using Sephadex G-200 gel) did not reach fruition. In the final stage of the preparation of the gel column, while the gel was being equilibrated with an 0.005M tris/maleate buffer (pH = 6.4) containing ferrous sulphate and cysteine, the upper third of the column began to turn yellow and then after several hours became reddish-brown. It is probable that the gel adsorbed either ferric ions themselves or the ferric ion - maleic acid

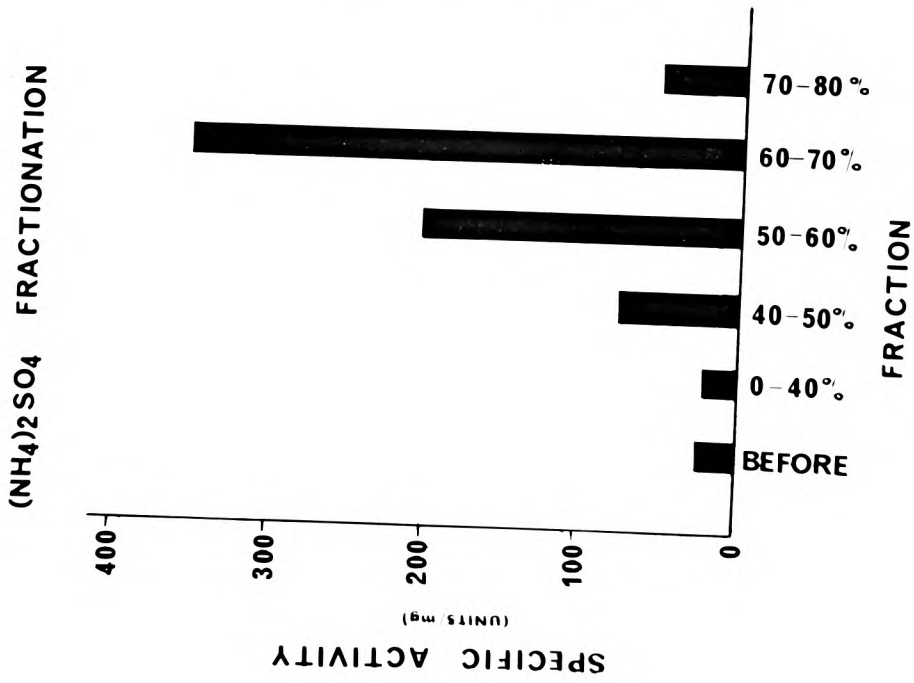
complex, both of which are yellow in colour. Under these circumstances it was decided not to continue with the experiment. Ogasawara *et al*. (1966) are the only workers to have purified 3-hydroxyanthranilic acid oxidase by Sephadex gel filtration but they used a buffer which did not contain ferrous sulphate.

FIGURE 15


ABSORPTION SPECTRA OF THE ENZYME EXTRACTS AT DIFFERENT STAGES OF PURIFICATION
1 — EXTRACT OF ACETONE POWDER
2 — AFTER ACIDIFICATION
3 — AFTER CHARCOAL
4 — AFTER $(NH_4)_2SO_4$
0.00005M TRIS/MALEATE BUFFER
ABSORBANCE
0.8
0.6
0.4
0.2
0
1
2
3
4
240
280
320
360
400
440
WAVELENGTH (mμ)

FIGURES 16 AND 17





(ii) SOME PROPERTIES OF 3-HYDROXYANTHRANILIC ACID OXIDASE

The present author has used the partially purified enzyme to investigate, spectrophotometrically, several features of the reaction catalysed by 3-hydroxyanthranilic acid oxidase, and to determine certain constants associated with the enzyme. The enzyme has been incubated with three compounds all closely related to the normal substrate (3-hydroxyanthranilic acid) - viz. 3-methoxyanthranilic acid, 4-methyl-3-hydroxyanthranilic acid, and 5-methyl-3-hydroxyanthranilic acid. Work on 6-methyl-3-hydroxyanthranilic acid has not yet been completed. Preliminary experiments soon revealed that both 3-methoxyanthranilic acid and 5-methyl-3-hydroxyanthranilic acid were not metabolised by the enzyme, whereas 4-methyl-3-hydroxyanthranilic acid was.

Fig. 18 shows the absorption spectra of an incubation mixture containing enzyme and 3-hydroxyanthranilic acid, determined at various intervals after the initiation of the reaction. It is seen that 3-hydroxyanthranilic acid has two absorption maxima in the buffer used - one at 312 mμ and one at 258 mμ. As soon as the enzyme is added a peak begins to appear round about 360 mμ, obviously due to the formation of the primary oxidation product of 3-hydroxyanthranilic acid. This latter peak continues to increase in size until approximately 45 minutes after the initiation of the reaction. The absorption maximum of the primary oxidation product in tris/maleate buffer (pH = 6.4) is 352 mμ. The 12.5 minute curve (Fig. 18) has begun to show a bulge round about 280 mμ, and, as the reaction proceeds and the 352 mμ

peak begins to decline, the 280 mμ peak increases in size. The latter peak has a maximum at 273 mμ, and this corresponds excellently with the absorption maximum of quinolinic acid (computed from Fig. 20 to be 272 - 275 mμ). Quinolinic acid is the first stable product of the 3-hydroxyanthranilic acid oxidase reaction. In summary, then, Fig. 18 shows the formation of quinolinic acid from 3-hydroxyanthranilic acid via an unstable intermediate, postulated to be 2-amino-3-acroleylfumaric acid.

A similar family of curves has been obtained for the enzyme reaction with 4-methyl-3-hydroxyanthranilic acid as substrate (Fig. 19). The absorption maxima of the latter compound are found at 308 mμ and 256 mμ. Once again, an unstable primary oxidation product, with an absorption maximum at 355 mμ, is initially produced. The latter, presumably the methyl ketone analogue of 2-amino-3-acroleylfumaric acid, is the precursor of the stable end-product of the reaction, which has an absorption maximum at 276 mμ. The latter maximum corresponds exactly with that of the expected end-product, 6-methylquinolinic acid (computed from Fig. 20 to be 274 - 278 mμ). Thus it appears that 4-methyl-3-hydroxyanthranilic acid is metabolised in a very similar way to 3-hydroxyanthranilic acid itself. It did appear, however, that the absorption coefficient at 355 mμ of the primary oxidation product of 4-methyl-3-hydroxyanthranilic acid was considerably greater than the corresponding constant at 352 mμ for 3-hydroxyanthranilic acid.

The Michaelis' constants (Fig. 21) for 3-hydroxyanthranilic acid (0.74×10^{-5}M) and 4-methyl-3-hydroxyanthranilic acid (0.79×10^{-5}M) are very similar. If simple Michaelis' kinetics are assumed, then $K_m = K_s$ (the equilibrium constant for the reaction: enzyme + substrate $\rightleftharpoons$ enzyme-substrate complex), and the virtual equivalence of the K_m values for the two substrates indicates a similar affinity of the enzyme for these substrates. However, the maximum velocity (V_{max}) of the reaction with 3-hydroxyanthranilic acid as substrate (455 units/minute) is somewhat greater than the corresponding reaction with 4-methyl-3-hydroxyanthranilic acid (345 units/minute). As the V_{max} is a measure of the velocity constant of the breakdown of the enzyme-substrate complex, it is obvious that the introduction of a methyl group at position 4 of the benzene ring, while not appreciably altering the affinity of the enzyme for the methylated substrate, does affect the breakdown of the enzyme-substrate complex. It is possible that this is caused by a lengthening of the period required to cleave the 3 - 4 bond in the substrate molecule. Exactly how the methyl group causes this is not known at present. Phenolytic oxygenation across the 3 - 4 bond can occur in a number of ways: one possibility is a heterolytic split of the bond with the electrons moving in one of two general directions - either towards C_3 or towards C_4. If the latter is the case, methylation of C_4 should reduce the V_{max} of the reaction because of the well known electron repelling properties of the methyl group. If, on the other hand, the split is towards C_3, methylation of C_4 should increase the

V_{max} of the reaction. On the basis of the kinetic evidence outlined above it appears that heterolysis of the 3 - 4 bond of the two substrates occurs, with electrons moving in the general direction of C_4. Obviously much more work must be done with other 4-substituted derivatives (both electron-attracting and electron-repelling groups should be introduced) of 3-hydroxyanthranilic acid before this very tentative hypothesis can be substantiated.

Unfortunately no kinetic constants were determined for 3-methoxyanthranilic acid, so it is not known whether this compound inhibits 3-hydroxyanthranilic acid oxidase or not. The fact that it is not metabolised, however, shows that a free hydroxyl group at position 3 is essential for the binding and/or cleavage of the molecule. Inhibition studies will have to be carried out to choose between the alternatives. For example, if 3-methoxyanthranilic acid is found to be a competitive inhibitor of 3-hydroxyanthranilic acid oxidase, this will indicate that, although binding to the active site occurs, cleavage of the 3 - 4 bond does not take place. Earlier work by Vescia and di Prisco (1962) indicated that the carboxyl and amino groups (but not the hydroxyl group) of 3-hydroxyanthranilic acid are essential for substrate binding.

The somewhat surprising competitive inhibition of 3-hydroxyanthranilic acid oxidase exhibited by 5-methyl-3-hydroxyanthranilic acid ($K_i = 1.26 \times 10^{-5}$M; Fig. 21) is inexplicable at the present time. The reason why 5-methylation prevents the cleavage of the 3 - 4 bond of the molecule must

await further analysis. In this regard the behaviour of 6-methyl-3-hydroxyanthranilic acid will be of interest.

In conclusion, it should be noted that the K_m for 3-hydroxyanthranilic acid has been determined by a number of workers (Table 3).

