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Author
Nunes, Mathews Anthony

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PILOCARPINE: STUDIES ON ITS BIOSYNTHESIS AND IN VITRO DEGRADATION

by

Mathews Anthony Nunes
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DISSERTATION

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ABSTRACT

The biosynthesis of pilocarpine in Pilocarpus pennatifolius was studied by the administration of radio-labeled precursors. Radioactive sodium acetate, histidine, histidinol, methionine and threonine were administered by the cut stem method. Histidine, methionine and threonine were administered together by a wick inserted through the stem of an intact plant. Sodium acetate and histidine were fed to root cuttings by suspending the roots in aqueous solutions of the precursors. After 64 to 75 hours, the roots were harvested and total alkaloid extracts made. These extracts were then fed to stem cuttings.

Of the cut stem feedings only L-methionine-(methyl-^{14}C) showed a significant incorporation of 0.06% into pilocarpine. Subsequent degradation showed 98% of the radioactivity located in the N-methyl group. The wick feeding showed no incorporation of radioactivity into pilocarpine after 7 days. The total alkaloid extracts from the root feedings showed significant radioactivity. However, the pilocarpine isolated after feeding of the extracts to the stems was not radioactive.

Many of the feedings could not be performed under ideal conditions. Three sources of plants were used.

One plant was in poor health and died within six months of feeding. Pilocarpine was found not to be the principal alkaloid produced by the plants used in another feeding. Thus, a lack of incorporation from these feedings is not definitive evidence that the precursor is not a reactant in the biosynthetic scheme.

The methyl group of methionine has been found to be the biological source of the N-methyl group of pilocarpine. This portion of the biosynthesis occurred within the leaves. At present it seems reasonable that pilocarpidine is biosynthesized in the roots and translocated to the leaves where it is N-methylated to pilocarpine.

The kinetics of the hydroxide-ion catalyzed hydrolysis and epimerization of pilocarpine in aqueous solution has been investigated utilizing pH-stat titrimetry and NMR spectroscopy. The mechanism of epimerization involves formation of a carbanion stabilized by resonance with the enolate hybrid. Both hydrolysis and epimerization follow pseudo first-order kinetics, and the appropriate rate constants and energies of activation have been calculated. Epimerization has been found to occur to a greater extent than previously assumed, and must be considered as one of the major pathways of degradation and inactivation of pilocarpine. The rate of hydroxide-ion catalyzed epimerization increases more rapidly with temperature than does the rate of hydrolysis, a fact

which should be taken into account if ophthalmic solutions of pilocarpine are sterilized by heat. It is suggested that isopilocarpine may not be a genuine jaborandi alkaloid, but an artifact produced by epimerization of pilocarpine during drying, storage and extraction of the plant material.

ACKNOWLEDGMENTS

To my wife Kathy and our children whose love, inspiration and endless patience have been invaluable to me. I also thank my parents for their encouragement throughout the years.

This work was performed under the supervision of Dr. Einar Brochmann-Hanssen to whom I am thankful. I wish to express my gratitude to Dr. Neal Castagnoli, Jr. whose counseling and discussions have guided me. I also thank Dr. Kent S. Marshall for his consultation and advice.

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PREFACE

The subject matter is presented in two parts. The first part describes the work which has been done on the elucidation of the biosynthetic pathway of pilocarpine. The second part deals with the analytical method for the determination of pilocarpine, and the kinetics of hydrolysis and epimerization in alkaline aqueous solution. Since the topics are distinct, they are presented with an individual introduction and bibliography for each.

PART I

BIOSYNTHETIC STUDIES

I. INTRODUCTION

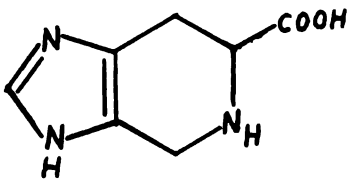
Since the isolation of morphine by Serturner in 1806, more than three thousand alkaloids have been isolated from natural sources. Alkaloids are most often found in higher plants, but also occur in animals and fungi, constituting the most diverse classification of natural compounds. Their diversity stems from the fact that the term alkaloid is not a chemical designation, but a name traditionally applied to a group of nitrogen-containing, basic substances from natural sources with varying chemical and physiological properties. More recently, N-heterocyclic compounds which originate from amino acids have been termed 'real alkaloids', while substances which originate from amino acids but have the nitrogen in an aliphatic linkage are called 'protoalkaloids'. In this proposed system the term 'pseudoalkaloids' is restricted to molecules which are formed by the addition of ammonia into normally non-nitrogen moieties such as isoprenoids (1).

The function of alkaloids in plants is still a matter of controversy. Some of the possibilities which have been proposed are that they serve as by-products of plant metabolism, as reserve materials capable of supplying nitrogen, as protective substances, discouraging animal or insect attack, and as plant stimulants or regulators similar to hormones (2). Although a few exceptions are known, there is only very slight evidence

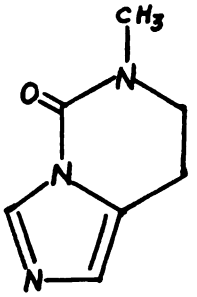
that alkaloid formation serves any function useful to the plant. It is probably best to regard alkaloid formation as a metabolic process which begins with substances essential to the plant and ends with compounds which do not necessarily serve an essential function.

The natural amino acid histidine and its decarboxylation product histamine are the most widely distributed imidazole derivatives in nature. In spite of this abundance, only a relatively few imidazole alkaloids have been discovered. In addition to the jaborandi alkaloids, spinacine (I), zapotidine (II), dolicotheline (III) and isomacrorine (IV) are among the few reported (3, 4, 5, 6). To date dolicotheline is the only imidazole alkaloid which has been shown by tracer studies to utilize histamine and, to a lesser extent, histidine (7, 8) in its biosynthesis.

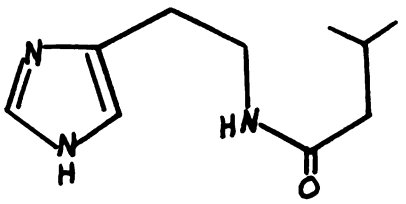
The biosynthesis of histidine is closely related to purine metabolism and has been clarified recently by Luckner (9) who based his conclusions on the cell free synthesis of D-erythro-imidazole glycerol phosphate ester by three enteric bacterial species (10). It was found that imidazole glycerol phosphate was formed from 5-phosphoribosyl pyrophosphate, the amide nitrogen of glutamine, and the N-1 and C-2 portion of the adenine ring of ATP as shown in Figure 1. The conversion of D-erythro imidazole glycerol phosphate (VII) to histidine proceeds first to imidazole acetol phosphate (VIII)



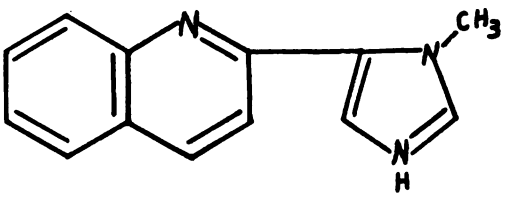
Spinacine
(I)



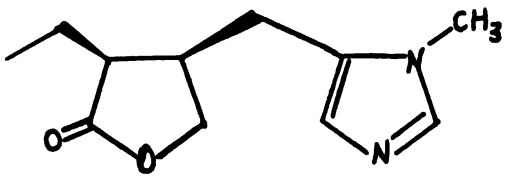
Zapotidine
(II)



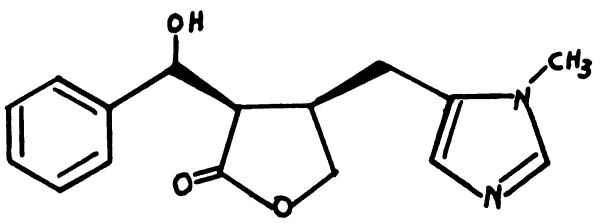
Dolicotheline
(III)



Isomacrorine
(IV)



Pilocarpine
(V)



Pilosine
(VI)

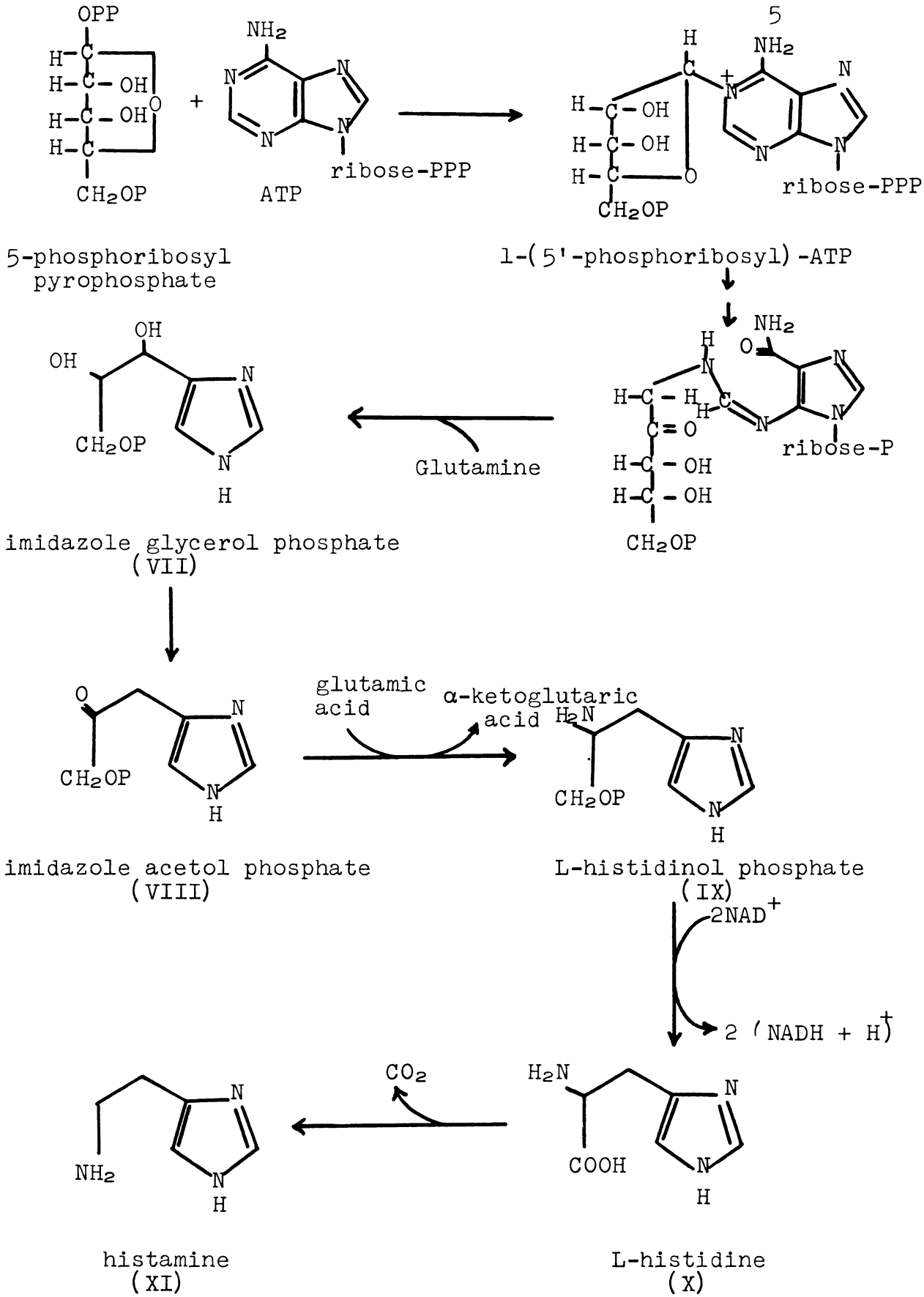


Figure 1. Biosynthesis of L-Histidine

by the enzyme imidazole glycerol phosphate dehydrase. This compound is then converted to L-histidinol phosphate (IX) through a transamination reaction with glutamic acid. After enzymatic cleavage of the phosphate, the L-histidinol thus formed is oxidized to L-histidine (X). This oxidation occurs in the presence of L-histidinol dehydrogenase and NAD^+. Histamine (XI) is produced when L-histidine undergoes enzymatic decarboxylation.

Due to the structural similarity between the jaborandi alkaloids and histidine, it is likely that the amino acid or a species in its biosynthetic or degradative pathway is involved. Since pilocarpine is the most abundant and the most physiologically important of the jaborandi alkaloids, our biosynthetic studies were directed toward it. Robinson (11) proposed in 1955 that pilocarpine arose from a combination of the histidine precursor imidazole acetol phosphate and either two molecules of acetate or a four carbon unit such as butyrate or acetoacetate. This postulate has been generalized by Boit (12) and is depicted with additional detail in Figure 2.

Reaction 1 is esterification of acetoacetyl CoA with the alcohol function of imidazole acetol phosphate. Reaction 2 is a type of aldol condensation which has been shown to occur in the plant biosynthesis of the butenolide ring of the cardiac glycosides (13). Reaction 3 is dehydration and is followed by a stereospecific reduction of the butenolide

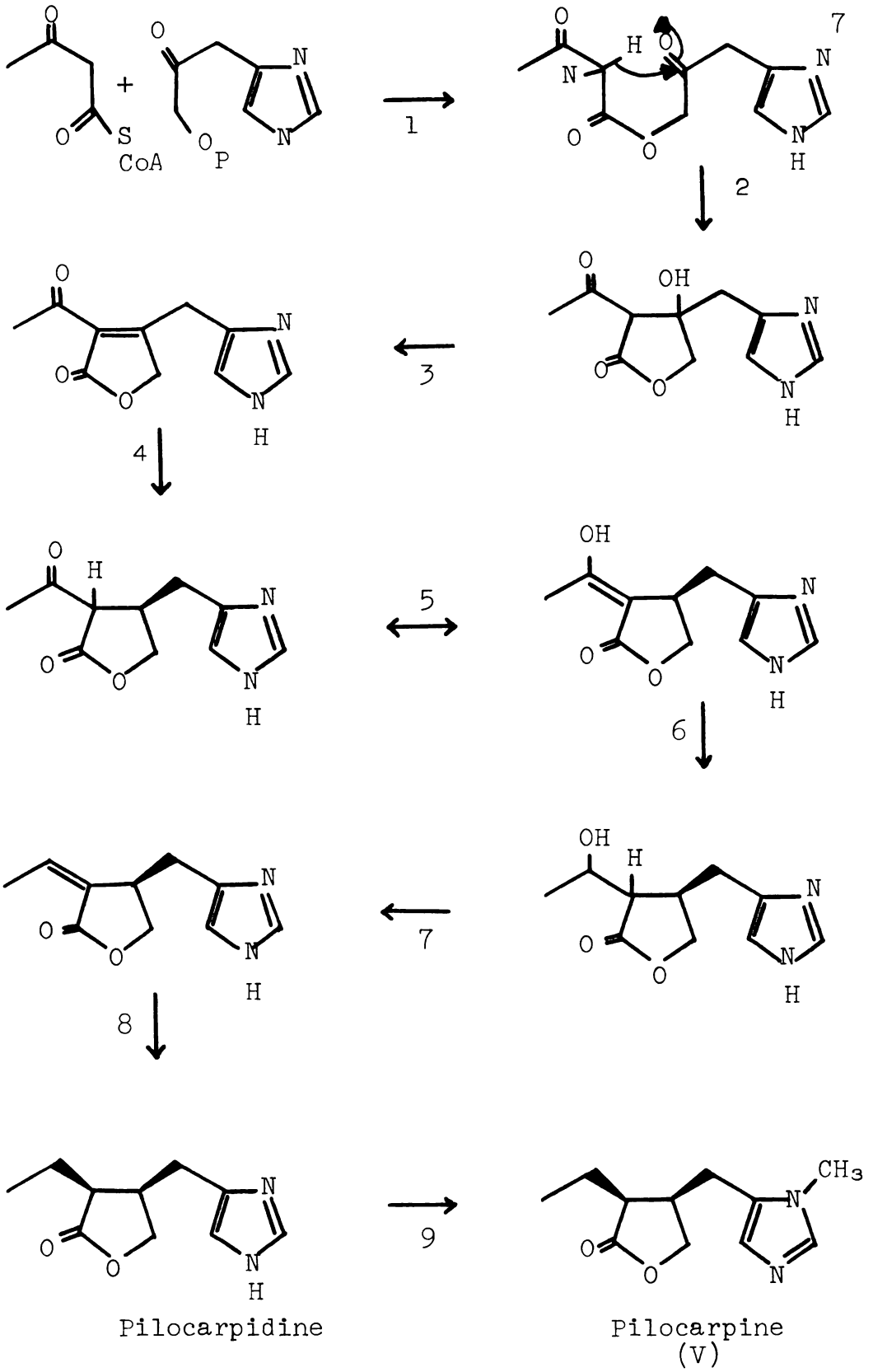


Figure 2. Proposed Biosynthetic Pathway for Pilocarpine (A).

produced (reaction 4). Reactions 5, 6, 7 and 8 are quite analogous to the steps for chain elongation of the fatty acids, and result in pilocarpidine. Methylation by S-adenosylmethionine yields pilocarpine (reaction 9). Chemically, as well as *in vivo*, methylation normally occurs at the 3-position of the imidazole. However N-1 methylation is known to occur in the production of isomacrorine (IV) in the shrub *Macrorungia longistrobus* (6). Pilosine (VI) which has a hydroxybenzyl group instead of the ethyl substituent on the lactone is also a naturally occurring jaborandi alkaloid. The presence of this hydroxyl group leads one to consider the possibility of threonine serving as a precursor for the four carbon unit of pilocarpine. A proposed biosynthetic pathway using the threonine metabolite α-ketobutyrate (XII) and the histidine metabolite urocanic acid (XIII) is illustrated in Figure 3. Condensation of these two metabolites would produce the hydroxydiacid (XIV) which could be reduced and dehydrated to the substituted maleic acid (XV). Selective reduction of one carboxyl to the alcohol (XVI) could then result in cyclization to the butenolide (XVII). A stereospecific reduction of the butenolide would give pilocarpidine (XVIII). Subsequent N-methylation by S-adenosylmethionine yields pilocarpine.