TABLE 3: MICHAELIS' CONSTANTS FOR 3-HYDROXYANTHRANILIC ACID

Enzyme source	K_m	Reference
Beef liver	2.6×10^{-5}M	Stevens and Henderson (1959)
Calf liver	2.1×10^{-5}M	Vescia and di Prisco (1962)
Beef liver	2.1×10^{-5}M	Decker *et al*. (1961)
Beef kidney	2.0×10^{-5}M	Ogasawara *et al*. (1966)
Rat and Ox liver	7.0×10^{-6}M	Iaccarino *et al*. (1961)
Calf liver	7.7×10^{-6}M	di Prisco *et al*. (1961)
Rat liver	7.4×10^{-6}M	Present author

FIGURE 18

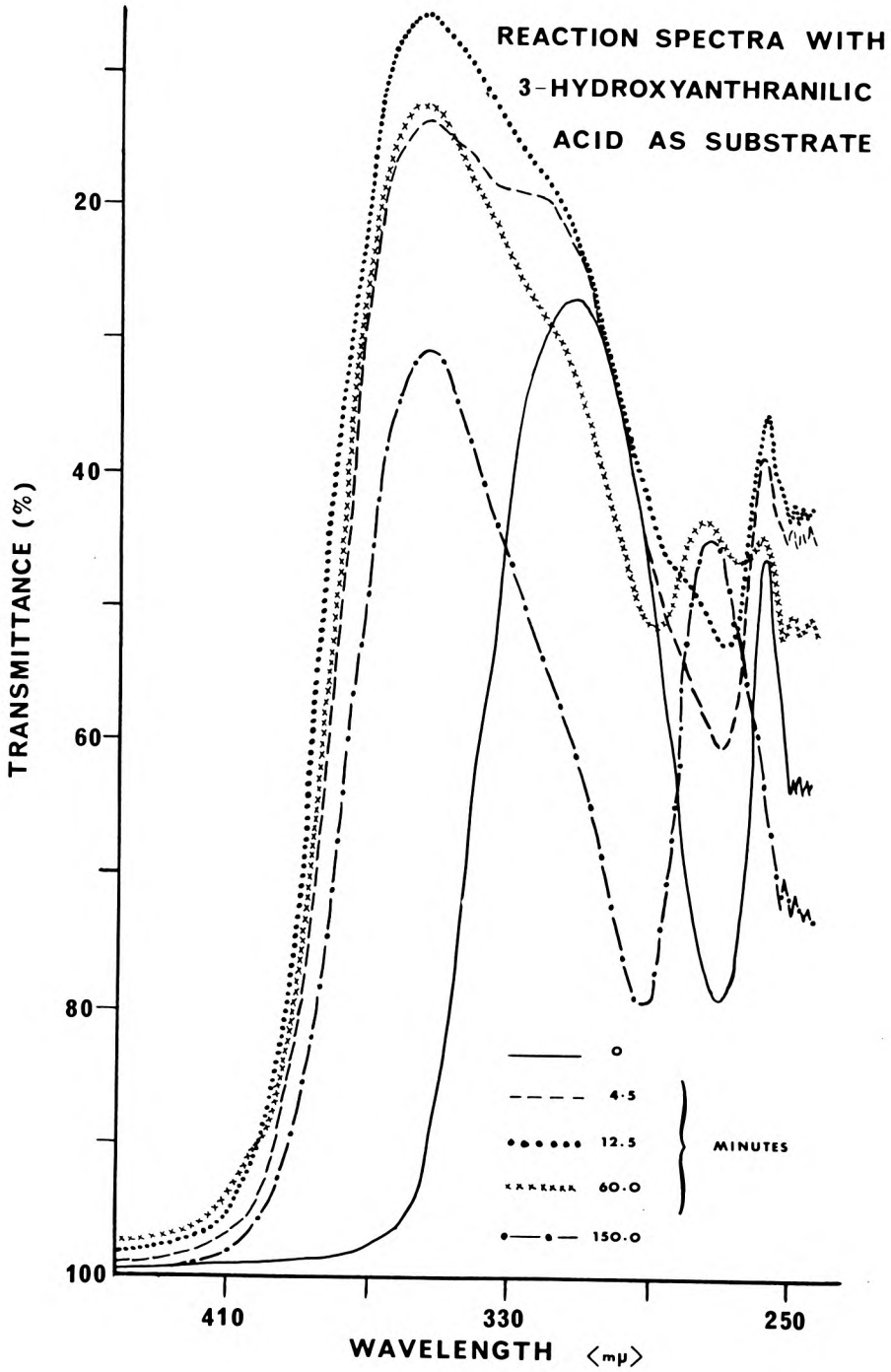

REACTION SPECTRA WITH
3-HYDROXYANTHRANILIC
ACID AS SUBSTRATE
TRANSMITTANCE (%)
20
40
60
80
100
410
330
250
WAVELENGTH (mμ)
0
4·5
12.5
60.0
150.0
MINUTES

FIGURE 19

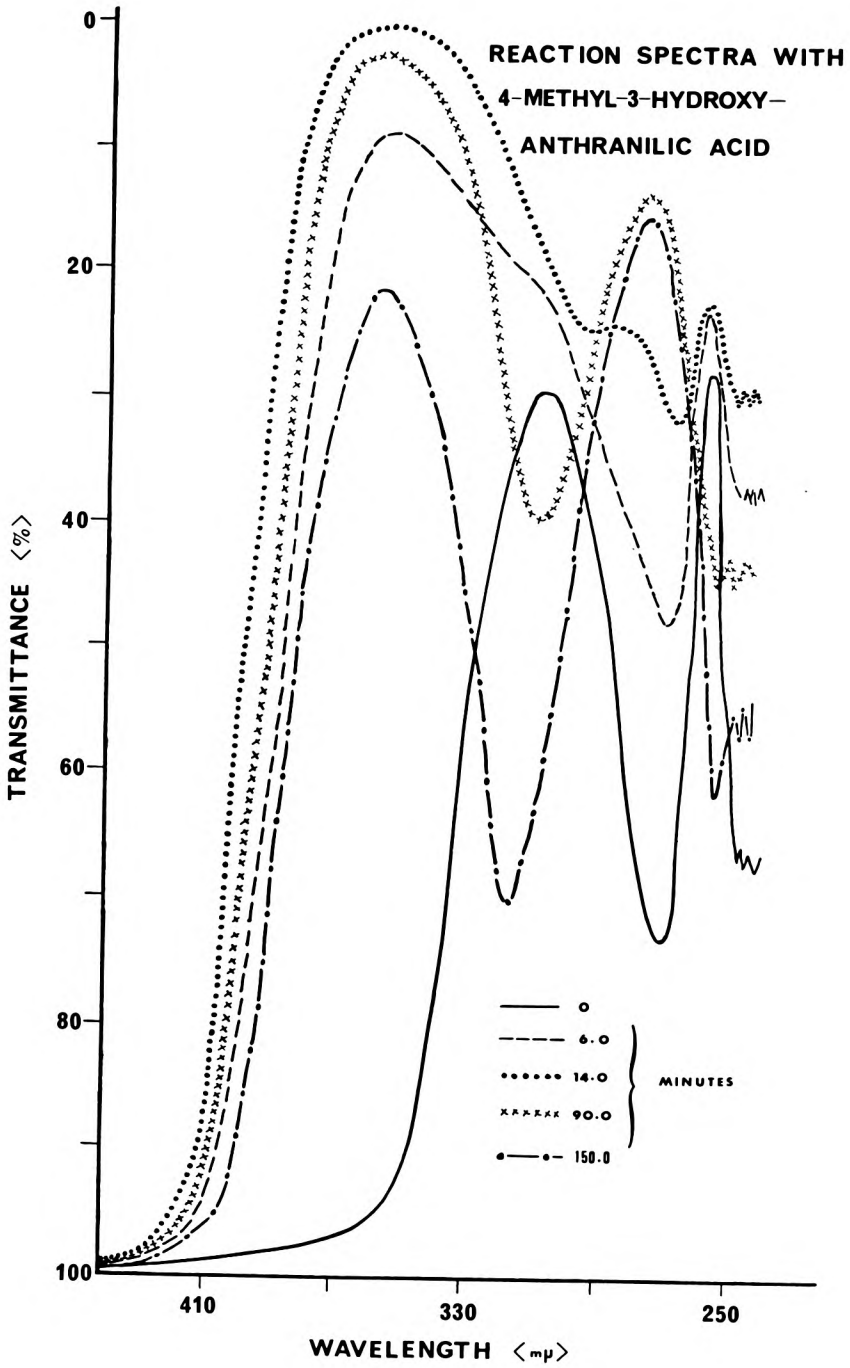

REACTION SPECTRA WITH
4-METHYL-3-HYDROXY-
ANTHRANILIC ACID
TRANSMITTANCE (%)
0
20
40
60
80
100
410
330
250
WAVELENGTH (mμ)
0
6.0
14.0
90.0
150.0
MINUTES

FIGURE 20

ULTRA-VIOLET SPECTRA OF PYRIDINE CARBOXYLIC ACIDS

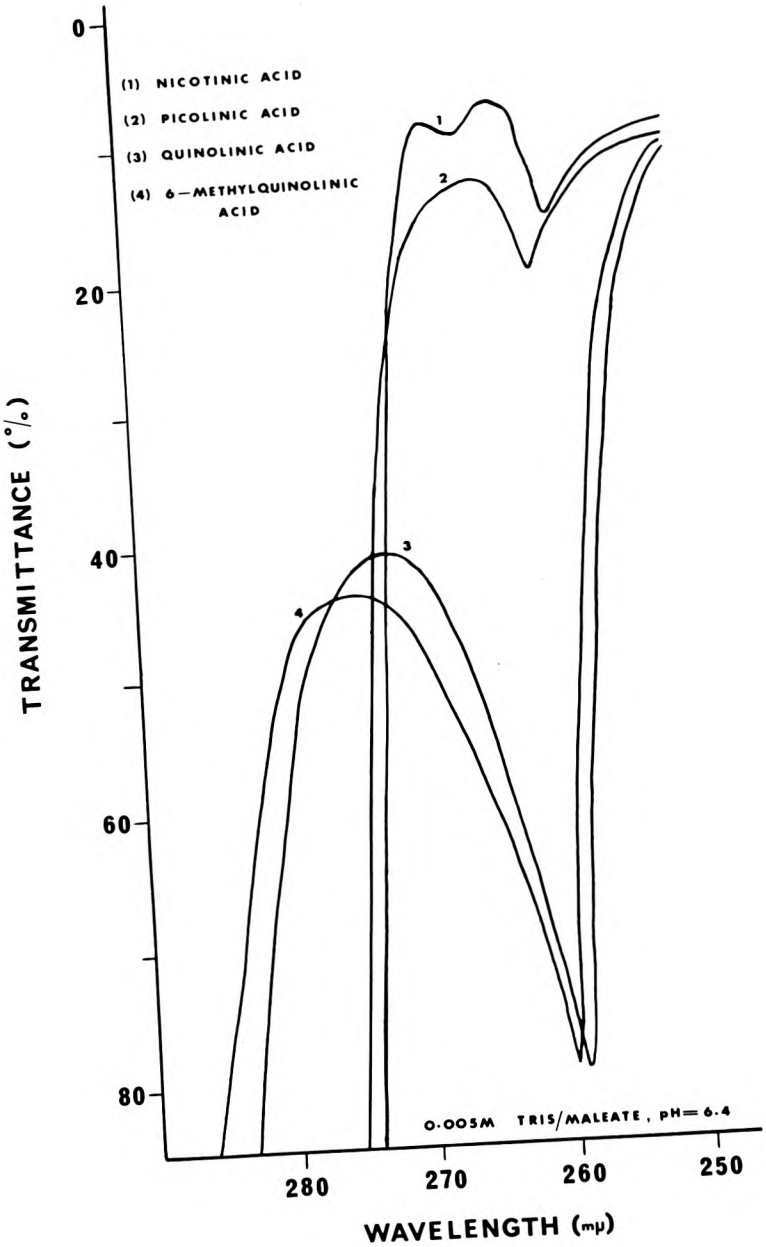
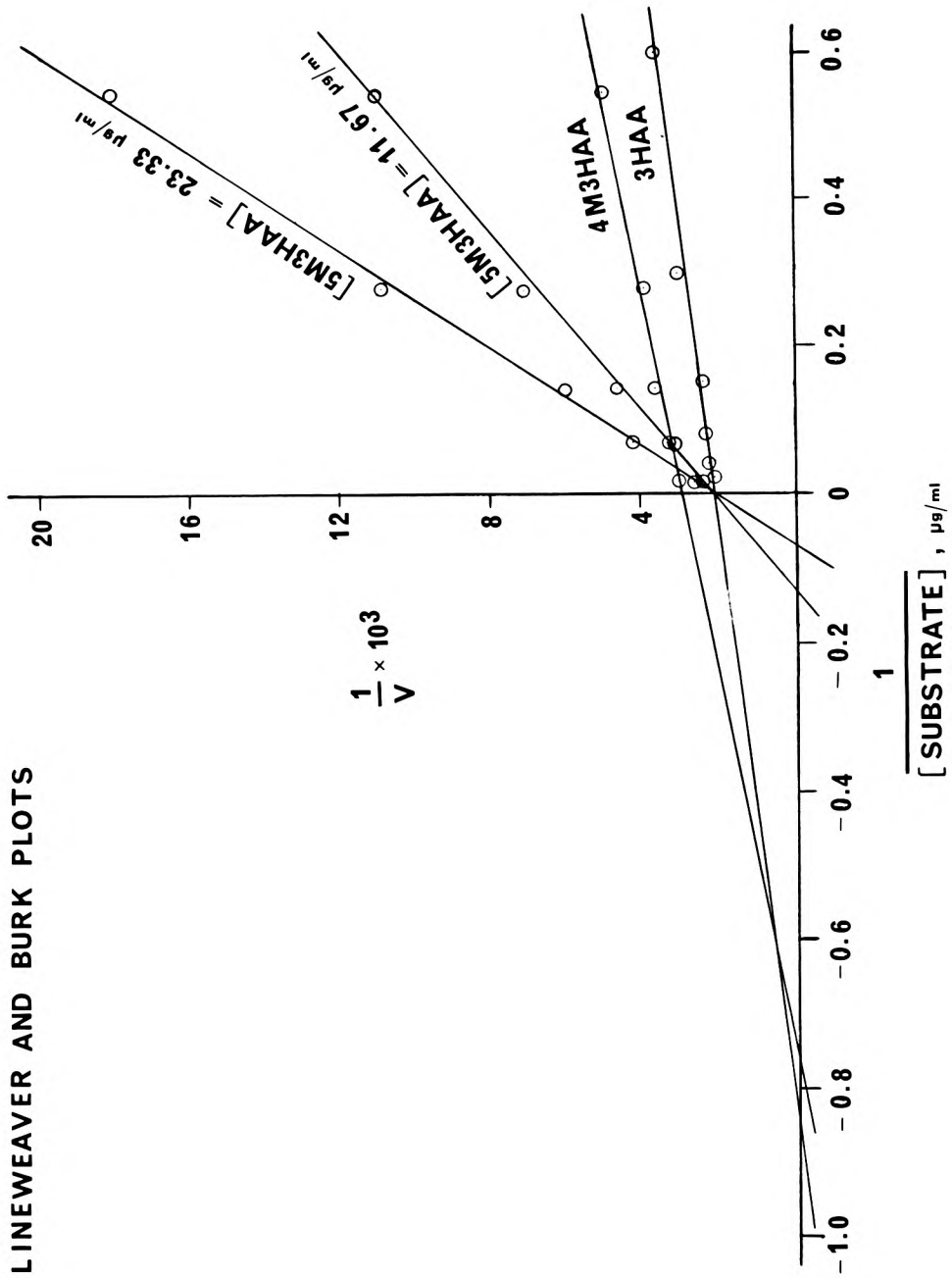


FIGURE 21


LINEWEAVER AND BURK PLOTS
[5M3HAA] = 23.33 µg/ml
[5M3HAA] = 11.67 µg/ml
4M3HAA
3HAA
20
16
12
8
4
1/V × 10³
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
1/[SUBSTRATE], µg/ml

EXPERIMENTAL PART

INSTRUMENTS

(i) Spectrophotometers: A Beckman DB-G Recording Spectrophotometer was used in the determination of the Michaelis' and Inhibitor constants and for the plotting of the reaction spectra. For the routine assay of 3-hydroxyanthranilic acid oxidase either a Beckman DB-G Recording Spectrophotometer or a Beckman DU Manual Spectrophotometer, (usually the latter) was used. Ultra-violet spectra were measured on one of the following: Beckman DB; Beckman DB-G; Unicam SP-800 Recording Spectrophotometers. Infra-red spectra were determined with a Perkin-Elmer Infracord Model 137 Spectrophotometer.

(ii) pH Meter: A Beckman "Expandomatic" pH meter was used throughout.

(iii) Centrifuges: A MSE Superspeed-50 Refrigerated Centrifuge was used throughout except in the case of the acetone fractionation where an International Model PR-1 Refrigerated Centrifuge was used.

REAGENTS

"Analar" reagents were used in all the enzyme work, and double distilled water was used in the preparation of all solutions. 3-Hydroxyanthranilic acid ("A" Grade) was obtained from Mann Research Laboratories and was used without further purification. The charcoal used was "Merck" brand Medicinal Charcoal, and the protamine sulphate (*ex* salmon) was obtained from L. Light and Company.

ANALYSES

The elemental analyses were performed by Janssen Pharmaceutica, Belgium, except for the analysis of Compound "X" which was carried out by the Department of Chemistry, University of the Witwatersrand, Johannesburg.

PART I

SYNTHESIS OF 3-HYDROXYANTHRANILIC ACID-3-^{14}C

Stage 1: Synthesis of Phenetole-1-^{14}C (II, Fig. 2).

Sodium phenoxide-1-^{14}C (25.54 g) (28 ± 2 c.p.m. / μmole phenol) was dissolved in water (300 ml) and ethyl-p-toluene sulphonate (51.2 g) was added. The mixture was refluxed for 1 hour, cooled, and extracted with ether. The ethereal extract was dried (Na_2SO_4), evaporated, and the residue distilled. 18.89 g phenetole-1-^{14}C, b.p. = 159-162°/630mm, was obtained. Yield = 73%.

Stage 2: Synthesis of o-Nitrophenetole-1-^{14}C (III, Fig. 2)

(a) Preparation of Benzoyl Nitrate (Francis - 1906):

The success of this preparation depends on the maintenance of completely anhydrous conditions throughout. The CCl_4 was dried with anhydrous $CaCl_2$, distilled, and stored in an airtight bottle. The $AgNO_3$ was ground to a fine powder in a mortar and dried for 24 hours over anhydrous $CaCl_2$ in a desiccator. The benzoyl chloride was fractionally distilled and stored in an airtight container.

Silver nitrate (65 g) was added slowly with continuous stirring to benzoyl chloride (37 ml) contained in a round-bottomed flask. The stirrer was fitted with a glycerol trap. The temperature was maintained at -10° throughout by the use of an acetone/dry ice bath. After the addition of all the $AgNO_3$ the solution was filtered rapidly under vacuum into a dry container, and the benzoyl nitrate so obtained used immediately.

(b) Nitration of Phenetole-1-^{14}C :

Phenetole-1-^{14}C (18.89 g), dissolved in CCl_4 (50 ml) was added dropwise, with constant stirring, to a solution of benzoyl nitrate (25.8 g) in CCl_4 (50 ml). The temperature was maintained at -15° throughout the addition. The solution was allowed to warm up slowly to room temperature, and left at this temperature for 12 hours. It was then extracted with saturated $NaHCO_3$ solution, washed several times with water (until the water phase was neutral to litmus), dried (Na_2SO_4), and the CCl_4 evaporated. The residue distilled at 255-260°/630 mm. Yield = 17.8 g (68.7%).

Stage 3: Synthesis of o-Phenetidine-1-^{14}C sulphate (IV, Fig. 2)

A mixture of o-nitrophenetole-1-^{14}C (17.8 g), absolute alcohol (40 ml), and Adams' platinum oxide catalyst (420 mg) was reduced with hydrogen at 70°/630 mm, utilising the apparatus described by Vogel (1956). The mixture was stirred magnetically throughout the reaction. It has been found that vigorous stirring speeds up the reaction considerably. When the reaction was complete the catalyst, previously colloidal, appeared to coagulate and settled to the bottom of the flask. The same phenomenon has been observed during the hydrogenation of several other aromatic nitro compounds and can be used to indicate the end-point of such reactions. As soon as the hydrogenation was complete, the solution was acidified with 1:1 H_2SO_4/absolute alcohol. The catalyst was removed by vacuum filtration and the filtrate was evaporated on a waterbath. A blue-grey waxy material (24.4 g) was obtained.

Stage 4: Synthesis of 8-Ethoxyquinoline-8-^{14}C (V, Fig. 2)

The amine sulphate (24.4 g), H_2SO_4 (102 g; 70% w/w), glycerol (47.8 g), and sodium m-nitrobenzene sulphonate (25.4 g) (Long and Schofield - 1953) were mixed together in a round-bottomed flask. The mixture was refluxed at 130° with mechanical stirring, and then left to cool overnight. The solution was treated with $NaNO_2$ (7.3 g) at 4°, and then boiled carefully for 15 minutes to convert any remaining o-phenetidine to the corresponding phenol. The solution was rendered alkaline with excess NaOH, the temperature being maintained at 4° throughout, and then extracted with ether. The ethereal extract was dried (Na_2SO_4) and evaporated to dryness. The greenish-yellow oil so obtained was distilled and the fraction boiling between 279 and 290°/630 mm collected. Yield = 5.4 g (29.3%, based on o-nitrophenetole).

Stage 5: Synthesis of N-Benzyl-8-ethoxyquinoline-8-^{14}C bromide (VI, Fig. 2)

Benzyl bromide (5.9 g; 10% excess) was added to a solution of the quinoline (5.4 g) in dry benzene (40 ml). The solution was refluxed on a waterbath for 12 hours during which time a solid precipitated. The crystals (9.47 g) were collected by vacuum filtration, washed with a small volume of dry benzene, and dried. Yield = 93%; m.p. = 143 - 145°.