Until a relatively short time ago, the metabolic fate of a compound could be followed only with great difficulty. Since the end of the Second World War, however, isotopes

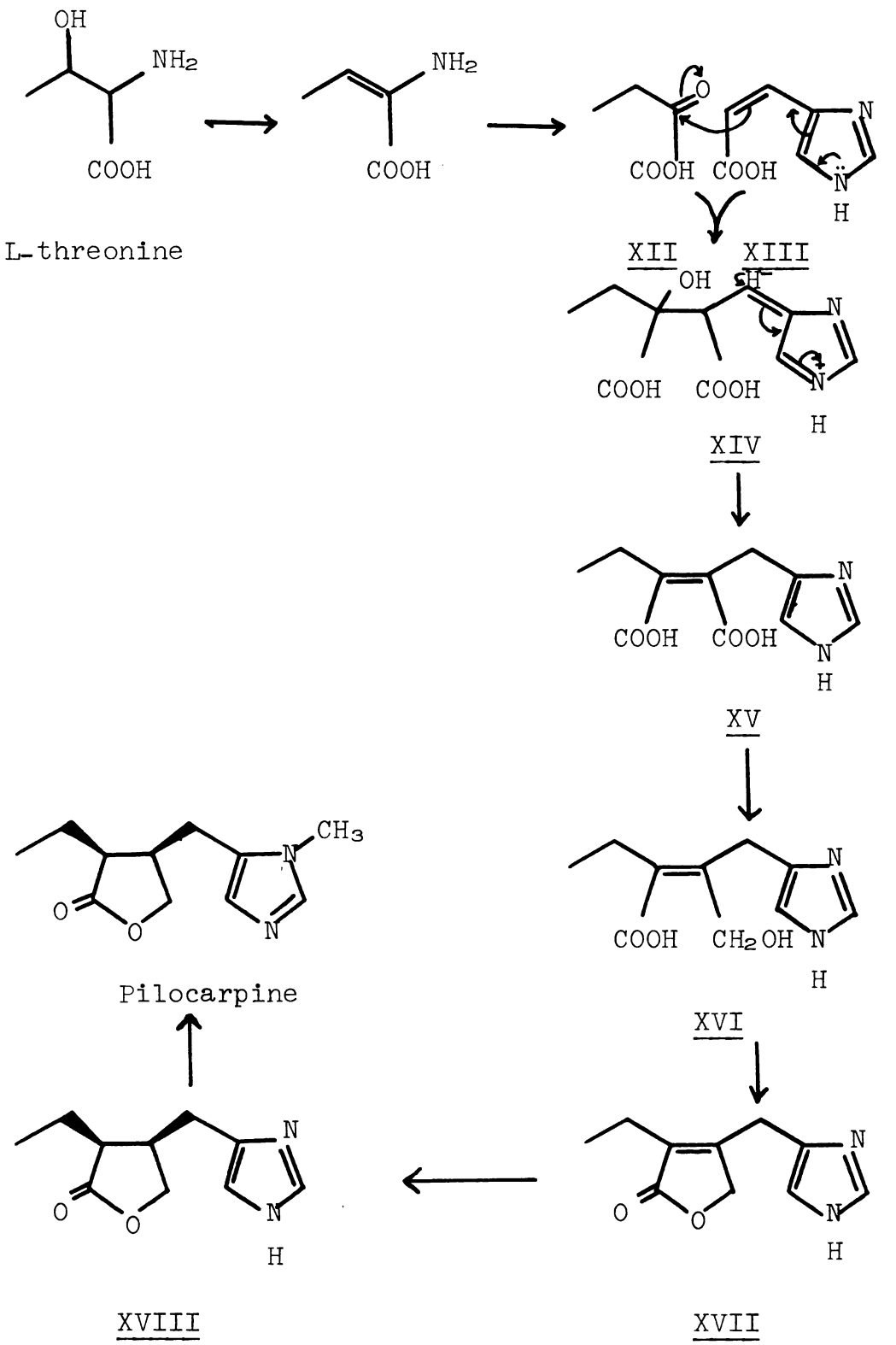


Figure 3. Proposed Biosynthetic Pathway for Pilocarpine (B).

have become increasingly available and now are the most powerful single tool for biosynthetic and metabolic studies. Administration of isotopically labeled compounds enables the investigator to determine into which substance the labeled portion has been incorporated and, equally importantly, to what extent it is incorporated. Success depends on the correct choice of precursor, effective distribution to the active site, and a selective degradation of the metabolite to determine the position of radioactive incorporation. One must be careful in choosing the labeling pattern so as to be able to recognize the pattern of the precursor in the metabolite. When doing in vivo experiments, consideration must also be given to the possibility that the precursor is treated in the same manner as endogenous compounds (i.e. little or no isotope effect is involved), and that the radioactivity is not of such a quantity as to cause radiation induced disruptions in the normal metabolism of the organism.

This study was undertaken to test the proposed biosynthetic scheme for pilocarpine. The intent was to feed specifically labeled precursors, and to extract and purify the alkaloid to determine the specific activity and extent of incorporation. The position of the label was to be determined by selective degradative procedures.

II. EXPERIMENTAL APPROACH

1. Introduction

Feeding experiments were conducted at the University of Munich, the University of Tubingen in Germany and at the University of California Medical Center. All feedings were performed with Pilocarpus pennatifolius. The plants at the Medical Center were grown from seeds obtained from the Munich plant, while the Tubingen plants were grown from seeds obtained from an independent source.

The difficulty of selecting the correct plant part is of major importance in examining a biosynthetic pathway. This may be circumvented by using the whole plant. However, we could not do this due to our extreme difficulty in obtaining plant material. Seeds of Pilocarpus jaborandi which were donated by the Brazilian agriculture department failed to germinate here. Requests from other sources also failed. Currently four mature Pilocarpus pennatifolius plants are growing in the Medical Center greenhouse. These are the products of numerous seeds from the plant grown in Munich. Since the greatest concentration of pilocarpine is found in the leaves, cut stems were first chosen for feeding experiments. As data from these feedings accumulated, feeding by a wick method and cut root sections were investigated.

2. Munich Feedings

These feedings and the isolation of alkaloids were graciously done by Dr. Clara Kiraly Olah, and employed the cut stem method of feeding (14). In this method, a branch of the plant was severed and immediately immersed in water. While still under water, an additional 1/2 inch was cut from the end and the stem carefully transferred, with a drop of water clinging to the end, to a test tube containing an aqueous solution of the radioactive precursor. It was necessary to keep the cut end wet since the transport system within the vascular cambium would be damaged by exposure to air. Distilled water was then added as needed and the feeding continued until the leaflets became quite wilted, usually two to four days. During these experiments the plant material was maintained under "daytime" conditions of 32° and 80% humidity for twelve hours and "nighttime" conditions of 22° and 80% humidity for twelve hours. The plant material was then harvested and, if extraction was not performed immediately, placed in plastic bags and stored in a freezer. After 24 hours of feeding approximately 98% of the radioactivity (determined by a Geiger-Muller counter) had been absorbed by the leaves, the maximum radioactivity appearing in the top leaflets. The radioactive precursors used in these feedings were sodium acetate-(2-^{14}C), and DL-histidine-(ring-2-^{14}C).

3. Tubingen Feedings

These feedings and the initial extractions were diligently performed by Dr. E. Brochmann-Hanssen and were done by two

methods: a) the cut stem method described previously, and b) the wick method (14). During the feedings, the plant material was maintained under "daytime" conditions of 28° and high humidity with fluorescent lighting for approximately twelve hours, and "nighttime" conditions of 22° and low humidity without light for twelve hours. The plant material for the cut stem feedings was kept in the radioactive solution for the "daytime" periods, and transferred to an aqueous solution of half-strength plant fertilizer at night. The cut stem feedings were continued for three to four days before being harvested. For the wick feeding, a plant about three feet tall was used, and a cotton wick inserted with a needle between the bark and the woody portion of the stem about one inch above the soil. The ends of the wick were placed in an aqueous solution of the radioactive precursors. The radioactive solution was drawn through the wick into the plant as water was required for metabolism and respiration. The primary advantage of this method was that, with the exception of the trauma involved with inserting the wick, the plant remained in its normal state. Thus, unless the vascular cambium, which is responsible for transport within the plant, had been seriously damaged, the precursor was distributed to the root system as well as the aerial portions (scion) of the plant. The wick feeding was continued for seven days prior to harvesting the scion.

The precursors chosen for the cut stem feedings were L-histidinol-(ring-2-^{14}C) which was synthesized in our

laboratory, L-methionine-(methyl-^{14}C) and DL-treonine-(2-^{14}C). Separate feedings were used for each of these potential precursors. Since the wick feeding was done at the expense of the whole plant, L-histidine-(ring-2-^{14}C), L-methionine-(methyl-^{14}C) and DL-threonine-(2-^{14}C) were all dissolved in the solution which was fed by this method.

4. U. C. Medical Center Feedings

Cut stem feedings were performed in our greenhouse employing the technique previously described. The plant material was maintained within the greenhouse under its normal light, temperature and humidity conditions. These feedings were performed with L-histidine-(ring-2-^{14}C), L-methionine-(ring-2-^{14}C), L-threonine-(U-^{14}C) and sodium acetate-(1-^{14}C). In addition, attempts were made to achieve incorporation by what we called an "excised root suspension". Approximately fifty grams of small root material was cut, immediately immersed in sterile distilled water, and washed to remove as much soil as possible. The roots were then rinsed with sterile distilled water and packed into an autoclaved test tube containing an aqueous solution of the potential precursor. A sterile cotton plug was placed in the top, and the tube kept in the dark for two to three days. The length of time depended on the extent of microbial growth as evidenced by the formation of a "scum" on the top of the water. The roots were harvested and homogenized in a Waring blender using acidified methanol (1 ml. of concentrated nitric

acid per liter of methanol). The homogenate was percolated with the acidified methanol until negative toward Dragendorff's reagent. The methanol was evaporated under reduced pressure, the brown residue dissolved in cold water, and brought to pH 9 with sodium bicarbonate. The bases were then extracted with cold chloroform. The chloroform extracts were made acidic with 0.1 N hydrochloric acid in methanol and evaporated to dryness. The residue was dissolved in 10 ml. of absolute methanol and a 0.1 ml. aliquot removed for scintillation counting. The radioactivity data of the extracts appears in Table II. The methanol was evaporated to give the total alkaloid extract. The extract was dissolved in water, adjusted to pH 6 and 20 mg. of non-radioactive L-methionine added. This was then used as the precursor solution for a cut stem feeding.

5. Synthesis of Labeled Precursor L-histidinol-(ring-2-^{14}C).--(15)

L-histidinol-(ring-2-^{14}C) (X) was benzoylated with benzoyl chloride under Schotten-Baumann conditions to give α-N-benzoyl-L-histidine (XI). The carboxyl group was esterified by saturating a solution of XI in methanol with hydrogen chloride gas to give α-N-benzoyl-L-histidine methyl ester (XII). The ester function was reduced in tetrahydrofuran with lithium aluminum hydride to α-N-benzoyl-histidinol (XIII). The benzoyl group was removed by hydrolysis in refluxing hydrochloric acid to L-histidinol-(ring-2-^{14}C) dihydrochloride (XIV). The synthetic sequence is illustrated in Figure 4.

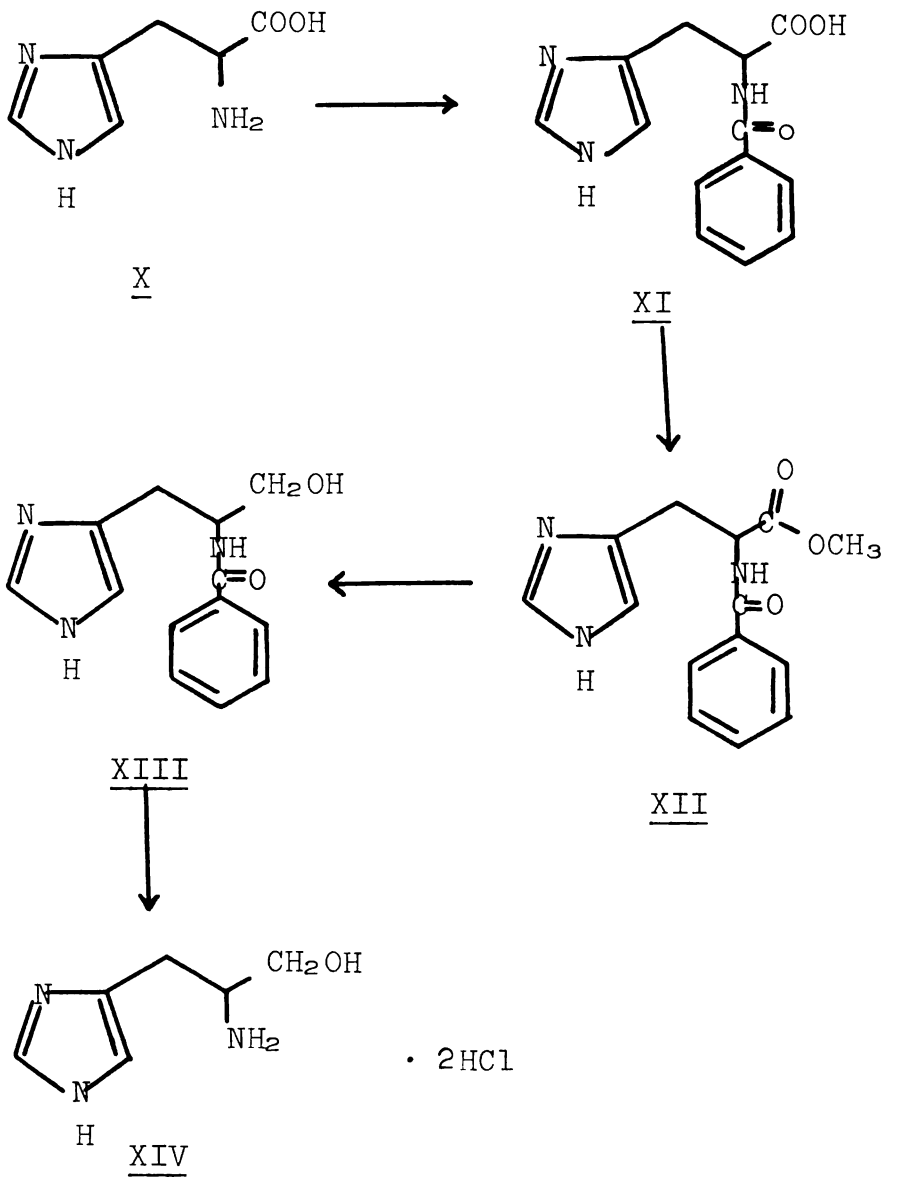


Figure 4. Synthetic Scheme of L-Histidinol Dihydrochloride.