Stage 6: Synthesis of N-Benzyl-3-ethoxy-N-formyl-anthranilic acid-3-^{14}C (VII, Fig. 2)

An approximately 5% solution of $Ca(MnO_4)_2.2H_2O$ was prepared by dissolving about 25 g of the salt in 500 ml water and boiling. The small amount of MnO_2 formed was removed by filtration of the cold solution. The solution was standardized against ferrous ammonium sulphate solution. A boiling solution of Ag_2SO_4 (4.3 g in 500 ml water) was added to an aqueous solution of N-benzyl-8-ethoxyquinoline bromide (9.47 g in 150 ml water). The precipitate of AgBr was removed by filtration and washed with water. The filtrate was cooled to 0° and the calculated quantity of calcium permanganate (16.1 g) was added dropwise with constant stirring over a period of ca. 2 hours. The mixture was brought to the boil, filtered, and the MnO_2 washed thoroughly with boiling water. The filtrate and washings were evaporated on a waterbath to small volume and filtered to remove the small amount of tar that had formed. The clear filtrate was cautiously acidified with 1:1 HCl until precipitation was complete. The acid (4.7 g) was collected by filtration and dried at 80° for 5 hours, m.p. = 160 - 163°. Yield = 57%. As this compound is more stable than 3-hydroxyanthranilic acid, it was stored in a stoppered test tube under N_2, and was converted into 3-hydroxyanthranilic acid-3-^{14}C, as required, by simultaneous hydrolysis and dealkylation with phosphorus and HI (Stage 7) (Diamond and Levy - 1965).

PART II

ATTEMPTED SYNTHESIS OF 4-METHYL-3-HYDROXYANTHRANILIC ACID-3-^{14}C

Stage 1: Synthesis of Salicylaldehyde (II, Fig. 3)

(a) (i) Method of Gattermann (1943):

Phenol (25 g) was added to a hot (80°) solution of NaOH (80 g NaOH in 80 ml water) contained in a 1 litre round-bottomed flask fitted with a thermometer and a condenser. The solution was cooled to 60 - 65°, chloroform (20 g) was added, and the contents of the flask were gently swirled. After a period of 10 minutes, during which the temperature of the mixture was maintained between 65 and 70°, a second portion of chloroform (20 g) was added and, once again, after the same interval, a third 20 g portion. At this stage the flask was frequently shaken. The reaction was completed by refluxing vigorously on a waterbath for 1 hour. The solution was steam distilled until chloroform failed to pass over, cooled to about 45°, acidified with dilute H_2SO_4, and steam distilled. The distillate was extracted with ether and the ether evaporated. The oily residue was shaken in a glass-stoppered bottle with two volumes of saturated $Na_2S_2O_5$ solution, the thick paste which formed was left to stand for 1 hour, collected by vacuum filtration, and washed well with alcohol and then with ether. When the bisulphite compound was decomposed by warming with dilute H_2SO_4 the salicylaldehyde separated out as an oil. It was taken up in ether, dried (Na_2SO_4), and the ether evaporated. The pure aldehyde so obtained distilled at 190 - 192°/630 mm. Yield = 5.8 g (17.9%).

(ii) The reaction was carried out essentially as in (a) (i) except that the chloroform (60 g) was added dropwise with stirring over a period of ca. 1 hour. The temperature was maintained at 60 - 65° throughout the addition. The yield was not improved.

(iii) The reaction was carried out as in (a) (i) except that KOH (112.2 g) was used instead of NaOH . Yield = 2.2 g (6.8%).

(b) Method of Armstrong and Richardson (1933):

A solution of trichloroacetic acid (41 g in 40 ml water) was added to a solution of phenol (18.8 g), KOH (112.2 g) and water (140 ml). The mixture was cautiously heated and, after the violent boiling had ceased (a few minutes), refluxed on the waterbath for 1 hour. The solution was then worked up as described in (a) (i), but the small amount of brown oil obtained decomposed during distillation.

(c) Method of Sten and Ray (1932):

A mixture of phenol (25 g), chloroform (25 ml) and ethanol (75 ml) was added dropwise with stirring to a hot (80°) solution of NaOH (50 g) in water (80 ml) to which pyridine (5 ml) had been added. The mixture was heated on a waterbath for 4 hours and worked up as previously described. No improvement in yield was obtained.

(d) Method of Brady and Jacobovits (1950):

Chloroform (21 ml) was added to a warm (60°) solution of sodium phenoxide (21 g phenol in 70 ml 15-N NaOH) and the

mixture was shaken vigorously for 15 minutes. After about 10 minutes shaking a yellowish solid crystallized out of solution. The reaction was completed by heating on a waterbath for 30 minutes. The solution was cooled, diluted with water until the solid dissolved and steam distilled to remove excess chloroform. Acidification with dilute HCl was followed by steam distillation , the distillate being worked up in the usual manner. Yield = 2.2 g (8.1%).

Stage 2: Synthesis of o-Cresol (III, Fig. 3)

(a) The Clemmensen Reduction (Martin - 1942):

A mixture of zinc (150 g), mercuric chloride (10 g), concentrated HCl (5 ml), and water (150 ml) was shaken for 5 minutes, the aqueous solution decanted, and water (150 ml) and concentrated HCl (200 ml) added. The mixture was warmed and, as soon as hydrogen began to be evolved, salicylaldehyde (25 g) was added dropwise over a period of *ca*. 1 hour. After the addition the mixture was refluxed vigorously for 3 hours, concentrated HCl (*ca*. 5 ml) being added every 15 minutes. The product was isolated by steam distillation, saturating the distillate with NaCl, and extracting the o-cresol from it with petroleum ether (b.p. = 40 - 60°). The extract was dried (Na_2SO_4), the petroleum ether evaporated, and the residue distilled. A colourless oil, b.p. = 186 - 200°/630 mm, which solidified on cooling was obtained. Yield = 2.5 g (11.3%).

(b) The Huang-Minlon Modification of the Wolff-Kishner Reduction (1946)

A solution of salicylaldehyde (10 g), 90% hydrazine hydrate (5.8 g) and NaOH (11.1 g) in diethylene glycol was refluxed for 1 hour. Sufficient distillate was then removed to allow the temperature of the solution to rise to 185°; the distillate was saved and added to the bulk of the solution once the reaction was complete. The solution was refluxed at 185° for 3 hours, cooled, acidified with dilute H_2SO_4, extracted with ether, dried (Na_2SO_4), and the ether evaporated. The brown residue was distilled (b.p. = 186 - 189°/ 630 mm) and a colourless liquid was obtained which crystallized on cooling. Yield = 5.3 g (60%).

Stage 3: Synthesis of 6-Nitro-o-cresol (IV, Fig. 3) (Gibson - 1925)

A mixture of HNO_3 (107 ml; density = 1.42) and glacial acetic acid (300 ml) was stirred in a freezing mixture at -15° while a mixture of o-cresol (100 g) and glacial acetic acid (100 ml) was added in the course of about 2 hours. By the end of the addition a thick magma of crystals had formed. After standing for two hours in the freezing mixture the mass was poured into water (ca. 5 - 6 litres) and the crystals, collected by vacuum filtration, were submitted to steam distillation. 6-Nitro-o-cresol containing a small proportion of 4,6-dinitro-o-cresol passed over, while 4-nitro-o-cresol mixed with a small proportion of the dinitro-compound remained behind. The crude distillate was fractionally steam distilled and those fractions with a melting point between 65 and 68° were combined and dried over $CaCl_2$. Yield = 43 g (30.3%)

Stage 4: Synthesis of 2-Methyl-6-nitrophenetole (V, Fig. 3):

Solid NaOH (12.3 g) was added to a suspension of 6-nitro-o-cresol (43 g) in water (150 ml). The mixture turned dark red as the sodium salt of the nitrocresol dissolved. Ethyl-p-toluene sulphonate (67.3 g) was added, the solution refluxed for 24 hours, cooled, extracted with ether, the ethereal extract washed three times with 50% NaOH and then with water, and the ether evaporated. The black liquid obtained was refluxed for 1 hour with 2-N KOH to decompose excess ethyl-p-toluene sulphonate, cooled, extracted with ether, dried (Na_2SO_4), and the ether evaporated. The residue distilled at 254 - 260°/630 mm. Yield = 34.3 g (66.8%). The unchanged 6-nitro-o-cresol was recovered by acidifying the aqueous extract and alkaline washings with 50% H_2SO_4, and collecting the crystals by vacuum filtration. The crystals (10 g) were dried over $CaCl_2$.

Stage 5: Synthesis of 2-Ethoxy-m-toluidine sulphate (VI, Fig. 3)

(a) Hydrogenation of 2-methyl-6-nitrophenetole:

A mixture of 2-methyl-6-nitrophenetole (10 g), absolute alcohol (25 ml), and platinum oxide catalyst (247 mg) was reduced with hydrogen at 70°/630 mm. As soon as the hydrogenation was complete, the solution was acidified with 1:1 H_2SO_4/absolute alcohol (6.5 g concentrated H_2SO_4). The catalyst was removed by vacuum filtration and the filtrate evaporated on the waterbath. A purple crystalline material was obtained. Yield = 13.1 g (95%).

(b) Preparation of Derivatives of 2-Ethoxy-*m*-toluidine:

A small quantity of 2-ethoxy-*m*-toluidine sulphate was dissolved in water, rendered alkaline with Na_2CO_3, extracted with ether, and the ether evaporated. A brown-coloured oil was obtained from which a picrate (m.p. = 142 - 143°) and a hydrochloride (sublimed from 160°; residue decomposed at 199 - 202°) were prepared. The picrate was prepared from benzene solution and recrystallized from alcohol/water. Both compounds were dried at 100° for 3 hours. Elemental analyses are shown in Table 4.

TABLE 4: CHARACTERIZATION OF THE PICRATE AND HYDROCHLORIDE OF 2-ETHOXY-*m*-TOLUIDINE

	Picrate		Hydrochloride	
	Calculated	Found	Calculated	Found
%C	47.37	47.23	57.60	57.36
%H	4 24	4.17	7.47	7.48
%N	14.76	14.64	7.47	7.57
%Cl	- -	- -	18.94	18.85

Stage 6: Synthesis of 7-Methyl-8-ethoxyquinoline (VII, Fig. 3)

Glycerol (8.7 g; dehydrated by heating to 180° for 10 minutes) and finely powdered iodine (0.29 g) were added to a solution of 2-ethoxy-*m*-toluidine sulphate (10.5 g) in concentrated H_2SO_4 (12.9 g), and the mixture heated to 180°, at which temperature vigorous evolution of SO_2 occurred. After 5 minutes at 180° the temperature was reduced to 140° and the solution refluxed for 3 hours. After cooling, the solution was

rendered alkaline with 20% NaOH solution, and steam distilled. The yellow-green steam distillate was extracted with ether, dried (Na_2SO_4), and the ether evaporated leaving behind a light green oil. The yield was of the order of 30%. A picrate was prepared (alcoholic solution) from this oil, recrystallized from alcohol/water, and dried at 80° (m.p. = 152 - 156°).

In one experiment the residue from the steam distillation was extracted with ether, dried (Na_2SO_4), and the ether evaporated. A small amount of a greenish-brown oil was obtained. This was steam distilled, the distillate extracted with ether, dried (Na_2SO_4), and the ether evaporated. A cloudy yellow oil was obtained which solidified in fine yellow needles when cooled. For analysis the compound was crystallized three times from alcohol/water and pure white crystals were obtained, m.p. = 69 - 70°.

Found	:	75.04%C	5.6%H	8.6%N
Calculated (for 7-methyl-8-hydroxyquinoline)	:	75.40%C	5.66%H	8.8%N

7-Methyl-8-hydroxyquinoline obtained by a different route had a m.p. = 68 - 69° (Levy - unpublished results). A mixed melting point of this substance and the material melting at 69 - 70° showed no depression.

Stage 7: Attempted Synthesis of N-Benzyl-4-methyl-8-ethoxyquinoline bromide (VIII, Fig. 3)

Benzyl bromide (7.41 g; 10% excess) was added to 4-methyl-8-ethoxyquinoline (6.3 g), and the mixture was heated on a waterbath for 12 hours, during which time a solid crystallized out. The solid was collected by vacuum filtration, washed with

dry benzene, and crystallized twice from ethanol/ether.

Yield = 3.0 g (24.9%).

Three such preparations were carried out and each time a solid with a different melting point was obtained (see below). The yields were of the order of 20 - 30% in each case.

Compound A : 187 - 189°

Compound B : 202 - 204°

Compound C : 175 - 178°

The three compounds were further characterized by thin-layer chromatography, ultra-violet and infra-red spectrophotometry, and in the case of Compound A, elemental analysis.

(a) Thin layer Chromatography:

A, B, and C were dissolved in absolute alcohol and applied to a silica gel plate. The sample spots were allowed to dry in air and the plate was run in a solvent composed of butanol-dioxan-absolute alcohol (6:3:1). The three compounds were located with an ultra-violet lamp and the R_F values of all three were identical (=0.73). A small faint residue was observed at the point of application of all three compounds.

(b) Ultra-violet Spectrophotometry:

The ultra-violet spectra of A, B, and C in absolute alcohol are shown in Fig. 4.

(c) Infra-red Spectrophotometry:

The infra-red spectra of A, B, and C (1.5 mg of each in 300 mg KBr) are shown in Fig. 5.

(d) Elemental Analysis of Compound A:

Found	: 51.90%C	5.37%H	4.82%N	27.50%Br
Calculated (for N-benzyl-4-methyl-8-ethoxyquinoline bromide)	: 63.65%C	5.59%H	3.92%N	22.38%Br

Stage 8: Attempted Synthesis of N-Benzyl-3-ethoxy-N-formyl-4-methyl-anthranilic acid (IX, Fig. 3)

(a) From Compound B:

An approximately 5% solution of $Ca(MnO_4)_2.2H_2O$ was prepared as described previously and standardized against ferrous ammonium sulphate. A boiling solution of Ag_2SO_4 (0.91 g in 200 ml water) was added to an aqueous solution of compound B (2.0 g in 30 ml water). The precipitate was removed by filtration and washed with water. The filtrate and washings were cooled to 0°, the calculated quantity of calcium permanganate (3.38 g) was added dropwise with constant stirring over a period of 1 hour, and the solution was left to warm up overnight. The mixture was filtered and the residue washed twice with boiling water. The filtrate and washings were combined, evaporated to small volume on a waterbath, and filtered. The residue was saved and the filtrate was acidified with 1-N HCl but no precipitation occurred. The acid solution was extracted with ether, dried (Na_2SO_4), the ether evaporated, and a small volume of brown tarry material obtained. All attempts to crystallize this material failed. The residue referred to above was dissolved in alkaline solution (Na_2CO_3) and filtered. A brown solution was obtained which was acidified with 1-N HCl, but no precipitation occurred.

(b) From Compounds A and C Combined:

Compounds A and C were subjected to exactly the same treatment as described in (a), with the same results.

PART III

ATTEMPTED ISOLATION OF THE PRIMARY OXIDATION PRODUCT OF 3-HYDROXYANTHRANILIC ACID

(a) Preparation of Beef Liver Acetone Powder:

Five pounds of fresh beef liver were minced in an electric meat mincer, mixed well with twice the volume of distilled water, and poured slowly, with constant stirring, into 5 volumes of acetone at -10°. After standing at -10° for half-an-hour the supernatant was decanted and the precipitate, collected by vacuum filtration, was washed three times by suspension in acetone at -10°, and then dried at room temperature for 1 hour. The dry powder was pulverized in a mortar and stored in a vacuum desiccator at -10°.

(b) Determination of the 3-Hydroxyanthranilic acid Oxidase Activity of the Acetone Powder:

Acetone powder (0.5 g) was extracted at 0° with 0.017M phosphate buffer (pH = 7.4) (25 ml) for 5 minutes. The mixture was filtered under vacuum, and the filtrate kept at 0°. 3-Hydroxyanthranilic acid (0.0050 g) dissolved in phosphate buffer (pH = 7.4) (10 ml) was added to the filtrate and the absorbance of an aliquot of the resulting solution was measured at 360 mμ

against a blank containing filtrate but no 3-hydroxy-anthranilic acid. The absorbance of the solution increased from 0.55 to 0.82 in one minute, and the acetone powder was deemed active.

(c) Isolation of the 2,4-Dinitrophenylhydrazone of the Primary Oxidation Product of 3-Hydroxyanthranilic Acid (Compound "X") (cf. Wiss and Bettendorf - 1957):

Acetone powder (50 g) was suspended in 0.017M phosphate buffer (pH = 7.4) (1 litre) and stirred for 30 minutes at 0°. 3-Hydroxyanthranilic acid (0.5 g) was added and the mixture stirred for a further 30 minutes. A 50% aqueous solution of trichloroacetic acid (100 ml) was added to the mixture, the precipitate collected in a refrigerated centrifuge, and discarded. A saturated solution of recrystallized 2,4-dinitrophenylhydrazine (m.p. = 201 - 202°) in 2-N HCl (200 ml) was added to the clear yellow supernatant at 0°. A very fine orange precipitate began to form immediately, and the solution was left to stand at 0° overnight. The precipitate was collected by centrifugation, dissolved in pure, dry ethyl acetate and placed in an evaporating basin inside a vacuum desiccator. The ethyl acetate was evaporated by continuous evacuation of the desiccator. The dark red crystals so obtained were dissolved in the minimum volume of pure, dry ethyl acetate and a few drops of cyclohexane were added to the warm solution, which was then cooled rapidly to 0°. The solution was left to stand at 0° for 2 hours, filtered under vacuum, and the fine red powder dried in a desiccator. Weight = 31 mg. The compound appeared to sublime continuously from ca. 180° and no definite melting point could be obtained.

(d) Characterization of Compound X:

(i) Thin-layer Chromatography:

Samples of Compound X and purified 2,4-dinitrophenylhydrazine were dissolved in ethyl acetate and applied to a silica gel plate. The sample spots were allowed to dry in air and the plate was run for 2 hours in a solvent composed of butanol-dioxan-absolute alcohol (6:3:1). The R_F values of the two compounds were 0.77 (Compound X) and 0.58 (2,4-dinitrophenylhydrazine).

(ii) Ultra-violet Spectrophotometry:

The ultra-violet spectra of Compound X (1.0 mg in 50 ml), 2,4-dinitrophenylhydrazine, and the 2,4-dinitrophenylhydrazone of acetone in absolute alcohol were recorded (Fig. 7)

(iii) Infra-red Spectrophotometry:

The infra-red spectrum of Compound X (1.5 mg in 300 mg KBr) was recorded (Fig. 8).

(iv) Elemental Analysis:

47.26%C 4.47%H 17.86%N

Unfortunately, these figures are valueless, because the compound could not be completely combusted.

PART IV

PURIFICATION OF 3-HYDROXYANTHRANILIC ACID OXIDASE

A. Buffers

(i) Three 0.033M collidine buffers of pH respectively 5.4, 6.0 and 6.5, were prepared according to Gomori (1946).

(ii) A 0.033M phosphate buffer (pH = 6.4) was prepared according to Geigy (1962).

(iii) The 0.05M tris/maleate buffers (pH = 6.4) were prepared according to Geigy (1962). A stock solution of 0.02M tris/maleate was autoclaved at 15 lbs. per square inch for 15 minutes and stored in sealed bottles at 0° until required.

B. Stability of $FeSO_4$ Solutions of Different pH

The absorbance of 1mM solutions of $FeSO_4.7H_2O$ in 0.033M collidine buffers (pH = 5.4, 6.0 and 6.5) was measured at 360 mμ against blanks containing only the relevant buffer. In order to standardize measurements as far as possible, the absorbance readings were timed from when the $FeSO_4$ solutions were first prepared (Fig. 9).

The absorbance of a 1mM $FeSO_4$ solution in 0.033M collidine buffer (pH = 6.5), containing 1.5 mM cysteine, was measured at 360 mμ against a blank containing 1.5mM cysteine in 0.033M collidine buffer (pH = 6.5). The absorbance remained constant for the period of 15 minutes during which it was measured. A transient purple colour appeared in the solution when the $FeSO_4$ and cysteine were first mixed together, but this disappeared within two or three minutes.

C. Solubility and Stability of 3-Hydroxyanthranilic acid in Different Solvents.

3-Hydroxyanthranilic acid is only sparingly soluble in 0.005M tris/maleate buffer (pH = 6.4), water, methanol, propanol, and dioxan, but is more readily soluble in acetone and absolute alcohol. In addition 3-hydroxyanthranilic acid is oxidized relatively quickly to cinnabarinic acid in all these solvents at room temperature. This is particularly the case with aqueous solvents. Consequently stock solutions of 3-hydroxyanthranilic acid in absolute alcohol were prepared, stored at -10°, and diluted considerably with buffer before use.

D. Assay Systems

(1) Oxidation of 3-Hydroxyanthranilic acid to Cinnabarinic acid

(a) Absorption Spectrum of Cinnabarinic acid:

A solution of 3-hydroxyanthranilic acid in 0.05M tris/maleate buffer (pH = 6.4) (15.4 µg/ml) was added to a solution of $NaIO_3$ (2.592 mg/ml) in the same buffer. After standing for 1 hour at room temperature the absorption spectrum of the orange-coloured solution was measured against a blank containing $NaIO_3$ but no 3-hydroxyanthranilic acid (Fig. 11). An absorption maximum occurs at 452 mµ.

(b) Rate of Formation of Cinnabarinic acid:

Fig. 13 shows the rate of formation of cinnabarinic acid produced by the oxidation of 3-hydroxyanthranilic acid (6.17 µg/ml) in 0.05M tris/maleate buffer (pH = 6.4) with $NaIO_3$ (8.640 mg/ml) in the same buffer. The reaction was started by the addition of 3-hydroxyanthranilic acid, and a blank containing $NaIO_3$ was used. A plateau occurs between 35 and 90 minutes.