6. Isolation of Pilocarpine

The plant material was cut with pruning shears and homogenized with acidic methanol (1 ml. concentrated nitric acid per liter of methanol) in a Waring blender. Non-radioactive pilocarpine nitrate was added for reverse isotope

dilution, and the material percolated with acidified methanol until the percolate was negative to Dragendorff's reagent. The methanol was removed using a rotary vacuum evaporator and the residue dissolved in water. Chlorophyll and other non-basic materials were removed by extraction with chloroform. The aqueous phase was cooled, brought to pH 9 with sodium bicarbonate, and extracted with cold chloroform to separate the alkaloids from any remaining impurities. The chloroform extract was acidified with methanolic nitric acid and evaporated under reduced pressure to give impure pilocarpine nitrate which was purified by repeated recrystallization from 95% ethanol to constant radioactivity. The extraction procedure is outlined in Figure 5.

7. Determination of Radioactivity

The radioactivity was determined by liquid scintillation counting using a toluene-dioxane-ethanol scintillation fluid. It was necessary to dissolve the pilocarpine nitrate in 0.1 ml. of absolute methanol prior to adding the scintillation fluid. Toluene-^{14}C was used as an internal standard. Only the pilocarpine nitrate isolated from the L-methionine-(methyl-^{14}C) feeding done at the Medical Center showed a significant radioactivity of 6,568 disintegrations $min.^{-1}$ $mg.^{-1}$ or 890 $\mu Ci.\ mole^{-1}$. The percentage of incorporation was 0.06.

8. Degradative Plan

The precursors used and the distribution of radioactivity which would result upon incorporation are shown in Figure 6.

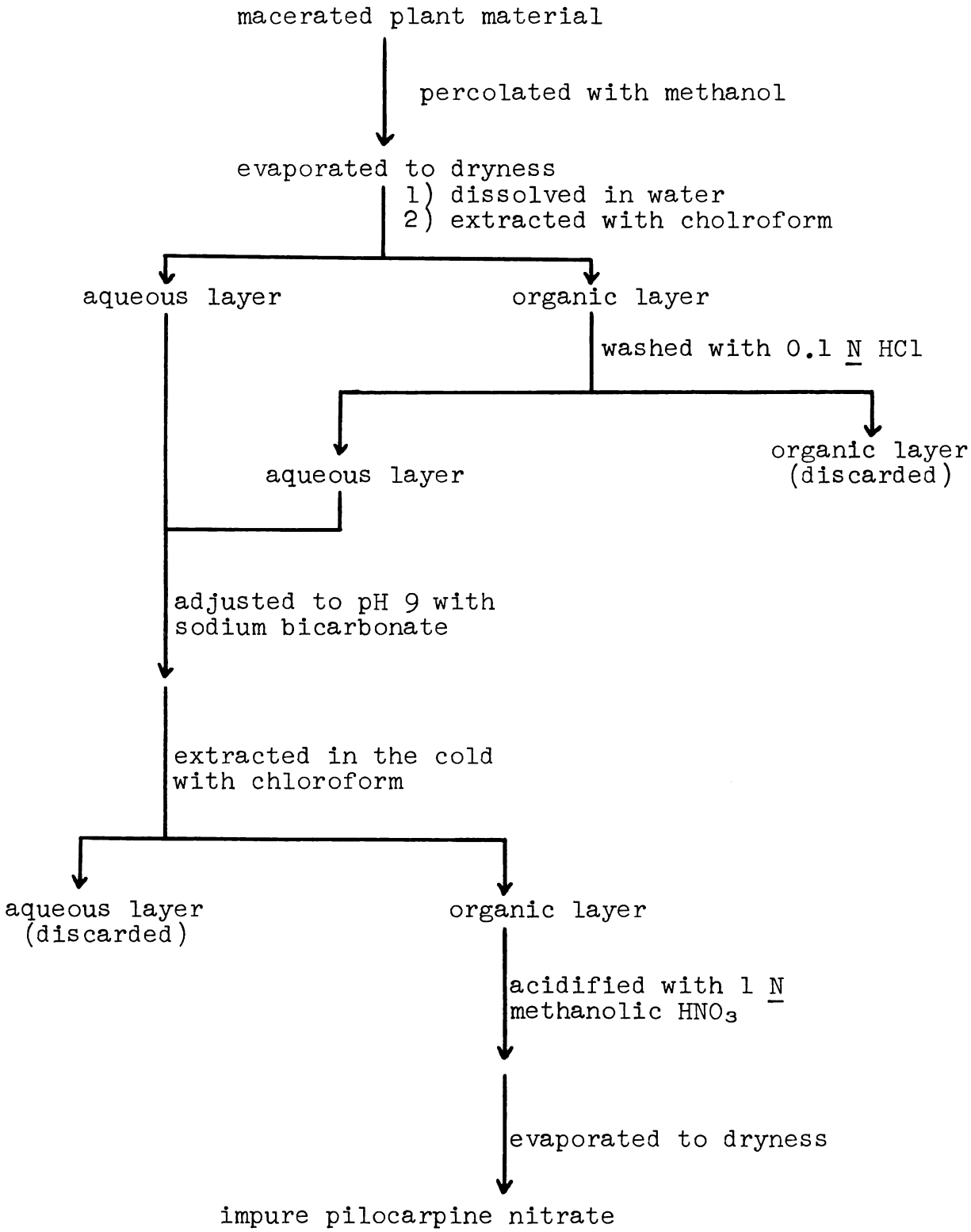


Figure 5. Extraction Scheme for Isolation of Pilocarpine Nitrate

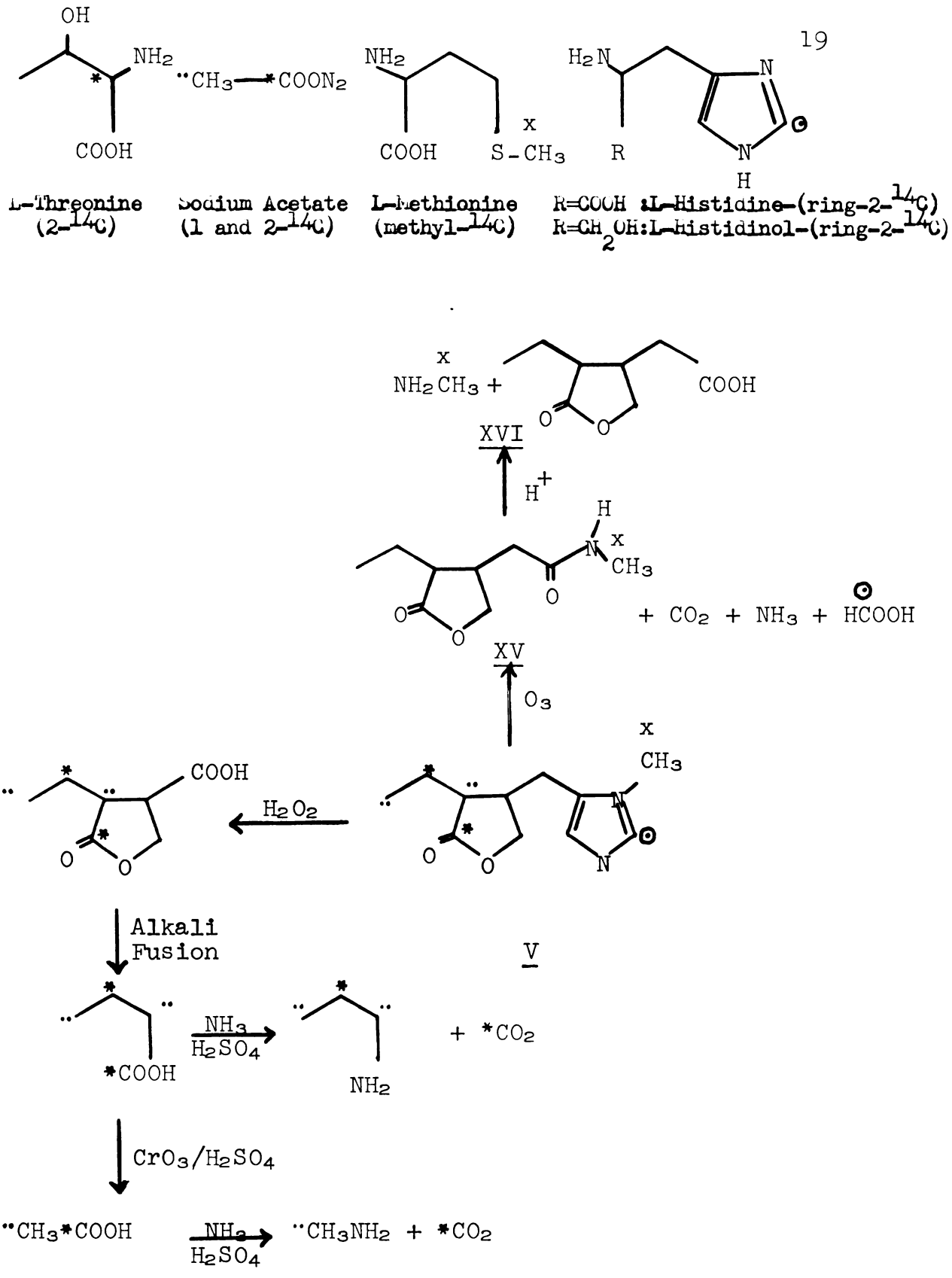


Figure 6. Labeled Precursors and Proposed Degradative Pathways.

The proposed degradative pathways are also illustrated.

Degradation of Radioactive Pilocarpine.--(16,17)

To show the position where L-methionine-(methyl-^{14}C) was incorporated, the radioactive pilocarpine nitrate from this feeding was degraded as illustrated in Figure 6. The radioactive pilocarpine nitrate was diluted with non-radioactive pilocarpine nitrate and recrystallized to give pilocarpine nitrate with a specific activity of 1,537 disintegrations $min.^{-1}$ $mg.^{-1}$ or 192 μCi. $mole^{-1}$. The free base was obtained by an alkaline extraction with chloroform and ozonized to give N-methyl-homopilopic acid amide (XV). The amide was subjected to acid hydrolysis to yield methylamine hydrochloride (XVI) which was derivatized with p-toluenesulfonyl chloride. The resultant N-methyl-(^{14}C)-p-toluenesulfonamide was recrystallized three times to a constant radioactivity of 2,210 disintegrations $min.^{-1}$ $mg.^{-1}$ or 188 μCi. $mole^{-1}$ (98% of the radioactivity of the starting pilocarpine nitrate).

III. RESULTS AND DISCUSSION

The radioactivity of the isolated pilocarpine nitrate from the feedings and the results of the degradation are given in Tables I and II.

Of all the precursors and methods investigated, only the L-methionine-(methyl-^{14}C) administered by the cut stem method at the Medical Center showed significant incorporation into pilocarpine. This feeding, followed by isotope dilution and isolation gave radioactive pilocarpine nitrate with a specific activity of 6,568 disintegrations $min.^{-1}$ $mg.^{-1}$ corresponding to an incorporation of 0.06% calculated on the basis of the amount of pilocarpine nitrate added. A controlled degradation showed that more than 98% of the radioactivity was located in the N-methyl group as expected. Thus, L-methionine is the methyl donor for N-methylation in the biosynthesis of pilocarpine and this step occurs in the leaves.

Since radioactive L-methionine showed incorporation into pilocarpine when fed at the Medical Center, but was not incorporated to a significant extent when fed to plants in Tubingen, the results of all of the Tubingen feedings were suspect. More plant material was requested in order to determine the pilocarpine content of these plants. Subsequent isolation of the alkaloids showed by thin layer chromatography that

	Precursor	Amount of Precursor Fed (mCi.)	Type of Feeding	Time of Feeding (hrs.)	Amount of Diluent Added (mg)	Specific Activity of Pilocarpine		Incorporation (%)
						dpm/mg.	μCi./mole	
Munich	Sodium Acetate-(2-^{14}C)	0.25	Stem	48	165.7*	<29≠	-	
	Sodium Acetate-(2-^{14}C)	0.25	Stem	72	80	<31≠	-	
	DL-Histidine-(ring-2-^{14}C)	0.25	Stem	48	165.6*	<11≠	-	
Tubingen	L-Histidinol-(ring-2-^{14}C)	0.134	Stem	96	150	23	3	7.8×10^{-6}
	L-Methionine-(methyl-^{14}C)	0.25	Stem	93	150	33	4	6.0×10^{-6}
	DL-Threonine-(2-^{14}C)	0.3	Stem	68	150	7.3	0.9	1.7×10^{-6}
	L-Histidine-(ring-2-^{14}C)	0.5	W I C K	7 days	200	0	0	0
	L-Methionine-(methyl-^{14}C)	0.25						
	DL-Threonine-(2-^{14}C)	0.2						

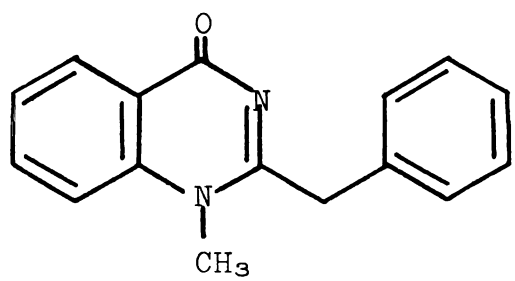
*Approximately 1:1 pilocarpine nitrate-isopilocarpine nitrate
≠Lowest value recorded, but not constant specific activity.

Table I. Results of Feeding Experiments of Pilocarpus pennatifolius with Labeled Precursors.

Precursor	Amount of Precursor fed (mCi.)	Type of Feeding	Time of Feeding	Amount of Diluent Added (mg)	Specific Activity of Alkaloid		Incorporation (%)
					dpm/mg.	μCi./mole	
L-Methionine-(methyl-^{14}C)	0.5	Stem	71	100	6,568	890	0.06
Sodium Acetate-(1-^{14}C)	2.0	Stem	72	100	88	11	2.09×10^{-4}
L-Threonine-(u-^{14}C)	0.5	Stem	72	100	25	3	2.27×10^{-4}
L-Histidine-(ring-2-^{14}C)	0.75	Stem	72	100	90	11	5.45×10^{-4}
Sodium Acetate-(1-^{14}C)	1.0	Root	64	0	1.33×10^{6} dpm/extract		0.06
		↓ Stem	76	100	0	0	0
Sodium Acetate-(1-^{14}C)	1.0	Root	68	0	1.13×10^{7} dpm/extract		0.51
		↓ Stem	100	100	0	0	0
L-Histidine-(ring-2-^{14}C)	1.25	Root	75	0	9.73×10^{6} dpm/extract		0.35
		↓ Stem	83	100	37	4.6	1.35×10^{-4}

Table II. Results of Feeding Experiments of Pilocarpus pennatifolius with Labeled Precursors.

pilocarpine was only a minor constituent and the principal alkaloid was not one of the known jaborandi alkaloids. Isolation and characterization revealed that the most abundant compound was the 4-quinazolone alkaloid arborine (XVII) which had previously been isolated by Chakravarti et al. (19) from various Glycosmis species. We have since shown that arborine is only a minor constituent of the plants grown in the Medical Center greenhouse, and that pilocarpine is the predominant alkaloid. Thus, it is impossible to draw conclusions from any of the experiments conducted in Tubingen. Verification that the Tubingen plants are indeed Pilocarpus has been requested, but has not been forthcoming.