(c) The Effect of the Concentration of $NaIO_3$ on the Formation of Cinnabarinic acid:

Fig. 22 shows the effect of the concentration of $NaIO_3$ on the production of cinnabarinic acid. The cinnabarinic acid solutions were obtained by treating a series of solutions of $NaIO_3$ in 0.05M tris/maleate buffer (pH = 6.4) of varying concentrations, with a solution of 3-hydroxyanthranilic acid (6.17 μg/ml) in the same buffer. The absorbance of the resulting solutions was measured at 452 mμ 1 hour later against a blank containing 3-hydroxyanthranilic acid but no $NaIO_3$. A concentration of $NaIO_3$ greater than ca. 5.0 mg/ml is necessary for the complete oxidation of 6.17 μg/ml of 3-hydroxyanthranilic acid.

(d) Standard Curve:

A standard curve showing the relation between the amount of 3-hydroxyanthranilic acid oxidized and the amount of cinnabarinic acid produced is given in Fig. 12. The reactants were all dissolved in 0.05M tris/maleate buffer (pH = 6.4) and the reaction was started by the addition of $NaIO_3$ to a final concentration of 25 mg/ml. The absorbance of the cinnabarinic acid solutions was measured at 452 mμ 1 hour later.

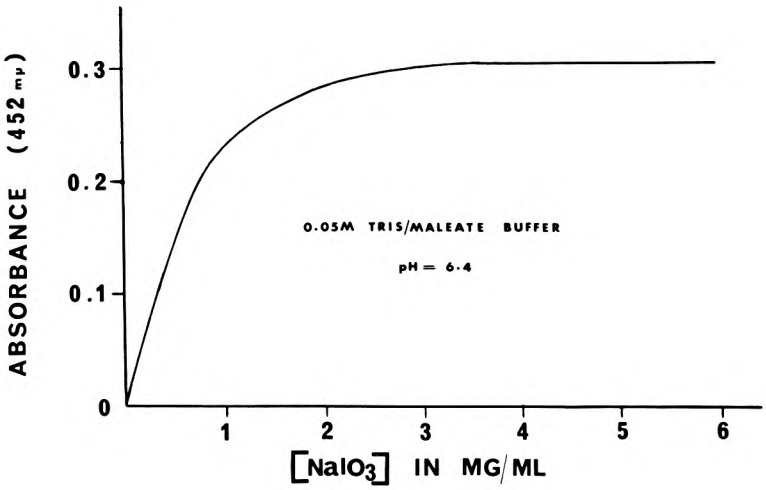
(e) The Cinnabarinic acid Assay System:

(i) 3-hydroxyanthranilic acid solution:

A stock solution of 3-hydroxyanthranilic acid in absolute alcohol (0.40 mg/ml) was prepared and stored at -10° until required. The stock solution was diluted 10 times with 0.05M tris/maleate buffer (pH = 6.4) for use in the assay system.

FIGURE 22

EFFECT OF THE CONCENTRATION OF $NaIO_3$ ON THE FORMATION OF CINNA-BARINIC ACID



(ii) $NaIO_3$ solution:

A solution of $NaIO_3$ in 0.05M tris/maleate buffer (pH = 6.4) (16.7 mg/ml) was stored at 0° until required.

(iii) $FeSO_4$ solution:

A solution of $FeSO_4.7H_2O$ in 0.05M tris/maleate buffer (pH = 6.4) (0.83 mg/ml) was prepared just prior to use and kept at 0°.

(iv) Enzyme solution:

The preparation of the enzyme is described in detail in section IV.G. For the purpose of the cinnabarinic acid assay system the enzyme was contained in an 0.05M tris/maleate buffer (pH = 6.4) containing $FeSO_4.7H_2O$ (0.83 mg/ml; 3×10^{-3}M).

(v) Method of assay:

Solution A : 4 ml diluted 3-hydroxyanthranilic acid solution (final concentration = 26.67 µg/ml)
1 ml $FeSO_4$ solution (final concentration = 138.33 µg/ml)
1 ml 0.05M tris/maleate buffer (pH = 6.4)

Solution B : 4 ml diluted 3-hydroxyanthranilic acid solution
1 ml $FeSO_4$ solution
0.5 ml 0.05M tris/maleate buffer (pH = 6.4)
0.5 ml enzyme solution

Solution A (i) : 3 ml 0.05M tris/maleate buffer (pH = 6.4)
1 ml solution A (final concentration of 3-hydroxyanthranilic acid = 6.67 µg/ml)

Solution A (ii)	:	3 ml $NaIO_3$ solution (final concentration = 12.525 mg/ml)
		1 ml solution A
Solution B (i)	:	3 ml 0.05M tris/maleate buffer (pH = 6.4)
		1 ml solution B
Solutions B (ii) B (iii) and B (iv)	:	3 ml $NaIO_3$ solution
		1 ml solution B

The enzyme solution (0.5 ml) was added rapidly to solution B at time = 0 seconds, and the latter was mixed well. At times = 30, 60, and 90 seconds 1 ml of solution B was withdrawn and pipetted rapidly with shaking into solutions B (ii) to B (iv) respectively. Immediately thereafter 1 ml of solution B was pipetted into B (i), 1 ml of solution A into A (i) and 1 ml solution A into A (ii). The absorbance of solutions A (ii) (A (i) as blank) and solutions B (ii) to B (iv) (B (i) as blank) were measured at 452 mμ, 30 - 35 minutes after the initiation of the reaction (Fig. 14). The standard curve was then used to calculate the amount of 3-hydroxyanthranilic acid present at times 0, 30, 60 and 90 seconds respectively.

(vi) Unit enzyme activity:

One unit of enzyme was defined as that amount which catalyzed the transformation of 1 mμmole of 3-hydroxyanthranilic acid per minute, at pH = 6.4, and temperature 23 - 25°. All rate measurements were based on the initial velocity of the enzyme reaction.

(2) Oxidation of 3-Hydroxyanthranilic acid to 2-amino-3-acroleylfumaric acid

(i) Buffer:

An 0.005M tris/maleate buffer (pH = 6.4) containing $FeSO_4.7H_2O$ (83 µg/ml; 3 x 10^{-4}M) and cysteine (72 µg/ml; 6 x 10^{-4}M) was used in the preparation of all the reagents for this assay system.

(ii) 3-Hydroxyanthranilic acid solution:

A stock solution of 3-hydroxyanthranilic acid in absolute alcohol (1.6 mg/ml) was prepared and stored at -10° until required. The stock solution was diluted 10 times with buffer for use in the assay system.

(iii) Enzyme solution:

For the purpose of this assay system the enzyme was contained in an 0.005M tris/maleate buffer (pH = 6.4) containing $FeSO_4.7H_2O$ (83 µg/ml) and cysteine (72 µg/ml).

(iv) Method of assay:

Solution A	:	2.5 ml buffer
		0.5 ml enzyme solution
Solution B	:	1.5 ml buffer
		0.5 ml enzyme solution
		1.0 ml diluted 3-hydroxyanthranilic acid solution (final concentration = 53.34 µg/ml)

After a 5 minute incubation period during which time the two solutions were allowed to attain room temperature, either the 3-hydroxyanthranilic acid or the enzyme was added to start the reaction. The absorbance of solution B at 360 mµ was measured for a period of 3 minutes using solution A as a blank. The

reaction usually remained linear for 1 to 2 minutes after the initiation of the reaction, and all velocity measurements were based on this initial linear period of reaction (Fig. 14).

(v) Unit of enzyme activity:

One unit of enzyme was defined as that amount of enzyme which caused an increase in absorbance at 360 mμ of 0.001 absorbance units per minute, at pH = 6.4, and temperature = 23 - 25°.

(vi) Specific activity:

This was defined as enzyme units per mg of protein.

E. Comparison of the Sensitivity of the Assay Systems

The two assay systems were modified slightly so that a sample of enzyme could be assayed under identical conditions by both. The concentration of 3-hydroxyanthranilic acid at the start of the reaction was 26.68 μg/ml and the protein concentration was 0.43 mg/ml. The results indicate that the measurement of 2-amino-3-acroleylfumaric acid is ca. 6 - 7 times more sensitive than the measurement of cinnabarinic acid (Fig. 14).

F. Protein Estimation

The enzyme solution was diluted with distilled water until it was either 0.0005M or 0.00005M with respect to the tris/maleate buffer. The absorbance of the diluted solution was measured at 280 mμ and 260 mμ against a blank containing diluted buffer, and the protein concentration was calculated from the following equation (Layne - 1957):

$$\text{Protein Concentration} = F(1.55 \times A_{280} - 0.76 \times A_{260}) \text{ mg/ml}$$

where, A_{280} and A_{260} are the absorbance at 280 and 260 mμ respectively, and F is the dilution factor. Fig 10 shows the

absorption spectra of:

(a) 0.005M tris/maleate buffer (pH = 6.4) containing $FeSO_4.7H_2O$ (83 µg/ml) and cysteine (72 µg/ml);

(b) 0.0005M tris/maleate buffer obtained by diluting (a) 10 times with distilled water;

(c) 0.00005M tris/maleate buffer obtained by diluting (a) 100 times with distilled water.

Distilled water was used as a blank in all three cases.

G. Purification of 3-Hydroxyanthranilic acid Oxidase

Note: (i) All assays reported in this section were based on the measurement of the formation of 2-amino-3-acroleylfumaric acid.

(ii) Unless otherwise stated all steps were carried out at 0°.

(iii) Unless otherwise stated the buffer used was 0.005M tris/maleate (pH = 6.4) containing $FeSO_4.7H_2O$ (83 µg/ml) and cysteine (72 µg/ml).

(i) Preparation of an Acetone Powder from Rat Liver

Six adult albino rats were killed by a sharp blow to the base of the skull, exsanguinated, and their livers removed immediately. The livers were cleaned of extraneous adipose and connective tissue, rinsed in distilled water, and dropped whole into purified acetone at -10°. After about 15 minutes the acetone was decanted and the livers minced in 3 - 5 volumes of purified acetone in a Waring Blendor for 2 minutes at -10°. The slurry so obtained was filtered under vacuum at room temperature, and the residue was washed twice at room temperature by suspension in purified acetone, and collected

by vacuum filtration. The cake of material so obtained was broken into small pieces, spread out in a large Petri dish, and allowed to dry at room temperature for 30 minutes by which time the bulk of the remaining acetone had evaporated. The dry material was pulverized in a mortar and stored in vacuo at -10° over anhydrous $CaSO_4$ until required. Weight = 10 - 15 g. The powder was generally used within a few days of its preparation. Fig. 23 shows the decrease in enzyme activity over a period of 51 days of an acetone powder stored under the above conditions.