XVII

The lack of incorporation of radioactivity during the Munich feedings also may not be significant. Each of the feedings was conducted with about six grams of plant material which is very little in comparison to the one to four hundred grams which have been used here. Even with these limited amounts, one would expect to isolate pilocarpine with some

radioactivity, even after isotope dilution, if the proper precursor were fed. It must be noted that the plant was twenty years old and not in good health. In fact it was later learned that the plant had died within six months of the time when the feedings were performed.

It is now left to interpret the feedings done here at the Medical Center. We have shown by isolation, and by incorporation of L-methionine-(methyl-^{14}C) that these plants do produce pilocarpine. There is every reason to believe that the precursors are reaching the site of action since all are normal plant constituents, and the levels of radioactivity were not so high as to disrupt the metabolism of the plant. Therefore, lack of incorporation must have been due to one or more of the following: 1) the precursor was not in the biosynthetic pathway, 2) the site of biosynthesis is not in the leaves, or 3) the plants were harvested either before there was sufficient time to achieve incorporation, or after the precursor was incorporated and the pilocarpine metabolized. The first choice is usually taken as the most logical conclusion. However, I feel that if biosynthesis is occurring in the leaves, at least the radioactive sodium acetate should have shown some incorporation. Since radioactive L-methionine did show incorporation, it is unlikely that there was insufficient time for biosynthesis or that the metabolic turnover rate was so high as to biosynthesize and metabolize all of the pilocarpine produced. Although

the content of pilocarpine is highest in the leaves, it is not unlikely that all or part of the biosynthesis occurs elsewhere and the products are translocated to the leaves. For example, nicotine has been shown to be biosynthesized in the root (20, 21, 22) and transported to the leaves where in some species it is demethylated to nornicotine. It was thought that an analogous situation may be present in Pilocarpus. Thus the attempt to do a root feeding by suspending cut root sections in aqueous solutions of the precursors. It is significant that all of the feedings to the roots showed considerable incorporation of radioactivity into the total alkaloid root extract, as shown in Table II. However, this is a total alkaloid extract and not necessarily the radioactivity resulting from pilocarpine or one of its precursors. In both the sodium acetate-(1-^{14}C) feedings, the radioactivity of the isolated pilocarpine was negligible, while the pilocarpine isolated from the L-histidine-(ring-2-^{14}C) feeding showed only a minimal radioactivity of 37 disintegrations min.$^{-1}$ mg.$^{-1}$. Since the root system is very extensive, with many fine root fibers, it would seem that the permeability of the root epidermis would be the only barrier to incorporation of a correct precursor.

IV. EXPERIMENTAL DATA

All melting points were determined with the Thomas-Hoover capillary melting point apparatus and were uncorrected. The nuclear magnetic resonance spectra were recorded at 100 MHz with a JOELCO JNM-4H-100 high resolution spectrometer. Ozonolysis was performed with a Welsbach model T-408 ozonizer. The radioactivity was determined by liquid scintillation counting with a Packard model 3375 Tri-Carb Liquid Scintillation Spectrometer. Substances were accurately weighed on a Cahn electrobalance and dissolved in 10 ml. of scintillation fluid. It was necessary to dissolve the samples of pilocarpine nitrate in 0.1 ml. of absolute methanol prior to adding the scintillation fluid. Toluene-^{14}C was used as an internal standard. Efficiencies were approximately 80%. The radioactivity was considered to be constant when two to three successive measurements differed by less than five percent. The scintillation fluid consisted of the following:

3.3 Gm. of "Premix P" containing 98% 2,5-diphenyloxazole (PPO) and 2% of 1,4-di-[2-(2-phenyloxazolyl)]-benzene (POPOP). This and 52 Gm. of naphthalene were dissolved in a mixture of 250 ml. of toluene, 250 ml. of dioxane and 150 ml. of absolute ethanol.

1. Synthesis of L-histidinol-(ring-2-^{14}C) Dihydrochloride. --(15)

(See Scheme IV)

The synthesis of L-histidinol dihydrochloride was carried out with both non-radioactive and radioactive L-histidine as the starting reagent. The non-radioactive L-histidine was purchased from Nutritional Biochemicals Corp. and the L-histidine-(ring-2-^{14}C) was purchased from Amersham Searle.

Synthesis of α-N-Benzoyl-L-histidine (XI). With stirring, 157.2 mg. (0.81 mmole) of L-histidine (X) hydrochloride was dissolved in 0.81 ml. of 2 N sodium hydroxide at 5°. To this solution, 0.1275 ml. (1.08 mmole) of benzoyl chloride and 1.75 ml. of 2 N sodium hydroxide were added dropwise over a two hour period (the benzoyl chloride was added by a Gilmont micropipette-burette). The mixture was then neutralized by addition of 0.55 ml. of 5 N hydrochloric acid followed by 0.03 ml. of glacial acetic acid to yield a white precipitate which was refrigerated for three hours. The mixture was extracted with chloroform and the aqueous phase concentrated with a stream of air to 2 to 3 ml. with the formation of white platelets. After standing overnight, the crystals were vacuum filtered and washed with cold water, followed by an ether washing. The precipitate was crystallized from hot water, collected by filtration and dried under reduced pressure at 65° for 3 hours. The yield was 155 mg. (74%), m. p. 241-242° (lit. (15) 240-243°).

Synthesis of α-N-Benzoyl-L-histidine Methyl Ester (XII). The α-N-benzoyl-L-histidine was suspended in 1.85 ml. of anhydrous

methanol in a two-neck flask fitted with a gas inlet tube and a reflux condenser (protected by a silica gel drying tube). The suspension was saturated with hydrogen chloride, without cooling and allowed to stand overnight. The mixture was evaporated under reduced pressure to a thick oil and traces of hydrochloric acid removed by repeated addition and evaporation, under reduced pressure, of methanol. The residue was dissolved in about 1 ml. of water and, with cooling, 10% ammonia solution added to make the mixture slightly alkaline. The ester separated as an oil which crystallized upon refrigeration for eight hours. The crystals were collected by filtration and recrystallized from hot water to yield 154 mg. (94%), m. p. 152-155° (lit. (14) 159°).

Synthesis of α-N-Benzoyl-L-histidinol (XIII). All glassware was heat dried prior to use. The methyl ester (XII) was dissolved with stirring and heat in 5 ml. of freshly distilled tetrahydrofuran (dried over sodium for one week) in a two-neck flask fitted with a reflux condenser (protected by a silica gel drying tube) and a dropping funnel. The solution was cooled below the boiling point and a mixture of 0.8 ml. of freshly distilled tetrahydrofuran and 0.8 ml. of lithium aluminum hydride solution[1] was added from the dropping funnel at a rate which kept the solution boiling. A pale yellow

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[1]Prepared by adding 10 ml. of anhydrous ether to 1 Gm. of lithium aluminum hydride and shaking periodically for one week.

suspension resulted and was refluxed for 45 minutes before cooling. The mixture was transferred to a centrifuge tube and wet ether, followed by water, was added until the excess lithium aluminum hydride had been destroyed. The mixture was centrifuged and the supernatant solution transferred to a separatory funnel where sodium chloride was added until the solution separated into an upper tetrahydrofuran phase and a lower aqueous layer which was returned to the centrifuge tube. The tetrahydrofuran was collected and the extraction repeated four times to give a final tetrahydrofuran volume of 20 to 25 ml. This solution was vacuum filtered and concentrated under reduced pressure to give an off-white solid which was suspended in cold water, filtered and washed with cold water. Following 3 hours drying under reduced pressure at 65° the yield was 88 mg. (64%), m. p. 206-208° (lit. (14) 210° for the recyrstallized product). This compound was used without further purification.

Synthesis of L-Histidinol Dihydrochloride (XIV). The N-benzoyl-L-histidinol was refluxed with 1.35 ml. of 5 N hydrochloric acid for 1.5 hours. The solution was cooled and the benzoic acid removed by extraction with ether. The aqueous solution was concentrated to a very small volume under reduced pressure and placed in a desiccator where it solidified to a brown solid. This solid was broken up, washed with cold absolute ethanol and ether before being recrystallized from 95% ethanol to yield 25.0 mg. (33%), m. p. 196-198° (lit.

(14) 198-199°). Elemental analysis calculated for $C_6H_{11}N_3O \cdot 2\ HCl$: C, 33.65; H, 6.13; N, 19.63; Cl, 33.12. Found: C, 33.80; H, 5.93; N, 19.77; Cl, 33.28.

When this synthesis was carried out with 1.50 mCi. of L-histidine-(ring-2-^{14}C), the resulting L-histidinol-(ring-2-^{14}C) dihydrochloride had a specific activity of 5.36 μCi. mg.$^{-1}$ or 1,147 mCi. mole^{-1}.

2. Degradation of Radioactive Pilocarpine Nitrate (16, 17). (See Scheme VI)

The degradation of pilocarpine nitrate was performed with non-radioactive pilocarpine nitrate, and with the radioactive pilocarpine nitrate isolated from the Medical Center cut stem feeding of L-methionine-(methyl-^{14}C). Prior to degradation, 14.4 mg. of the radioactive pilocarpine nitrate was diluted with 46 mg. of non-radioactive pilocarpine nitrate as a reverse isotope diluent. This mixture was recrystallized from 95% ethanol to yield pilocarpine nitrate whose specific activity was 1,537 disintegrations min.$^{-1}$ mg.$^{-1}$ or 1.92 μCi. mole^{-1}.

Preparation of N-Methyl-homopilopic Acid Amide (XV). Pilocarpine nitrate (41.3 mg.) was dissolved in a small amount of cold water and brought to pH 9 with sodium bicarbonate. Pilocarpine free base was extracted from this solution with cold chloroform and the extracts dried by filtration through anhydrous sodium sulfate. The chloroform was concentrated to about 4 ml. with a stream of

nitrogen and the solution cooled in an ice bath. Approximately 1% ozone was bubbled through the solution at a rate of 0.5 l. $min.^{-1}$ for 45 minutes. The evaporated chloroform was replaced, 1 ml. of water was added and the biphasic solution refluxed for 1/2 hour. The mixture was concentrated to an amorphous solid using a rotary vacuum evaporator. This solid was recyrstallized twice from 95% ethanol to yield 20.0 mg. (71%) of N-methyl homopilopic acid amide m. p. 103.5-104° (lit. (16) 104°). Chemical ionization mass spectrum, mH^{+} 186.1130 (ΔmMu 0.2) for $C_8H_{16}NO_3$.

When the diluted, radioactive pilocarpine nitrate was ozonolyzed in this manner, the N-methyl-(^{14}C)-homopilopic acid amide had a constant specific activity of 1,907 disintegrations $min.^{-1}$ $mg.^{-1}$ or 160 μCi. $mole^{-1}$.

Preparation of N-Methyl-p-toluenesulfonamide. The N-methyl homopilopic acid amide (XV) was combined with its mother liquors and hydrolyzed with refluxing 6 N hydrochloric acid for 6 hours. The slightly yellow solution was evaporated to an oil with a rotary vacuum evaporator. This oil was dissolved in 1 ml. of 1:1 dioxane-water and cooled in a salt/ice bath to -8°. One milliliter of 2.2 N sodium hydroxide was added with stirring and 75 mg. of p-toluenesulfonyl chloride in 0.5 ml. of dioxane added dropwise over fifteen minutes. A white solid was present which dissolved upon warming. The solution was refluxed for 1/2 hour and evaporated to dryness under reduced pressure. The amorphous residue was

dissolved in a minimal amount of 2.2 N sodium hydroxide. Upon acidification with concentrated sulfuric acid, a white solid formed and was extracted with carbon tetrachloride. The extracts were filtered through cotton and evaporated to dryness under reduced pressure. The while residue which remained was crystallized three times from a small volume of carbon tetrachloride to yield 13.4 mg. (67%) of N-methyl-p-toluenesulfonamide, m. p. 78-78.5° (lit. (18) 75°). Chemical ionization mass spectrum, mH^+ 186 for $C_8H_{11}NO_2S$.

The NMR spectrum showed a doublet for the aromatic protons ortho to the sulfonamide group at 7.74 ppm, a doublet for the meta protons at 7.33 ppm, and a broad signal at 4.71 ppm for the amide N-H proton. The N-methyl protons appeared as a doublet at 2.67 ppm and the methyl on the aromatic ring absorbed at 2.47 ppm. The integration was in agreement with the assignments.

When this degradation was carried out using the diluted, radioactive pilocarpine nitrate, the resulting N-methyl-p-toluenesulfonamide had a constant specific activity of 2,210 disintegrations $min.^{-1}$ $mg.^{-1}$ or 188 $\mu Ci.$ $mole^{-1}$. This was 98% of the radioactivity of the starting pilocarpine nitrate.

V. CONCLUSIONS AND PROPOSALS

The S-methyl group of L-methionine has been found to be the biological source of the N-methyl group of pilocarpine. This portion of the biosynthesis occurred within the leaves. The feeding of sodium acetate-(1 and 2-^{14}C), DL- and L-threonine-(2-^{14}C), L-histidine-(ring-2-^{14}C) and L-histidinol-(ring-2-^{14}C) by various routes showed no incorporation of radioactivity. Many of the feedings could not be performed under ideal conditions, and a lack of incorporation is not definitive evidence that the precursor is not a reactant in the biosynthetic scheme. As a higher plant, Pilocarpus pennatifolius is a complex organism and is continually undergoing growth, development and differentiation. With respect to secondary metabolism this means that a specific substance (e.g. pilocarpine) may be synthesized only in a particular part of the plant, at a particular stage of its development and at a particular time in its seasonal progression.

The development of an efficient synthesis of imidazole acetol would provide an excellent choice for a radioactive precursor. Future experiments should, if possible, be done by feeding the precursors to the roots of whole plants by hydroponics or other suitable means. If additional plants cannot be obtained for whole plant feedings, plant tissue cultures may provide a good alternative. At present it seems

reasonable that pilocarpidine is biosynthesized in the roots and translocated to the leaves where N-methylation occurs. The utility of pilocarpidine as a carrier alkaloid would make its somewhat involved synthesis worthwhile.

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PART II

DEGRADATIVE STUDIES

I. INTRODUCTION

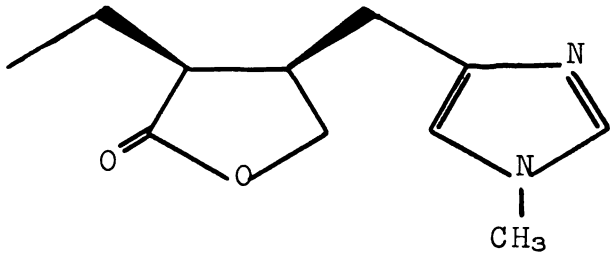
Decoctions of jaborandi leaves have long been used by Brazilian natives for their diaphoretic effect. It was not until 1874, however, that an extract of jaborandi was first sent to Europe for use as a medicinal agent. Pilocarpine is the principal alkaloid isolated from the leaflets of the South American shrubs known as Pilocarpus jaborandi and Pilocarpus microphyllus (Family Rutaceae). Jaborandi leaves were official in the ninth revision of the United States Pharmacopeia (1910) and in the British Pharmacopeia as early as 1878, but have been replaced by pilocarpine salts.

The first investigation of the physiological properties of pilocarpine appears to have been performed by Weber (1) in 1876, when he published his observations of its actions on the sweat and salivary glands. Four years later Harnack and Meyer (2) made a more detailed study, and since that time there have been numerous contributions to the pharmacology of this drug.

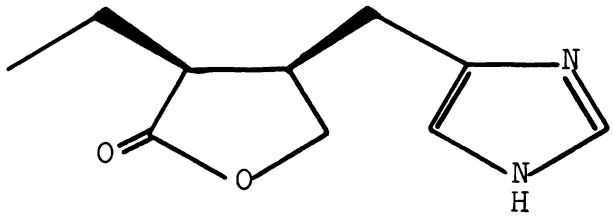
In general, the pharmacological activity of pilocarpine is cholinergic, acting on the parasympathetic receptors much in the same way as muscarine. Although pilocarpine has been assumed to act exclusively via direct autonomic effector cell stimulation, responses such as hypertension (3) and release of epinephrine from the adrenal medulla (4) can only

be accounted for by ganglionic stimulation. Wasser (5) postulated that the attachment of muscarinic agents to cholinergic receptors in smooth muscle involved two oxygen atoms and a quaternary nitrogen approximately 4 Å from the ether or carbonyl oxygen. Jones (6) proposed that pilocarpine could assume a conformation similar to that postulated for the muscarinic receptor in ganglia, however, the structure presented for pilocarpine is actually that of neo-pilocarpine (I).

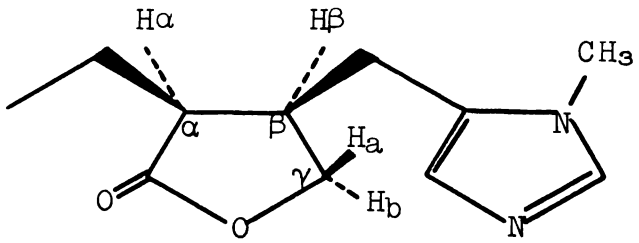
Pilocarpine has been used internally as a strong diaphoretic and as an antidote to lower doses of atropine. Its current usage is limited to the treatment of wide angle glaucoma. Topical application to the eye causes constriction of the pupil and dilation of the canal of Schlemm thereby promoting drainage of humor and relief of intraocular pressure. For this purpose sterile solutions or ointments of from 0.5 to 4 percent pilocarpine nitrate are used (there being no therapeutic advantage to using a greater concentration). Pilocarpine alginate for solid dose cul-de-sac deposition is a recent development in ophthalmic medication (7). Studies have shown that pilocarpine alginate flakes in the cul-de-sac provide enhanced constriction of the pupil and a miotic duration which is longer than that of pilocarpine hydrochloride or pilocarpine alginate solutions. It is also interesting to note that pilocarpine is reputed to stimulate hair growth, and extracts are sometimes used in the formulation of hair tonics.



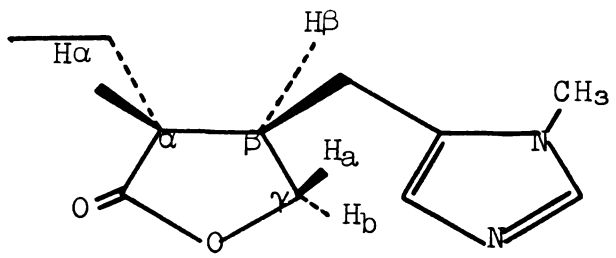
Neo-pilocarpine (I)



Pilocarpidine (II)



Pilocarpine (III)



Isopilocarpine (IV)

The relationship between the chemical structure and the physiological action has been reported by various authors. Brochmann-Hanssen et al. (8) found that various amine analogs, substituted for the imidazole moiety produced compounds with reduced activity. Ben Bassat and coworkers (9) indicated that quaternization of pilocarpine with a benzyl group confers anticholinergic activity. The intact lactone ring and the α-ethyl substituent on the lactone have both been reported to be essential for cholinergic activity. Pilocarpidine (II) has been found to possess similar but reduced activity (10) as has isopilocarpine (IV). There seems to be a difference in the relationship between dose and response for pilocarpine and isopilocarpine. In small doses pilocarpine was six to eight times more potent than isopilocarpine, whereas when administered in larger doses, the relationship was twenty-to-one.

Rabuteau (12) and Byasson (13) initiated the phytochemical investigations of jaborandi leaves by showing the presence of alkaloids. Pilocarpine (III) was shortly thereafter isolated independently by Gerrard (14) and Hardy (15), and Gerrard prepared several of its salts in crystalline form (16-18). In 1897 Petit and Polonovski (19) isolated an alkaloid which they called pilocarpidine and said that it was isomeric with pilocarpine, since pilocarpine was converted to pilocarpidine by alkali and heat. Merck (20) had also investigated a

compound which he called pilocarpidine and thus there arose numerous discrepancies among authors due to the differences in nomenclature during the late 1890's. Merck used the name pilocarpidine for a compound of formula $C_{10}H_{14}N_2O_2$. In 1900 Jowett (21) clarified the situation by confirming Petit and Polonovski's work with the pilocarpine isomer and gave it the name isopilocarpine (IV). He also confirmed the work of Merck, and hence the name pilocarpidine was given exclusively to Merck's C-10 compound (II). The leaves of Pilocarpus jaborandi contain from 0.5 to 1.0 percent total alkaloids, of which about one-half is pilocarpine.

The elucidation of the chemical structures of the jaborandi alkaloids is due primarily to the work of Jowett (21) and Pinner (22,23). In order to achieve separation of pilocarpine from isopilocarpine, Jowett crystallized successively the nitrates, hydrochlorides, hydrobromides and again the nitrates, some forty recrystallizations in all. The salts of both alkaloids are dextrorotatory, and mixtures containing from 50 to 66 percent isopilocarpine give nitrates of constant melting point (24). Pilocarpine and isopilocarpine behave as monoacidic bases with dissociation constants of 1×10^{-7} (25) and 0.68×10^{-7} (26), respectively. There are two asymmetric centers in the lactone portion of the molecule and Jowett (27,28) assumed the relationship between pilocarpine and isopilocarpine to be stereochemical (cis-trans isomers). This assumption was supported by the work of Langenbeck (29),

and proven in the 1930's when both epimers were synthesized using different pathways (30-32). Recently, DeGraw (33) has published a more efficient synthetic approach. It was not until 1966, however, that Hill and Barcza (34) established the absolute stereochemistry as αS:βR for pilocarpine (III) and αR:βR for isopilocarpine (IV).

Pilocarpine is the most abundant and the only therapeutically important jaborandi alkaloid. The base is usually a colorless oil, which has been crystallized, m.p. 34°. Its salts with acids crystallize well. The nitrate is most commonly used because it is obtained directly in the process of isolation, and is not hygroscopic, whereas the hydrochloride is hygroscopic.

Isopilocarpine also forms a syrupy liquid which is difficult to crystallize. Being the more stable epimer, it may be obtained from pilocarpine by heating the free base or its hydrochloride salt at 200° in the dry state, or by treatment with sodium alkoxide followed by acidification (34-36).

Two degradative pathways of pilocarpine are hydrolysis to pilocarpic acid and epimerization to isopilocarpine, both mechanisms resulting in loss of pharmacological activity. Hydrolysis of pilocarpine in aqueous solution is an equilibrium process which is catalyzed by hydrogen ions and hydroxide ions (37). Many methods have appeared in the literature for the quantitative determination of pilocarpine. Among these are the perchromate colorimetric method (38,39), the hydroxamic

acid colorimetric method (40), an infrared spectroscopic method (41), a nonaqueous titrimetric method (42), the optical rotation method of Anderson (43), a kinetic assay (44), and an NMR spectroscopic method (45). All these methods, however, suffer from the disadvantage that they do not distinguish pilocarpine from isopilocarpine. Although the two are diastereoisomers, there has been no simple way of separating them quantitatively or of determining one epimer in the presence of the other. A recent publication utilizing gas-liquid chromatography claims separation of the free bases (46). However, data other than the column packing and dimensions are not presented. Our work with this procedure indicates that a separation is possible, but column temperatures in the region of 200° are required in order to achieve retention times of about 15 minutes. At these temperatures pilocarpine free base is known to epimerize. Thus, when either pilocarpine free base or pilocarpine nitrate was injected at 195°, a leading shoulder was noted on the pilocarpine peak. This shoulder appeared with a retention time between that of pilocarpine and isopilocarpine, thus suggesting epimerization within the column. Injection of either isopilocarpine free base or isopilocarpine nitrate produced single peaks with retention times which were equal and less than those of pilocarpine.

Several investigations have studied the hydrolytic decomposition of pilocarpine at various pH values and temperatures. Blok (47) reported in 1945 that an alkaline borax-boric

acid buffer solution of pH 7.8 contained only 55 percent of the original pilocarpine, the other 45 percent being attributed to hydrolytic products. Riegelman and Vaughn (48) found that in order to achieve constant drug activity, pilocarpine solutions in a Hind and Goyan phosphate buffer at pH 6.8 should be replaced once a month. Anderson (49) investigated pilocarpine solutions adjusted to pH values between 6.5 and 7 with sodium borate, sodium hydroxide and sodium bicarbonate. His conclusions, based on optical rotation measurements were that the shelf-life of these solutions at 25° was approximately one month. Since ophthalmic solutions need to be sterile, Morrison and Truhlsen studied the stability of ophthalmic drugs toward autoclaving (50). They reported that pilocarpine hydrochloride solutions in boric acid-carbonate buffer at pH 7.6 lost 25 percent of their effectiveness after one autoclaving and almost 50 percent after two autoclavings. Baeschlin and coworkers utilized NMR spectroscopy to investigate the kinetics of hydrolysis of pilocarpine solutions (45,51) and concluded that solutions buffered to pH 6.1 gave the best balance of activity, stability and tolerance by the patient (52). A detailed kinetic study of hydrolysis utilizing pH stat titrimetry has been reported by Chung, Chin and Lach (37). They also found evidence that pilocarpine undergoes some epimerization at alkaline pH, based on the differences in specific rotation of the two epimers. Dopke and d'Heureuse

(36) showed that the epimerization of pilocarpine to isopilocarpine under anhydrous alkaline conditions occurs at the α-carbon of the lactone ring.

The rates of hydrolysis and epimerization are both pH dependent. Since they occur simultaneously, they should be considered as competing pathways of inactivation. A study of the kinetic parameters must, therefore, take into account both mechanisms since the hydrolysis is reversible upon acidification, but the epimerization is irreversible. Isolation and purification of the alkaloids from the plant material require extraction of the base at alkaline pH, and any epimerization which occurs during this step will lead to inactive isopilocarpine which is not readily removed by recrystallization.

The purpose of the study reported in this dissertation was to evaluate the extent of epimerization and to determine the rates of hydrolysis and epimerization of pilocarpine in aqueous solution at different pH values and temperatures. An NMR procedure was developed which was capable of quantitating either epimer when both were present in a mixture. This procedure in conjunction with pH-stat titrimetry was then utilized to determine the kinetic parameters for hydrolysis and epimerization.

II. EXPERIMENTAL

1. Reagents

Pilocarpine nitrate[1], m.p. 174-175° [lit. (42) 174-179°], $[\alpha]_D^{23}$ +80° (c = 2, water) [lit. (42) + 79.5 to +82.0° (c = 2, water)]; isopilocarpine nitrate[2], m.p. 158-158.5° [lit. (53) 159°], $[\alpha]_D^{23}$ +39.4° (c = 2, water) [lit. (54) +38.8° (water)]; deuterium oxide[3] (D_2O), 99.85 mole %; sodium deuteroxide[4] (NaOD), 40% in D_2O; deuterium chloride[4] (DCl), 38% in D_2O; methanol-[OD][4]; nitrogen gas 99.996+% pure. All other chemicals were of analytical reagent grade.

2. Apparatus

The following were used: Radiometer pH-stat, consisting of TTT 11 titrator, SBR2C titrigraph, SBU1a syringe burette, TTA3 titration assembly, GK2301C combination electrode and B104 5 ml. syringe; Radiometer PHM-28 pH meter, and Sola 115 volt constant voltage transformer. The reactions were carried out in a water-jacketed reaction vessel of 75 ml. capacity and stirred with a magnetic stirrer. Solution temperature was

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[1]Sigma Chemical Co.

[2]Pierce Chemical Co.

[3]Bio-Rad Laboratories

[4]Stohler Isotope Chemicals

maintained by means of a 25 liter circulating water bath with a Fisher proportional temperature control. The temperature of the reaction solution was recorded by a Wheatstone bridge assembly with a 51A thermistor[5] as the probe and a Barber-Coleman Series 8000 recorder as depicted in Fig. 7. The NMR spectra were prepared with a JOELCO JNM-4H-100, 100 MHz spectrometer. The expanded spectra were integrated by means of a duPont 310 curve resolver.

3. Procedure

A. NMR Spectra

All 100 MHz NMR spectra were recorded in deuterium oxide with tetramethylsilane as external reference. For the analysis of the proportion of epimers, the NMR spectrum of the terminal methyl group of the α-ethyl substituent was recorded with a ten-fold scale expansion and an offset of 1.0 ppm. The methyl triplet of each epimer was then integrated with a duPont curve resolver. This curve resolver functioned as a multi-channel analog computer. Each channel was adjusted to reproduce the standard Lorentzian shape of NMR signals. Each channel was then assigned to a peak of the overlapping triplets and made to reproduce the spectrum by varying the height and width of the channel. A Wheatstone bridge meter was brought into balance at 100 percent of scale when all of the channels were

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[5]Victor Engineering Co.

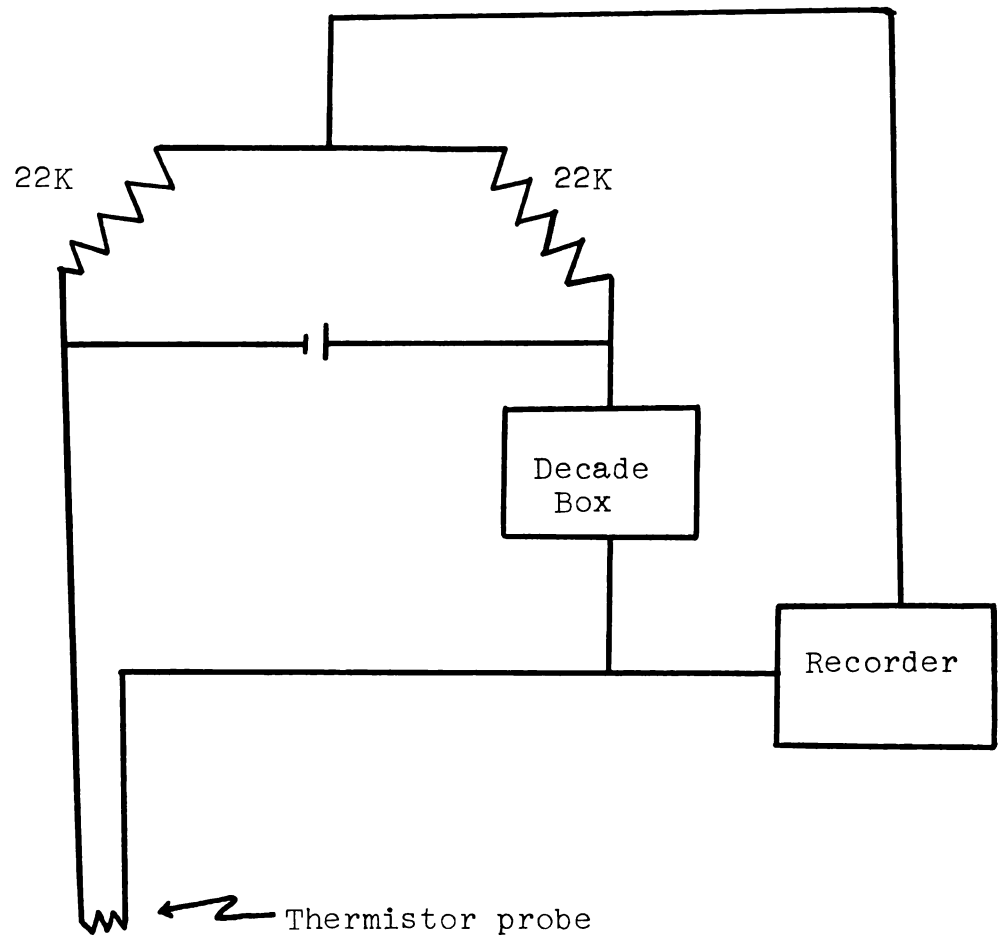

Figure 7. Circuit Diagram of Temperature Recording System.

on and the spectrum duplicated. The meter was then adjusted to zero percent when all of the channels were off and only the baseline was present. The three channels corresponding to the epimer being integrated were then turned on and the percentage of the total which was due to that epimer read directly from the meter. To evalute the accuracy of this method, mixtures containing from 10 to 35 percent isopilocarpine nitrate in pilocarpine nitrate were prepared and the expanded NMR spectrum of the terminal methyl group recorded. These spectra were integrated using the curve resolver and found to be within one percent of the actual composition.