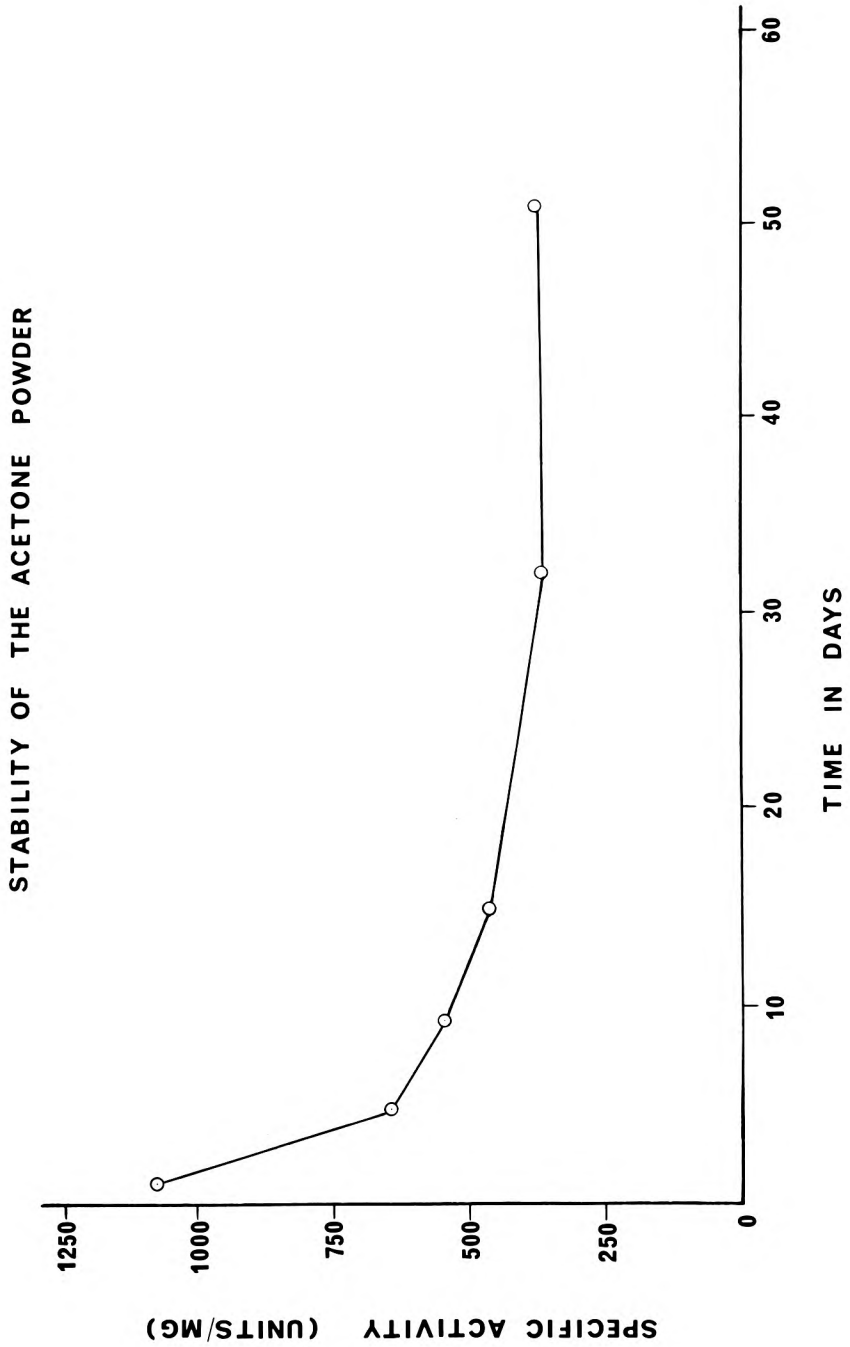
(ii) Extraction of the Acetone Powder

Acetone powder (10 g) was extracted for 30 minutes with 100 ml of buffer. The extract was centrifuged at 10,000g for 1 hour, the sediment discarded, and the clear dark red supernatant liquid (ca. 80 ml) stored at 0°. For use in the cinnabarinic acid assay system the powder was extracted with 0.05M tris/maleate buffer (pH = 6.4) containing $FeSO_4.7H_2O$ (0.83 mg/ml).

(iii) Precipitation of Protein by Acidification

The supernatant liquid obtained in (ii) was adjusted to pH = 3.5 with 1-N HCl, left at this pH for 30 minutes, and then adjusted to pH = 6.65 with 1-N NaOH. The solution was stirred magnetically throughout the addition of the acid and alkali. After standing for 15 minutes at pH = 6.65 the solution was centrifuged at 2,500g for 15 minutes and the precipitate, which contained no enzyme activity, discarded. The clear red supernatant liquid (ca. 70 ml) was stored at 0°.

FIGURE 23


STABILITY OF THE ACETONE POWDER
SPECIFIC ACTIVITY (UNITS/MG)
1250
1000
750
500
250
0
10
20
30
40
50
60
TIME IN DAYS

(iv) Acetone Fractionation

The apparatus used was based on that described by Dixon and Webb (1964) except that separate acetone/dry ice baths were used for the acetone and the enzyme extract. Fractionation was achieved by slowly adding pure, dry acetone at -25° down the side of a conical flask containing the enzyme solution obtained in (iii). The rate of addition did not exceed ca. 5 ml/minute/100 ml of enzyme extract. At the beginning, the enzyme extract was maintained at 0°, but as the acetone content of the extract increased the temperature was lowered. By the time 40% saturation with acetone had been reached the temperature of the extract had been dropped to -10° and this temperature was maintained throughout the remainder of the fractionation. The solution was stirred mechanically throughout the addition of the acetone. The enzyme extract was brought successively to 40, 60, and 80% saturation with acetone and the three precipitates so obtained were collected by centrifugation (3,000 g, 45 minutes, -12°) and dissolved in buffer. The specific activity of these fractions as compared with that of the starting material is shown in Table 5.

TABLE 5: ACETONE FRACTIONATION

Fraction	Specific Activity (units/mg)
Enzyme extract after acidification	1,150
0 - 40% saturation	14
40 - 60% saturation	57
60 - 80% saturation	81

(v) Precipitation of Nucleic Acids with Protamine Sulphate

The enzyme extract obtained in (iii) was adjusted to pH = 5.9 with 1-N HCl and a 1% solution of protamine sulphate in 0.005M tris/maleate buffer (pH = 5.0) containing $FeSO_4.7H_2O$ (83 μg/ml) and cysteine (72 μg/ml) was added dropwise with stirring. The white precipitate was collected by centrifugation at 6,500g for 15 minutes and discarded. The ratio of the absorbance of the enzyme extract at 280 mμ to that at 260 mμ was determined both before (0.898) and after (0.891) treatment with protamine sulphate. The specific activity before and after treatment with protamine sulphate was practically the same.

(vi) Adsorption of Nucleic Acids with Charcoal

Charcoal (20 mg) was suspended in ca. 5 ml buffer and added dropwise to 20 ml of the enzyme solution obtained in (iii). The mixture was stirred magnetically throughout the addition and thereafter for a further 15 minutes. The extract was centrifuged at 10,000g for 30 minutes and the clear orange supernatant liquid carefully pipetted out of the centrifuge cups so as not to disturb the very fine sedimented charcoal. The supernatant liquid so obtained was treated with another 20 mg charcoal and worked up as before. The specific activities of the charcoal-treated supernatant solutions and the original enzyme solution are shown in Table 6.

TABLE 6: ADSORPTION OF NUCLEIC ACIDS WITH CHARCOAL

Fraction	$R_{\frac{280}{260}}$	% Nucleic Acid	Protein Conc. (mg/ml)	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)
Before Charcoal	0.785	7.0	5.3	20	106	874
1st Sup.	1.000	3.0	4.8	-	-	835
2nd Sup.	0.920	4.2	3.6	-	-	786

(vii) Coagulation of Protein by Heating

(a) The effect of Different Temperatures on Enzyme Activity:

Three aliquots of the charcoal-treated solution (20 mg charcoal for every 100 mg protein) were heated rapidly with continuous stirring in a waterbath (80°) to 55, 60, and 65° respectively. The three solutions were maintained at these temperatures for 5 minutes by immersion in oil baths at the required temperature. During the entire heating procedure nitrogen was blown gently onto the surface of the solutions. After 5 minutes in the oil baths the solutions were cooled rapidly to 0°, allowed to stand at this temperature for 30 minutes, and the precipitates, which had formed during the heating, collected by centrifugation at 6,500g for 30 minutes and discarded. The specific activities of the three supernatant solutions so obtained were compared with that of the unheated enzyme solution (Table 7).

TABLE 7: THE EFFECT OF HEATING AT DIFFERENT TEMPERATURES ON ENZYME ACTIVITY

Fraction	Protein Conc. (mg/ml)	Specific Activity (units/mg)
Charcoal-treated supernatant	4.62	276
Heated for 5 minutes at 55°	3.95	710
Heated for 5 minutes at 60°	2.72	122
Heated for 5 minutes at 65°	2.40	19

(b) The Effect of the Duration of Heating on Enzyme Activity

Three aliquots of charcoal-treated enzyme solution were heated at 55° for 5, 10, and 15 minutes respectively, and worked up in the same way as described in (a). The results are shown in Table 8.

TABLE 8: THE EFFECT OF DURATION OF HEATING ON ENZYME ACTIVITY

Fraction	Protein Conc. (mg/ml)	Specific Activity (units/mg)
Charcoal-treated supernatant	3.22	294
Heated at 55° for 5 minutes	2.57	408
Heated at 55° for 10 minutes	2.53	191
Heated at 55° for 15 minutes	2.53	199

(viii) Ammonium Sulphate Fractionation

Solid $(NH_4)_2SO_4$ was added with continuous stirring to the charcoal-treated supernatant solution. The rate of addition never exceeded *ca.* 1g/10 ml solution/minute. After the addition of the desired amount of salt the solution was stirred for 30 minutes and then centrifuged at 23,000*g* for 30 minutes. The precipitates were dissolved in the minimal volume of buffer and dialyzed against 100 volumes of buffer for 12 hours. The cellophane dialysis tubing was soaked in buffer for 30 minutes before use. The weight of $(NH_4)_2SO_4$ required to bring the enzyme solution to a particular percentage saturation was calculated from the formula described by Kunitz (1952):

$$x = \frac{51.5(S_2 - S_1)}{1.0 - 0.272(S_2)}$$

where, x is the amount of $(NH_4)_2SO_4$ in g to be added to 100 ml of solution at saturation S_1 to bring it to saturation S_2. Fig. 16 shows the results of the fractionation. As a routine procedure the fraction precipitating between 50 and 70% was collected and dialyzed.