B. Hydroxide-Ion Catalyzed Reactions

The experimental procedure employed for the determination of the rate of base consumption was similar to that of Chung, Chin and Lach (37), except that potassium hydroxide was used in place of sodium hydroxide to minimize the sodium ion effect on the electrode at the higher pH values. Also, considerably higher concentrations of pilocarpine were used in order to facilitate sampling for NMR spectroscopy. Two and one-half grams of pilocarpine nitrate were dissolved in fifty milliliters of redistilled water in a water-jacketed reaction vessel containing the combination electrode, the thermistor probe and the delivery tube of the pH-stat system. This vessel was surrounded by a styrofoam insulator. The vessel

was sealed from air and continuously flushed with a stream of nitrogen which had passed through a series of three gas washing towers containing ten percent sulfuric acid, ten percent sodium hydroxide and distilled water, respectively. When the desired temperature was reached, the solution was rapidly brought to just below the preset pH by adding 2 N potassium hydroxide with a pipette and the final pH equilibrium achieved with the pH-stat system. The pH-stat recorder was then returned to zero and the consumption of base recorded at constant pH. The progress of the reaction was recorded over a period of from eight to ten half-lives. The temperature was found to vary less than $\pm 0.025°$ during this time. Pseudo first-order rate constants were obtained from Guggenheim plots (55) of the base consumption using a least squares (56) program and a Hewlett-Packard Model 9100 programable calculator. The Guggenheim method utilized a plot of the logarithm of the difference in volume at time t and the volume at time $(t+\Delta t)$[6] versus time. This method was chosen because of the difficulty in obtaining an accurate estimate of t_o and because instrument drift may cause a significant error in the determination of the endpoint for slow reactions. It is possible, however, to have linear Guggenheim plots for reactions which are not strictly pseudo first-order and it was necessary to demonstrate

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[6] Δt is a constant unit of time which was between two and three half-lives.

pseudo first-order kinetics by an independent method. At pH values of 11 and 12 the reactions were fast enough for accurate endpoint determination. For these elevated pH values, log $(V_\infty - V_t)$, where V is the volume of base consumed, when plotted against t, yielded straight lines with slopes of one, verifying first-order dependence on hydroxide ion. The rate constants obtained in this manner were in good agreement with those obtained when the data were treated by the Guggenheim method.

III. RESULTS AND DISCUSSION

The 60 MHz NMR spectrum of pilocarpine has been reported by Baeschlin, Etter and Mol (45) who used NMR spectroscopy to determine the rate of hydrolysis of pilocarpine solutions which were buffered to pH 6.1. The NMR spectrum of isopilocarpine has not been reported previously, and Link and Bernauer (46) were unable to detect any change in the NMR spectrum of pilocarpine after partial epimerization.

The 100 MHz NMR spectra of pilocarpine nitrate and isopilocarpine nitrate are shown in Figs. 8 and 9 respectively. The principal differences in the spectra of the two epimers are due to the γ-protons of the lactone ring and terminal methyl protons of the α-ethyl substituent. The γ-protons of both epimers show the doublet of doublets characteristic of an AMX pattern. For pilocarpine nitrate H_a is centered at 4.55 ppm (J_{gem} = 9.0 Hz, J_{vic} = 3.0 Hz) and H_b is centered at 4.80 ppm (J_{gem} = 9.0 Hz, J_{vic} = 6.0 Hz). Isopilocarpine nitrate also presents an AMX pattern with H_a absorbing at 4.48 ppm (J_{gem} = 10.0 Hz, J_{vic} = 6.2 Hz) and H_b occurring at 4.93 ppm (J_{gem} = 10.0 Hz, J_{vic} = 6.3 Hz). Gutowsky and coworkers (57) have calculated the relationship between the bond angle of geminal protons and their coupling constant.

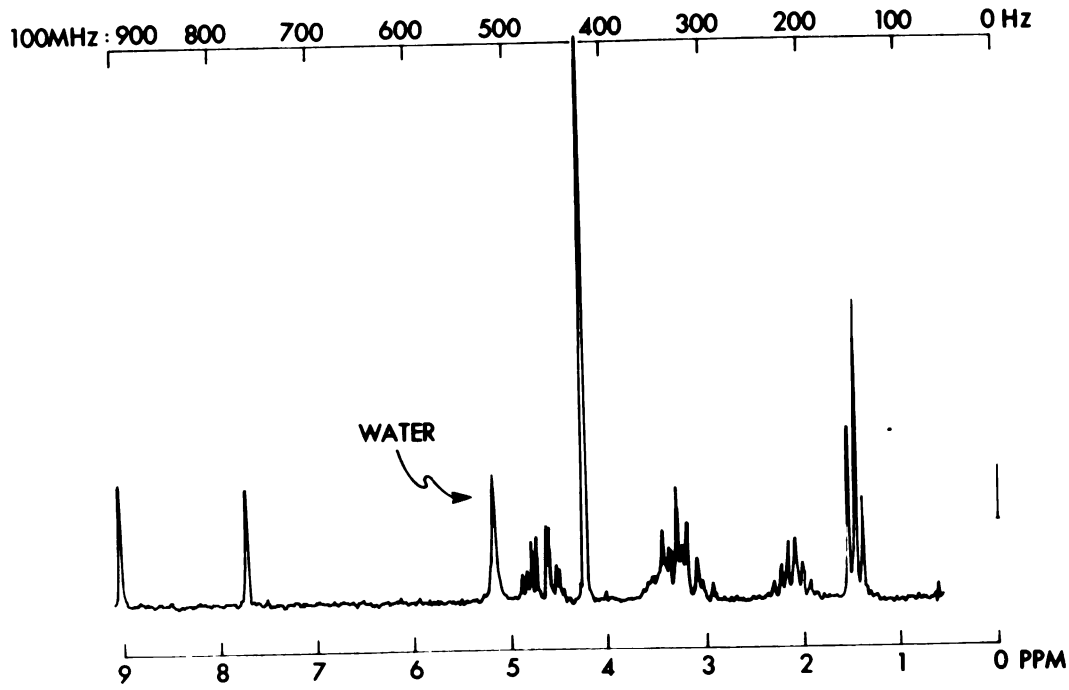

Figure 8 -- 100 MHz NMR Spectrum of Pilocarpine Nitrate in D_2O.

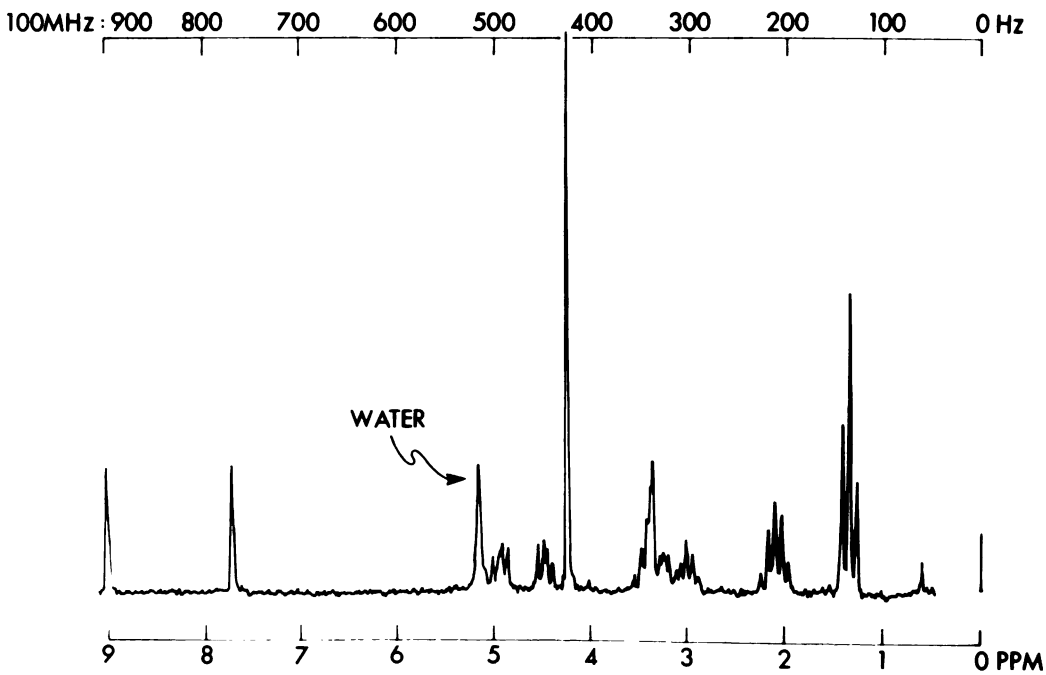

Figure 9 -- 100 MHz NMR Specturm of Isopilocarpine Nitrate in D_2O.

Relating their work to the γ-protons of the lactone ring, it can be stated that since the geminal coupling constant for pilocarpine is less than that of isopilocarpine, the geminal proton angle is greater for pilocarpine. Thus the β-C/γ-C/O bond angle is less in the lactone ring of pilocarpine. This would be the case if the ring were more puckered than the ring of isopilocarpine. For isopilocarpine, H_a resonates 7 Hz upfield from its resonance position in pilocarpine. Therefore, H_a is more shielded (more eclipsed) by the methylene bridge bond than in pilocarpine, indicating an approach toward planarity and a relief of ring strain for isopilocarpine. The terminal methyl group of the α-ethyl substituent appears as an A_3X_2 triplet with a coupling constant of 7.25 Hz. However, the chemical shifts differ by 0.1 ppm for the two epimers, pilocarpine resonating downfield relative to isopilocarpine. The spectral data are summarized in Table III.

Pilocarpic acid and isopilocarpic acid as well as their respective salts are all encountered during the course of hydrolysis and recyclization. For the purpose of spectral identification of these intermediates, pilocarpine nitrate and isopilocarpine nitrate were hydrolyzed with base to the sodium salts of the respective γ-hydroxy acids, and the NMR spectra recorded. The solutions were then adjusted with deuterium chloride to pH 3.5 to give the deuterochloride salts of the hydroxy acids. At this pH the rate of lactone formation

Table III - 100 MHz. NMR Spectral Data of Pilocarpine Nitrate (III), Isopilocarpine Nitrate (IV), Pilocarpate (V), Pilocarpic Acid (VI), Isopilocarpate (VII) and Isopilocarpic Acid (VIII) in D_2O.

	$\underline{CH_3}$-CH_2-	CH_3-$\underline{CH_2}$-	Hα	Hβ	-CH_2-bridge	N-CH_3	H_a	H_b	C-5 proton of imidazole	C-2 proton of imidazole
III	1.46(t)	2.07(m)	←	3.27(m)	→	4.25(s)	4.55(dd)	4.80(dd)	7.72(s)	9.06(s)
IV	1.36(t)	2.08(m)	←	3.21(m)	→	4.25(s)	4.48(dd)	4.93(dd)	7.72(s)	9.06(s)
V	1.31(t)	1.86(m)	2.26(m)	2.65(m)	3.04(m)	3.99(s)	←	3.98(m) →	7.18(s)	7.92(s)
VI	1.31(t)	2.05(m)	←	2.71(m) →	3.21(m)	4.25(s)	←	4.04(m) →	7.72(s)	9.06(s)
VII	1.31(t)	1.98(m)	2.36(m)	2.72(m)	3.12(m)	4.02(s)	←	3.96(s) →	7.18(s)	7.92(s)
VIII	1.31(t)	2.06(m)	←	2.76(m) →	3.25(d)	4.27(s)	←	4.02(d) →	7.72(s)	9.06(s)

is slow (37), and permitted recording of the NMR spectra prior to lactonization. Finally, acid was added to a pH of less than one in order to effect cyclization of the lactones. These reactions are illustrated in Figs. 10 and 11. Deuterated reagents and solvents were used in order to avoid the large resonance signal of water in the NMR spectra.[7] This resulted in partial deuterium exchange for the α-proton as the reactions proceeded. The NMR spectra also showed that the proton at C-2 of the imidazole ring slowly exchanged under alkaline conditions. When pilocarpine nitrate and isopilocarpine nitrate were hydrolyzed in alkaline aqueous solution to the anions of the γ-hydroxy acids (V and VII), the NMR triplets of the terminal methyl group of the ethyl substituent shifted upfield to 1.31 ppm for both epimers. The γ-protons (H_a and H_b) also shifted upfield and overlapped the N-methyl group, indicating that the lactone ring had been hydrolyzed. The C-2 and C-5 protons of the imidazole and the N-methyl group also absorbed farther upfield as expected when the imidazole ring is not ionized. In the spectra of the hydroxy acids (VI and VIII), the methyl

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[7]The pH measurements were performed with the pH meter standardized with aqueous buffers and expressed in pH units, thus ignoring the difference between the ionization products of water and deuterium oxide.

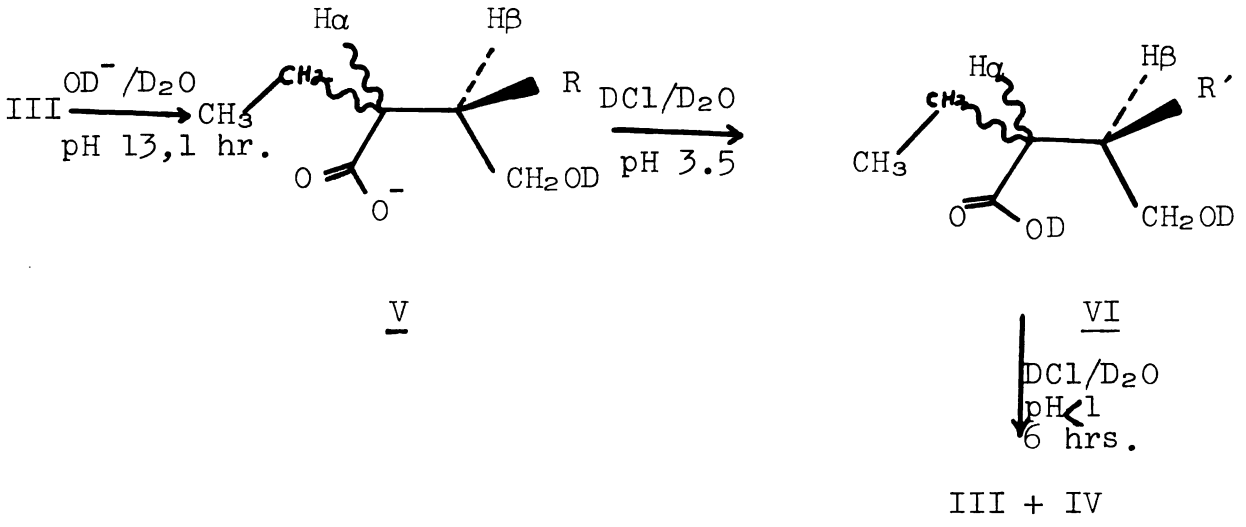
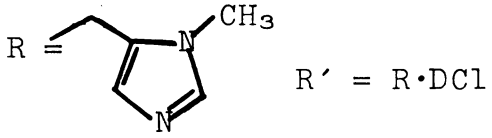


Figure 10. The Effects of Base on Pilocarpine (III) and Recyclization of the Lactone in Acid Medium.



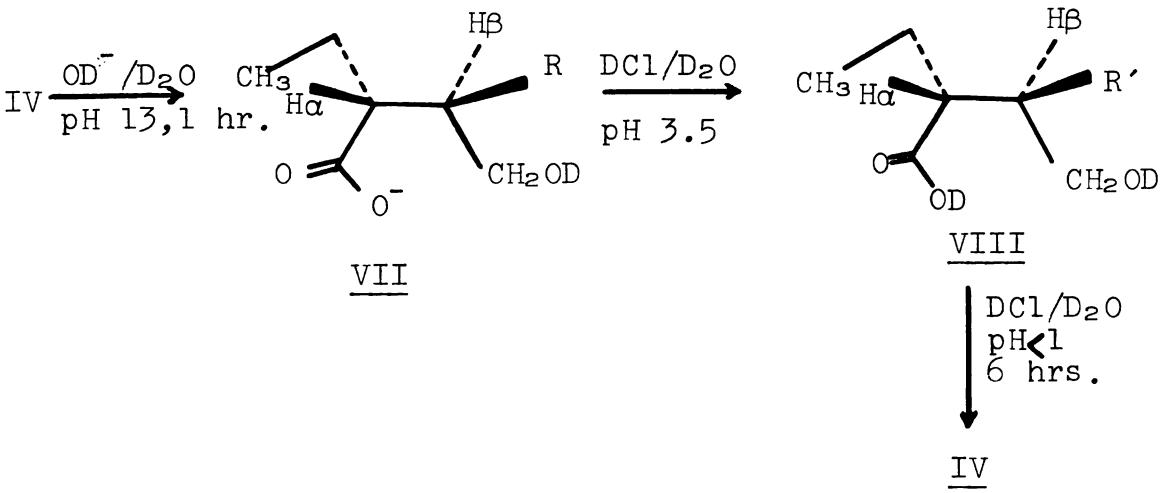
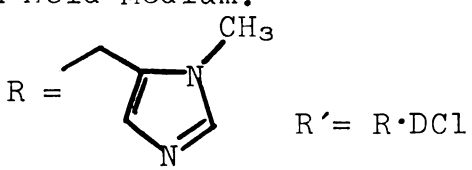


Figure 11. The Effect of Base on Isopilocarpine (IV) and Recyclization of the Lactone in Acid Medium.



triplets of the ethyl substituents were in the same position as in the anions, whereas the γ-protons had shifted slightly downfield toward their position in the closed lactones: resonating at slightly above 4 ppm. The N-methyl group and the C-2 and C-5 protons had shifted back to their resonance positions for the protonated imidazole ring. After complete hydrolysis and recyclization of pilocarpine as in Fig. 10, the spectrum of the product was significantly different from that of the original pilocarpine salt. Each γ-proton still appeared as the doublet of doublets for pilocarpine; however, there were also peaks corresponding to the γ-protons of isopilocarpine. The multiplet due to H_a, H_b, and the methylene bridge between the two rings was also different from either pure pilocarpine or isopilocarpine salt. The signal for the methyl group of the ethyl substituent clearly showed six peaks corresponding to the methyl triplets of pilocarpine and isopilocarpine. These two triplets were chosen as the best means for quantitating epimerization. For this purpose the NMR spectrum of the triplets was recorded with a ten-fold scale expansion and an offset of 1.0 ppm as illustrated in Fig. 12. Recyclization was complete under the experimental conditions described, as evidenced by the chemical shifts of the γ-protons and the methyl group of the ethyl substituent of the lactone ring. Using the curve resolver, integration of the two triplets corresponding to pilocarpine and isopilocarpine showed that the mixture produced

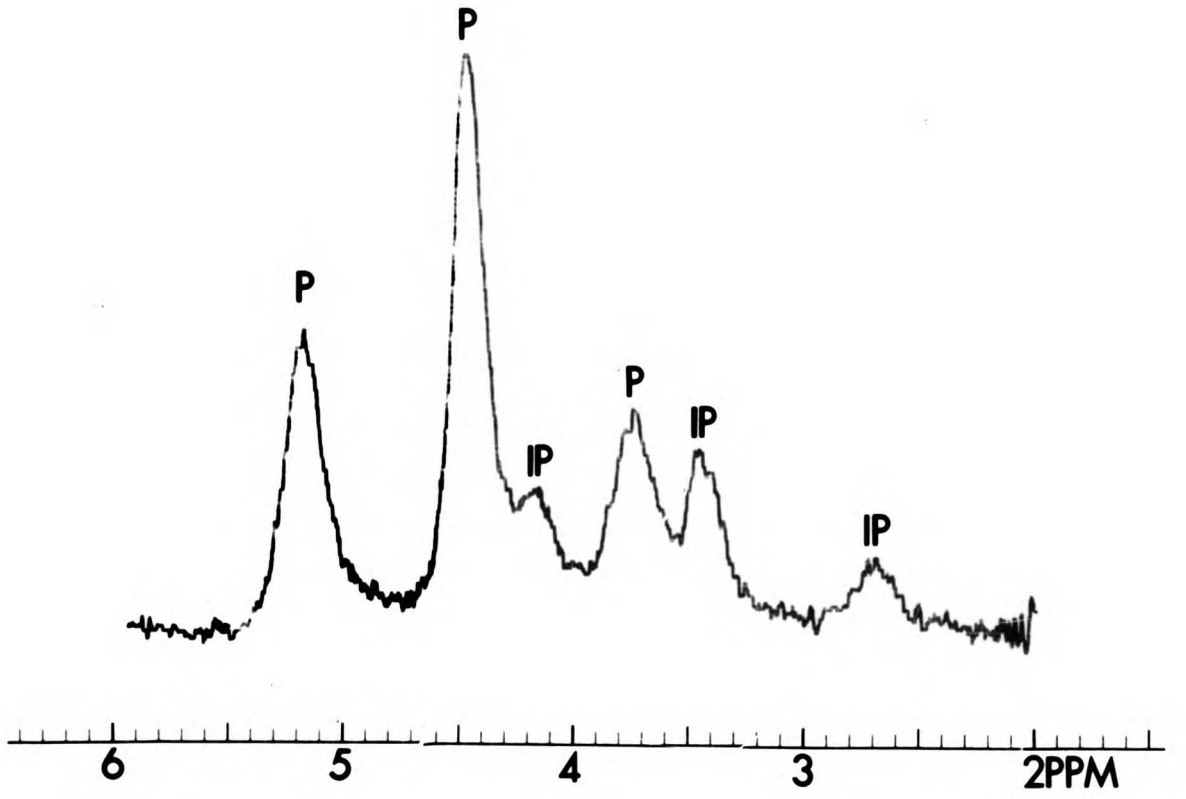


Figure 12 -- Expanded 100 MHz NMR spectrum of the Terminal Methyl Group of the α-Ethyl Substituent of Pilocarpine Deuterochloride (P) After Partial Epimerization to Isopilocarpine Deuterochloride (IP).

according to the scheme of Fig. 10 was comprised of about 80% pilocarpine deuterochloride and 20% iospilocarpine deuterochloride. Several workers have shown that once the lactone is hydrolyzed and the hydroxy acid is in the anion form, no further epimerization occurs (35,36). In agreement with this, NMR spectroscopy revealed that the ratio of pilocarpine to isopilocarpine was constant at a given temperature when solutions were completely hydrolyzed and kept at pH 13 for periods ranging from a few minutes to two days. To determine if epimerization occurred in the hydroxy acid species which was encountered during the recyclization process, a completely hydrolyzed solution was separated into two portions. One portion was quickly brought to a pH of less than one to insure rapid recyclization while the other was adjusted to and maintained at pH 3.5 for six hours before being brought to a pH of less than one. Upon integration of the NMR spectrum of the methyl region of the two samples, the ratio of pilocarpine to isopilocarpine was found to be the same in both solutions. Therefore, epimerization does not occur during the recyclization process, and the spectra of V and VI in Fig. 10 and Table III do not represent pure pilocarpate and pilocarpic acid, but mixtures of the epimers in the same ratio as that determined after recyclization of the hydrolyzed lactone. It has been observed that the equilibrium optical rotation of a pilocarpine nitrate solution at pH 1 is

less than that of a solution which has not been acidified (37). To determine if this decrease was due to epimerization, a solution of pilocarpine nitrate was maintained at a pH of less than one for two days. The NMR spectrum of this solution did not show the presence of the epimer. Therefore, epimerization is not detectable by NMR spectroscopy in a strongly acidic solution. When isopilocarpine was completely hydrolyzed and recyclized as in the scheme of Fig. 11, the spectrum of the product was identical to that of the isopilocarpine salt before hydrolysis. This indicated that isopilocarpine did not epimerize under these conditions to an extent which could be detected by NMR spectroscopy. Therefore, the equilibrium constant for epimerization is overwhelmingly in favor of the trans epimer, isopilocarpine.

The epimerization of pilocarpine requires the abstraction of the α-proton to produce a planar carbanion which is stabilized by delocalization to the enolate (36). Subsequent reprotonation produces the thermodynamically more stable trans epimer. The α-protons of γ-lactones have been shown to be more acidic than those of the corresponding open esters (58). Utilizing infrared spectroscopy, Dopke and d'Heureuse (36) have demonstrated the existence of the enolate as an intermediate during the epimerization of pilocarpine in alcoholic sodium ethoxide. In such an anhydrous medium, the epimerization is quantitative and proceeds smoothly without significant opening of the lactone ring. Isopilocar-

pine will also produce the same carbanion under these conditions as we have demonstrated by deuterium exchange. Isopilocarpine nitrate was refluxed with sodium methoxide in methanol-[OD] for eighteen hours before acidification with deuterium chloride. The solution was evaporated to dryness and the residue dissolved in deuterium oxide. The NMR spectrum showed that the multiplet corresponding to H_a, H_b, and the methylene bridge integrated for three protons, indicating exchange of one proton. At the same time, the multiplet corresponding to the methylene protons of the α-ethyl side chain changed to a first-order A_3X_2 quartet with a coupling constant of 7.25 Hz, indicating splitting only by the terminal methyl group as would be expected from complete exchange of the α-proton.

The overall effect of aqueous base on pilocarpine may be considered to be the result of three principal reactions: hydrolysis to pilocarpate, and epimerization to isopilocarpine followed by hydrolysis to isopilocarpate. These reactions are illustrated in Fig. 13, in which Eq. 1 represents the hydroxide-ion catalyzed hydrolysis of pilocarpine to pilocarpate, Eqs. 2 and 3, the base catalyzed epimerization of pilocarpine, and Eq. 4 the hydrolysis of isopilocarpine. Eq. 2 is shown as being an irreversible reaction. This approximation is based on the previously mentioned work of Dopke and d'Heureuse (36) who formed the enolate (P^-) in sodium ethoxide. Subsequent addition of deuterium oxide produced no pilocarpine, indicating that equilibrium favors isopilo-

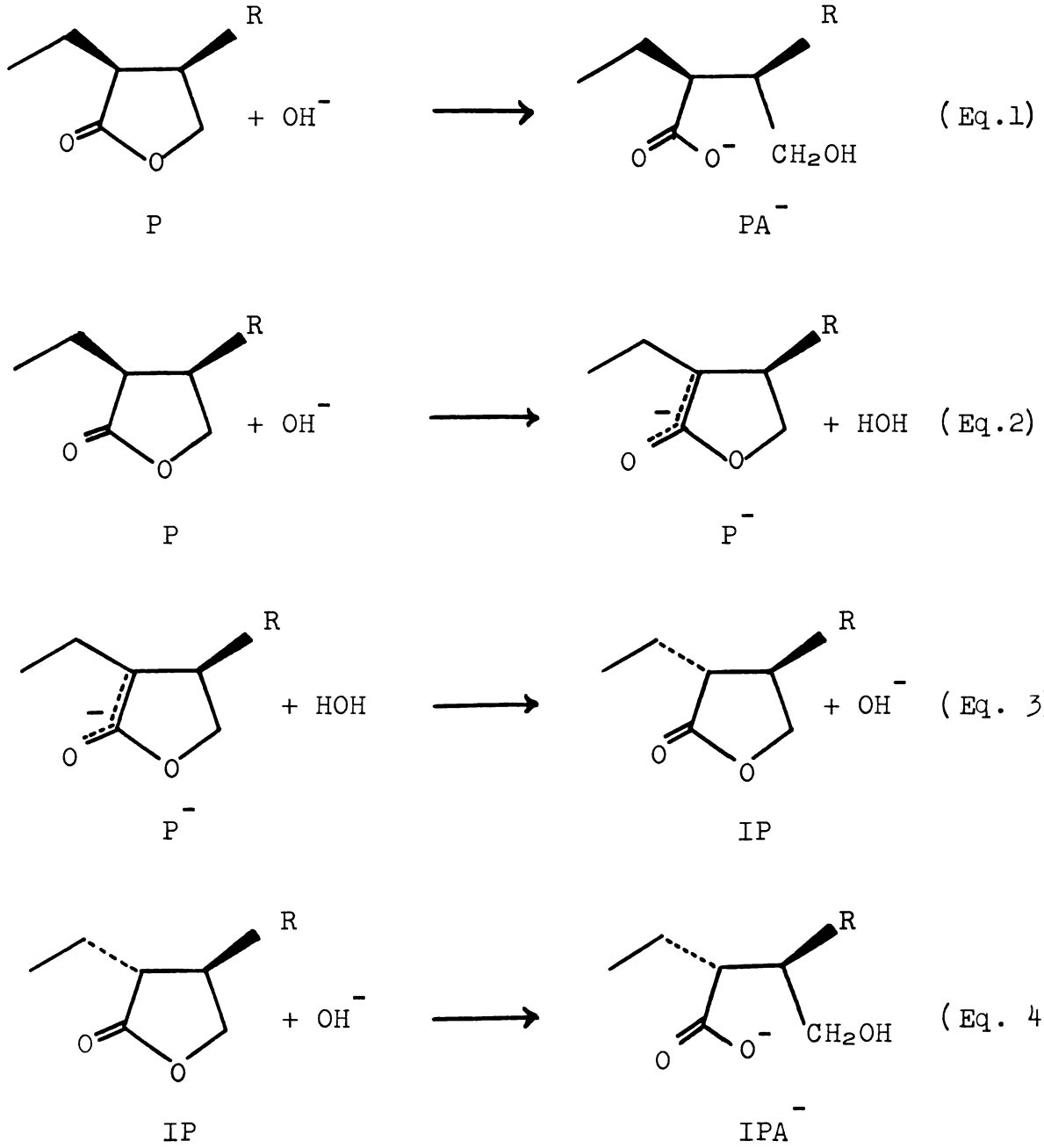
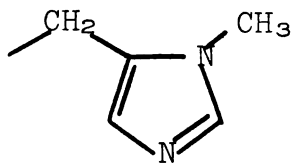


Figure 13 - Hydroxide-ion Catalyzed Hydrolysis and Epimerization of Pilocarpine (P); PA^- = Pilocarpate; P^- = Delocalized Carbanion = IP^-; IP = Isopilocarpine; IPA^- = Isopilocarpate;

R = 

carpine. Therefore, the net rate of formation of isopilocarpine from the enolate is very much greater, and the rate of formation of pilocarpine (Eq. 2) is insignificant by comparison.