(ix) Cγ-Alumina Gel Adsorption

(a) Adsorption of the Enzyme onto the Gel

A suspension of Cγ-alumina gel in distilled water was added dropwise with continuous stirring to the dialyzed enzyme solution obtained in (viii). For the purposes of the gel adsorption experiments the $(NH_4)_2SO_4$-fractionated enzyme was dissolved in 0.005M tris/maleate buffer (pH = 5.4) containing $FeSO_4.7H_2O$ (83 μg/ml) and cysteine (72 μg/ml). After the addition of the desired amount of gel the solution was stirred for 15 minutes and then centrifuged at 6,500g for 30 minutes. The gel was suspended in 0.005M tris/maleate buffer (pH = 5.4) containing the usual amounts of $FeSO_4$ and cysteine. The amount of gel added to the enzyme solution was expressed as the Gel/Protein ratio which is defined as:

$$\frac{\text{Dry weight of gel (mg)}}{\text{Total weight of protein (mg)}}$$

Aliquots of the dialyzed enzyme solution were treated with the gel suspension and the Gel/Protein ratios brought to 0.24, 0.36, 0.47, 0.71, and 0.94 respectively. The specific activities of the supernatant solutions obtained are shown in Fig. 17.

(b) Removal of the Enzyme from the Gel

The gel-fractions containing the adsorbed enzyme were combined and washed by suspension in a series of different solvents. The suspension was then centrifuged at 6,500g for 30 minutes, and the supernatant liquid assayed for enzyme activity. Washing with 0.005M tris/maleate buffer (pH = 5.4) containing the usual amounts of $FeSO_4$ and cysteine, distilled water, and 0.005M tris/maleate buffer (pH = 7.6), failed to remove the enzyme from the gel

surface. An 0.005M tris/maleate buffer (pH = 7.6) containing 10% $(NH_4)_2SO_4$ removed the enzyme but only in very poor yield.

(x) Sephadex Gel Filtration (cf. Andrews - 1964, 1965)

Sephadex G-200 gel (1.5 g) was suspended in 500 ml tris/maleate buffer (pH = 6.4) and allowed to swell for 3 days at 0°. The slurry was poured carefully into a 1.5 x 30 cm column filled with the same buffer. Any air pockets which formed were removed under vacuum. The gel column was supported by a glass-wool plug covered with a 2 cm thick layer of pure sea sand. The column was kept at 0 - 5° by pumping alcohol from a refrigerated alcohol bath at 0° through a jacket which completely surrounded both the column and its reservoir. The gel was allowed to settle for 24 hours during which time 0.005M tris/maleate buffer (pH = 6.4) was allowed to perculate through the column. After about 24 hours the flow rate remained constant at ca. 15 ml/hour, provided that the 500 ml reservoir was kept full. Once the flow rate had stabilized the 0.005M tris/maleate buffer (pH = 6.4) was replaced with one of the same molarity and pH but containing $FeSO_4.7H_2O$ (83 µg/ml) and cysteine (72 µg/ml). The column was allowed to equilibrate with the new buffer for 48 hours. Unfortunately the sephadex gel appeared to concentrate ferric hydroxide from the buffer and the column turned a deep reddish-brown colour.

(xi) Summary of the Final Purification Scheme

Table 9 outlines the purification scheme finally adopted for routine purposes. Fig. 15 shows the absorption spectra of the enzyme solutions at each of the four stages of purification referred to in Table 9. Each enzyme solution was diluted 100 times with distilled water before the spectrum was recorded.

TABLE 9: PURIFICATION PROCEDURES ADOPTED BY THE PRESENT AUTHOR

Procedure	Volume (ml)	Conc. (units/ml)	Total Units	R $\frac{280}{260}$	% Nucleic Acid	Protein Conc. (mg/ml)	Specific Activity (units/mg)	Yield %	Purification (times)
Supernatant from acetone powder extraction	84	8,300	697,200	0.92	4.5	26.0	320	100	1
Supernatant after acidification to pH = 3.5	74	8,500	629,000	0.77	7.5	8.7	978	90	3
Supernatant after treatment with 20 mg charcoal per 100 mg protein	70	7,300	511,000	0.98	3.5	8.0	913	73	3
Fraction collected between 50 and 70% saturation with $(NH_4)_2SO_4$	40	31,500	1,260,000	1.20	1.75	3.1	10,170	180	32

PART V

SOME PROPERTIES OF 3-HYDROXYANTHRANILIC ACID OXIDASE

Note: (i) The enzyme used in this section was purified by acidification, charcoal adsorption, and ammonium sulphate fractionation (Table 9).

(ii) All assays in this section were based on the measurement of the formation of 2-amino-3-acroleylfumaric acid or of the methyl ketone (p. 56) derived from 4-methyl-3-hydroxyanthranilic acid. In all cases measurements were made at 360 mμ at a temperature of 23 - 25°.

(iii) The buffer used throughout was 0.005M tris/maleate (pH = 6.4) containing $FeSO_4.7H_2O$ (83 μg/ml) and cysteine (72 μg/ml).

(iv) In all cases the reaction was started by the addition of the enzyme.

(i) Reaction Spectra with 3-Hydroxyanthranilic acid as Substrate

The absorption spectrum of a solution initially containing 26.67 μg/ml of 3-hydroxyanthranilic acid and enzyme (final protein concentration = 0.080 mg/ml) in 3 ml buffer was measured at various intervals after the reaction had been initiated against a blank containing enzyme and buffer but no 3-hydroxyanthranilic acid (Fig. 18).

(ii) Reaction Spectra with 4-Methyl-3-hydroxyanthranilic acid as Substrate

A stock solution of 4-methyl-3-hydroxyanthranilic acid in absolute alcohol (0.0088 g/5 ml) was stored at -10° until required. The stock solution was diluted considerably with buffer before use in the assay system.

The absorption spectrum of a solution initially containing 29.33 μg/ml of 4-methyl-3-hydroxyanthranilic acid and enzyme (final protein concentration = 0.149 mg/ml) in 3 ml buffer was measured at various intervals after the reaction had been initiated against a blank containing enzyme and buffer but no 4-methyl-3-hydroxyanthranilic acid (Fig. 19).

(iii) Ultra-violet Spectra of Some Pyridine Carboxylic Acids

The ultra-violet spectra of quinolinic, 6-methylquinolinic, nicotinic, and picolinic acids were measured in buffer (Fig. 20).

(iv) 3-Methoxyanthranilic acid

A stock solution of 3-methoxyanthranilic acid in absolute alcohol (0.0088g/5ml) was stored at -10° until required. The stock solution was diluted considerably with buffer before use in the assay system.

The absorption spectrum of a solution containing 29.33 μg/ml of 3-methoxyanthranilic acid showed no change after enzyme had been added, indicating that 3-methoxyanthranilic acid is not metabolised by the enzyme.

(v) Determination of the Michaelis' Constants for 3-Hydroxyanthranilic acid and 4-Methyl-3-hydroxyanthranilic acid

The Michaelis' constants were determined by measuring the initial velocity of the enzyme reaction at different substrate concentrations, varying from 1.67 μg/ml to 53.33 μg/ml for 3-hydroxyanthranilic acid and from 1.83 μg/ml to 58.67 μg/ml for 4-methyl-3-hydroxyanthranilic acid. Fig. 21 shows the Lineweaver and Burk (1934) reciprocal plots obtained from these measurements. The same enzyme solution was used in both the K_m determinations (Final protein concentration = 0.034 mg/ml). The blanks contained no substrate. Table 10 summarizes the results obtained.

TABLE 10: COMPARISON OF 3-HYDROXYANTHRANILIC ACID AND 4-METHYL-3-HYDROXYANTHRANILIC ACID AS SUBSTRATES FOR 3-HYDROXYANTHRANILIC ACID OXIDASE

Substrate	K_m	V_{max}
3-Hydroxyanthranilic acid	0.74×10^{-5}M	455 units/minute
4-Methyl-3-hydroxyanthranilic acid	0.79×10^{-5}M	345 units/minute

(vi) Determination of the Inhibitor Constant for 5-Methyl-3-hydroxyanthranilic acid

A stock solution of 5-methyl-3-hydroxyanthranilic acid (Diamond and Levy - unpublished results) in absolute alcohol (0.0088 g/5ml) was stored at -10° until required. The stock solution was diluted considerably with buffer before use in the assay system.

The absorption spectrum of a solution containing 29.33 µg/ml of 5M3HAA showed no change after enzyme had been added, indicating that 5-methyl-3-hydroxyanthranilic acid is not metabolised by the enzyme.

The K_i for 5-methyl-3-hydroxyanthranilic acid was determined by measuring the initial velocity of the enzyme reaction with concentrations of 3-hydroxyanthranilic acid varying from 1.67 µg/ml to 53.33 µg/ml in the presence of two fixed concentrations of inhibitor, viz., 23.33 µg/ml and 11.67 µg/ml. The Lineweaver and Burk reciprocal plots (Fig. 21) show typical competitive inhibition. The blanks contained 5-methyl-3-hydroxyanthranilic acid and enzyme but no 3-hydroxyanthranilic acid. The same enzyme solution as was used in (v) was utilized (final protein concentration = 0.034 mg/ml).

In fact, the determinations of the Michaelis' and Inhibitor constants were carried out as quickly as possible, one after the other, within the space of about 4 hours. The K_i for 5-methyl-3-hydroxyanthranilic acid is 1.26×10^{-5}M.

S U M M A R Y

(i) 3-Hydroxyanthranilic acid-3-^{14}C has been synthesized from phenol-1-^{14}C.

(ii) An attempt has been made to synthesize 4-methyl-3-hydroxyanthranilic acid-3-^{14}C from phenol-1-^{14}C, but the preparation of N-benzyl-4-methyl-8-ethoxyquinoline bromide from 4-methyl-8-ethoxyquinoline was unsuccessful.

(iii) A 2,4-dinitrophenylhydrazone of the primary enzymic oxidation product of 3-hydroxyanthranilic acid (or of one of the acid degradation products of the primary oxidation product) has been prepared. The characteristics of this derivative have been compared with those of a similar compound obtained by Wiss and Bettendorf (1957).

(iv) Partial purification of rat liver 3-hydroxyanthranilic acid oxidase has been achieved: the successful purification techniques used were acidification, charcoal adsorption, and ammonium sulphate fractionation. The purified enzyme is thirty times more active than an extract of rat liver acetone powder.

(v) The Michaelis' constants for 3-hydroxyanthranilic acid and 4-methyl-3-hydroxyanthranilic acid have been determined. Both 3-methoxyanthranilic acid and 5-methyl-3-hydroxyanthranilic acid are not metabolised by 3-hydroxyanthranilic acid oxidase; the 5-methyl compound is a competitive inhibitor of the enzyme.

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