The rate of disappearance of pilocarpine in alkaline solution has been shown to follow pseudo first-order kinetics (37). Therefore, the rate of epimerization and subsequent hydrolysis of the isopilocarpine produced must also be governed by a rate-limiting pseudo first-order reaction. Thus, the rate of disappearance of pilocarpine in alkaline, aqueous solution is controlled by two competing pseudo first-order reactions, and Fig. 13 may be abbreviated to:

$$P + OH^- \xrightarrow{k_H} PA^- \qquad \text{(Eq. 5)}$$

$$P + OH^- \xrightarrow{k_E} IPA^- \qquad \text{(Eq. 6)}$$

The rate law for this system is:

$$-\frac{d(P)}{dt} = (k_H + k_E)(OH^-)(P) \qquad \text{(Eq. 7)}$$

$$= k_{Exp}(OH^-)(P) \qquad \text{(Eq. 8)}$$

$$= k'_{Exp}(P) \qquad \text{(Eq. 9)}$$

where: k_H is the second-order rate constant for the specific hydroxide-ion catalyzed hydrolysis of pilocarpine.

k_E is the second-order rate constant for the specific hydroxide-ion catalyzed epimerization and hydrolysis of the isopilocarpine produced.

k_{Exp} is the experimentally determined second-order rate constant.

k'_{Exp} is the experimentally determined pseudo first-order rate constant ($k'_{Exp} = k_{Exp}(OH^-)$).

A plot of log k'_{Exp} versus pH gave a straight line with a slope of one, verifying that pseudo first-order kinetics are followed. The pH profiles at various temperatures are illustrated in Fig. 14. The experimentally determined second-order rate constant (k_{Exp}) was 6.98×10^2 l. mole^{-1} hr.$^{-1}$ at 25°, or eight percent lower than the value reported by Chung, et al. (37). To determine if this difference was due to a concentration effect, control experiments were performed in which the concentration of pilocarpine nitrate ranged from 0.02% to 6.0%. The observed second-order rate constants were found to be the same over the entire concentration range. The experimentally determined second-order rate constants (k_{Exp}) at the temperatures investigated are presented in Table IV.

Since the hydroxide-ion catalyzed reactions of pilocarpine behave as competing pseudo first-order reactions, the

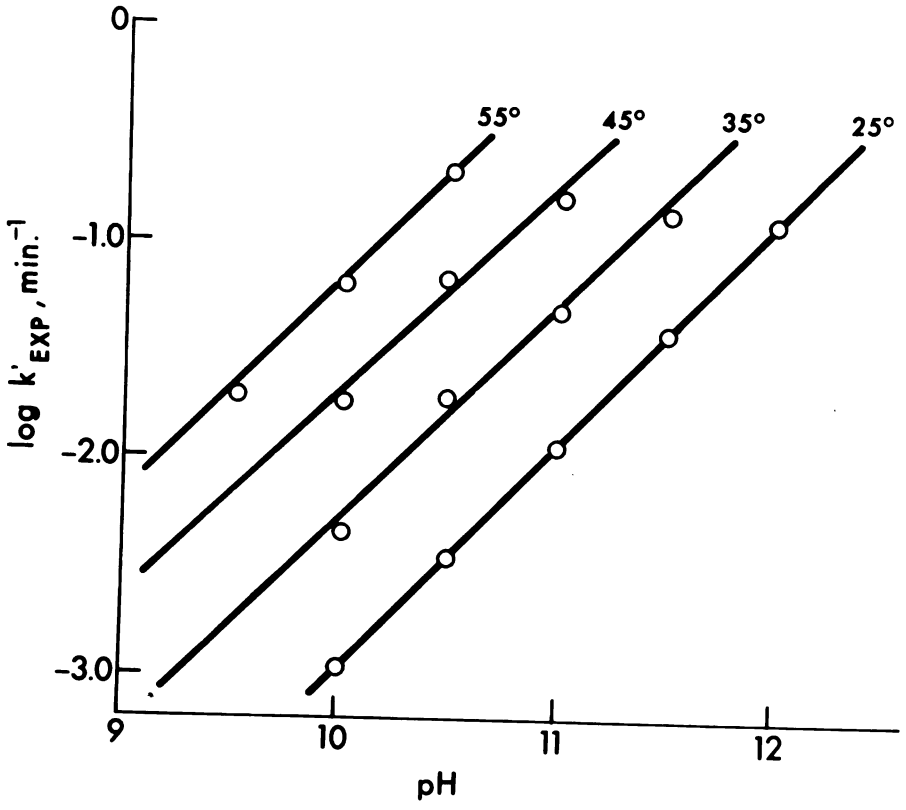


Figure 14 -- pH Profile of the Disappearance of Pilocarpine at Alkaline pH.

Table IV - Second -order Rate Constants at the Temperatures Investigated.

(k's in units of l. $mole^{-1}$ $hr.^{-1}$)

Rate Constant \ Temp.	25°	35°	45°	55°
k_{Exp}	6.98×10^{2}	2.97×10^{3}	1.10×10^{4}	3.90×10^{4}
k_{H}	5.53×10^{2}	2.19×10^{3}	7.75×10^{3}	2.65×10^{4}
k_{E}	1.45×10^{2}	0.78×10^{3}	3.35×10^{3}	1.25×10^{4}

fraction of pilocarpine which disappears by each pathway during the course of the reaction is constant, as is the ratio of products formed from each pathway. Consequently, the ratio of pilocarpate to isopilocarpate at the end of the reaction is equal to the ratio of the rate constant for the hydrolysis of pilocarpine (k_H) to the rate constant for the epimerization to isopilocarpine and its subsequent hydrolysis (k_E), i.e.,

$$\frac{(PA^-)}{(IPA^-)} = \frac{k_H}{k_E} \qquad \text{(Eq. 10)}$$

We have shown that epimerization does not occur after the lactone ring has been hydrolyzed, whether in the anion or the free hydroxy acid form. Therefore, the NMR spectrum recorded after complete hydrolysis and recyclization, when integrated, is an accurate reflection of the ratio of hydrolyzed epimers in the solution at any time during the hydroxide-ion catalyzed reactions. These spectra showed that the percentage of isopilocarpate formed during these reactions was independent of pH, but dependent upon temperature, as presented in Table V. At least three separate experiments were performed at each pH value and temperature, and two samples of each hydrolyzed solution were analyzed for extent of epimerization. Thus, the percentages of isopilocarpine presented in Table III are the average values from six or more spectra. The coefficient of variation was 5.0%.

Table V - Epimerization of Pilocarpine to Isopilocarpine After Complete Hydrolysis and Recyclization (%)

pH \ Temp	25°	35°	45°	55°
9.5	--	--	29.6	32.3
10.0	20.5	26.3	30.4	32.0
10.5	20.8	27.0	29.5	32.3
11.0	20.0	25.9	30.3	--
11.5	21.0	25.7	--	--
12.0	20.8	--	--	--

Since the ratio (X) of pilocarpine to isopilocarpine as determined from the NMR spectra is equal to the ratio of products formed during the reactions, we may also let it equal the ratio of rate constants, i.e.:

$$\frac{k_H}{k_E} = X \qquad \text{(Eq. 11)}$$

Using $k_{Exp} = k_H + k_E$ (eqs. 7 and 8), and substituting into Eq. 11 gives:

$$k_{Exp} = k_H + \frac{k_H}{X} \qquad \text{(Eq. 12)}$$

which is solved for k_H:

$$k_H = k_{Exp} \frac{X}{X + 1} \qquad \text{(Eq. 13)}$$

Similarly, k_E may be expressed as:

$$k_E = \frac{k_{Exp}}{X + 1} \qquad \text{(Eq. 14)}$$

Using Eqs. 13 and 14, k_H and k_E were calculated at the temperatures investigated and are presented in Table IV.

Preliminary experiments have been performed on the base catalyzed reactions of isopilocarpine. At 25° the rate of disappearance of isopilocarpine under pH-stat conditions was

also found to follow pseudo first-order kinetics. The experimentally determined second-order rate constant was 9.36×10^{2} l. mole^{-1} hr.$^{-1}$ at 25°. Since pilocarpine was not produced during this reaction, this value is the second-order rate constant for the specific hydroxide-ion catalyzed hydrolysis of isopilocarpine (Eq. 4), and is greater than k_E by a factor of 6.5. Therefore, in the conversion of pilocarpine to isopilocarpate, the epimerization of pilocarpine to isopilocarpine (Eqs. 2 and 3) must be rate limiting. Since the basicity of the pilocarpine carbanion (P^-) is much greater than the basicity of the hydroxide ion, it is logical to assume that the rate of carbanion protonation (Eq. 3) will be greater than the rate of abstraction of the α-proton by hydroxide ion (Eq. 2). Thus, it may be postulated that k_E is the second-order rate constant for the abstraction of the α-proton of pilocarpine.

Arrhenius plots for hydrolysis of pilocarpine and for its epimerization to isopilocarpine are illustrated in Figs. 15 and 16, respectively. The slopes of these plots were obtained by the method of least squares. The energy of activation for the hydrolysis was calculated to be 25.02 Kcal mole^{-1} (Correl. coeff. 0.9995) and for the epimerization, 28.48 Kcal mole^{-1} (Correl. coeff. 0.9998). The magnitude of the epimerization rate constant together with its comparative increase with temperature leads to the conclusion that epimerization represents a major pathway of degradation and inactivation of pilocarpine in alkaline aqueous solution.

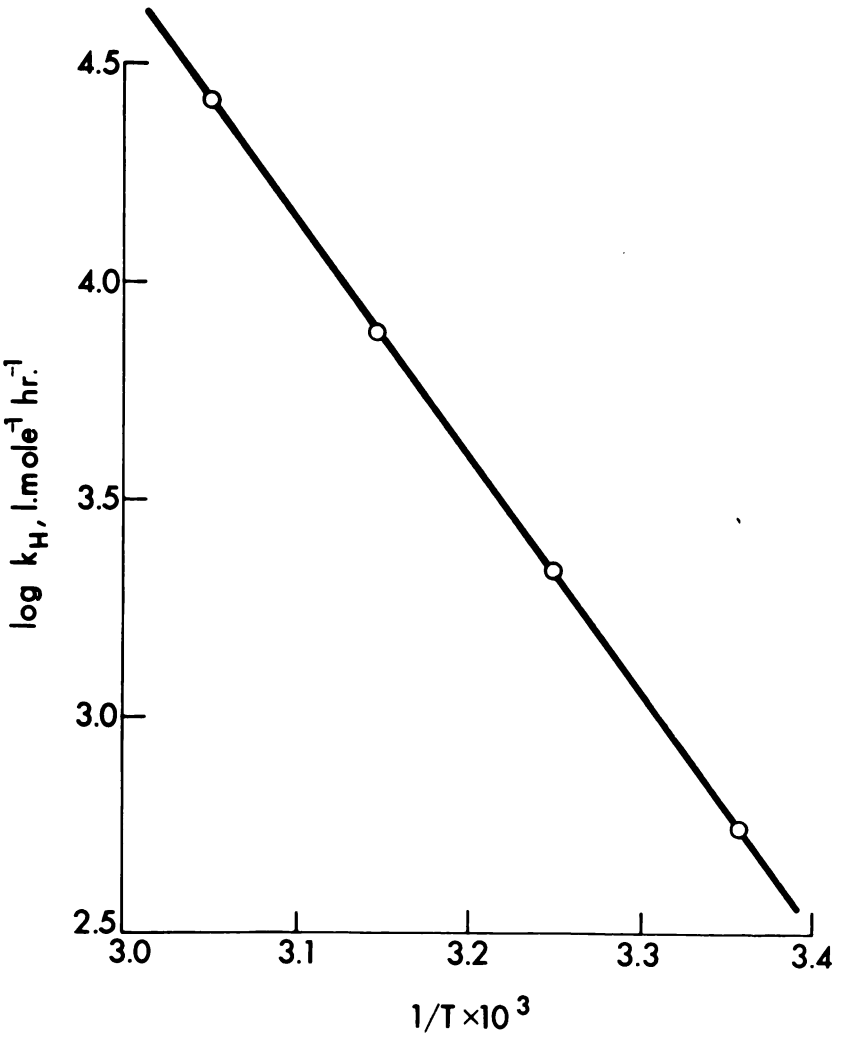


Figure 15 -- Arrhenius Plot of Hydroxide-ion Catalyzed Hydrolysis of Pilocarpine.

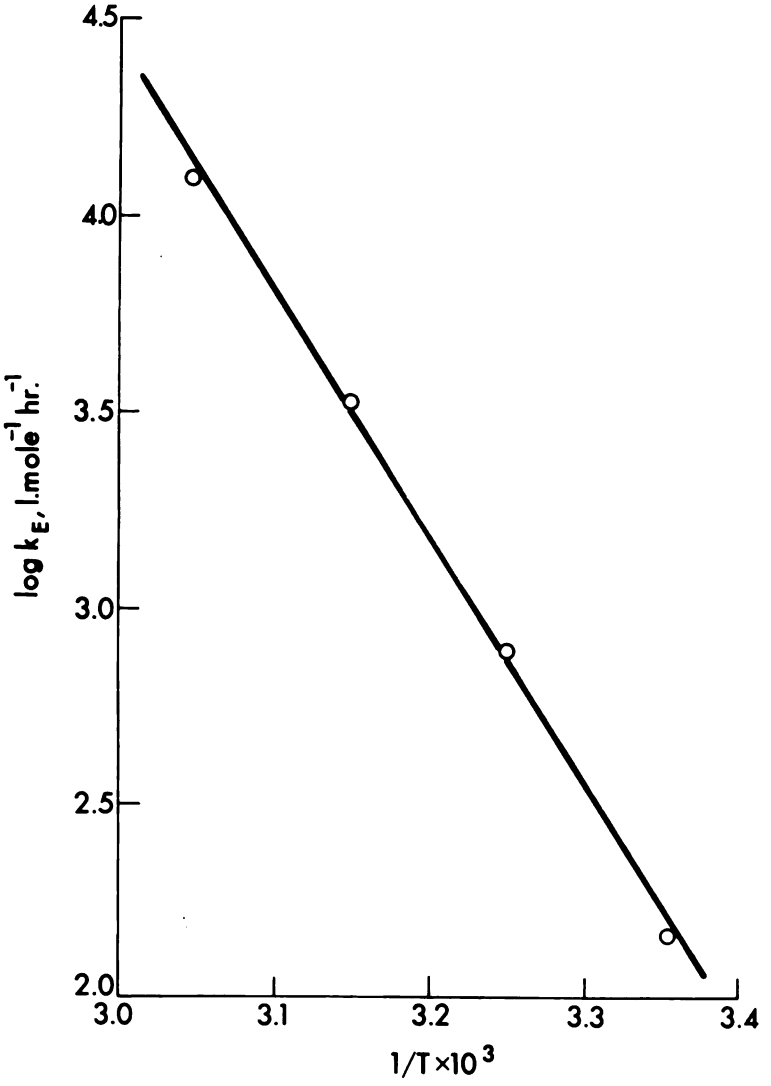


Figure 16 -- Arrhenius Plot of Hydroxide-ion Catalyzed Epimerization of Pilocarpine.

IV. SUMMARY AND CONCLUSIONS

The purposes of this investigation have been fulfilled by the development of an NMR method for the quantitation of pilocarpine or isopilocarpine when both are present in a mixture. Using this method in combination with pH-stat titrimetry the kinetic parameters associated with the alkaline, aqueous degradation of pilocarpine have been evaluated. Both hydrolysis and epimerization follow pseudo first-order kinetics and are competitive in nature. The epimerization of pilocarpine has been found to occur to a greater extent than previously assumed and must be considered as one of the major pathways of degradation and inactivation of pilocarpine. Since the rate of hydroxide-ion catalyzed epimerization increases more rapidly with temperature than does the rate of hydrolysis, ophthalmic solutions sterilized by heat may suffer significant loss of activity by this mechanism. It may also be surmised that it is not possible to isolate or extract pilocarpine base from its salts or from jaborandi leaves without some concurrent epimerization. It is, therefore, conceivable that isopilocarpine may not be a product of alkaloid biosynthesis in the living plants, but an artifact produced during drying, storage, extraction of the plant material and/or during the isolation of the alkaloids.

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