THE ROLE OF DELTA-1-PYRROLINE -5-CARBOXYLIC ACID IN THE BIOSYNTHESIS OF PYRROLIDINE RING OF NICOTINE

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ABSTRACT

THE ROLE OF Δ^1-PYRROLINE-5-CARBOXYLIC ACID IN THE BIOSYNTHESIS OF PYRROLIDINE RING OF NICOTINE

by Victor Krampl

Ornithine-2-C^{14}, glutamic acid-2-C^{14}, proline-C^{14}, and putrescine-1,4-C^{14} have been found to be efficient precursors for biogenesis of the pyrrolidine ring of nicotine. All these compounds exhibited an identical labeling pattern in the nicotine molecule; namely, that the total radioactivity was found to be equally distributed between carbons 2 and 5 of the pyrrolidine ring. It has been established in microorganisms and mammals that Δ^1-pyrroline-5-carboxylic acid (Δ^1-PC-5) is an intermediate in the conversion of glutamic acid or ornithine to proline. Similar experimental evidence with respect to higher plants was not available. It was anticipated that Δ^1-PC-$_5$ could function also in higher plants in these transformations. The objective of this study was to investigate the participation of Δ^1-PC-5 in the biogenesis of nicotine.

Dl-Δ^1-pyrroline-5-carboxylic acid-5-C^{14} has been synthesized, isolated as a hydrochloride and fed hydroponically to intact 3 months old tobacco plants.

Each plant received 1 to 2 mg of dl-Δ^1-PC·HCl-5-C^{14}, having the radioactivity of 2.0 to 4.0 x 10^6 c.p.m. The isolated nicotine was found to be radioactive. The specific activities varied between 4.5 and 0.6 x 10^4 c.p.m. per mmole, which corresponded to 0.14 to 0.02 per cent incorporation of the precursor. Variation in the incorporation of the precursor can be attributed to seasonal effects on the plants.

A "dry" feeding technique has been used for the administration of the precursor; namely, the total quantity of the precursor intended to be fed per plant, was administered in a single dose, in 1 to 2 ml solution. After the absorption of this volume to apparent dryness, three 1 to 2 ml aliquots of water followed, to achieve the highest possible uptake of the precursor. Approximately 40-50 per cent of the administered radioactivity after this treatment still remained unabsorbed. The precursor absorbed during the "dry" feeding period appeared to be the only material giving rise to the radioactivity found in nicotine. The remaining precursor in the nutrient solution was absorbed by the roots at a very slow rate. After expiration of the feeding period (7 days), approximately 25 per cent of the radioactivity originally fed to the plants was still recoverable. Scanning of the chromatograms and electrophorograms of the recovered residual feeding solution disclosed that the main peak of the recorded radioactivity still corresponded to that of the original solution fed. It could be tentatively suggested that one of the isomers of racemic Δ^1-PC·HCl-5-C^{14} could be absorbed by the roots at a much slower rate than its optical antipode and that this isomer does not participate in the biosynthesis of nicotine. Another possibility to consider is the polymerization of dl-Δ^1-PC·HCl-5-C^{14}.

Degradation of the nicotine resulted in the isolation of carbons 2 and 5 of the pyrrolidine ring as barium carbonate possessing 30 and 30 per cent of the total radioactivity, respectively, and carbon from the N-methyl group of nicotine isolated as methyltriethylammonium iodide, having 29 per cent of the total radioactivity. The relatively high incorporation of radioactivity into the N-methyl group is the only significant difference from the labeling pattern obtained with other known precursors of the pyrrolidine ring of nicotine.

Equal distribution of the radioactivity between carbons 2 and 5 presupposes, a priori, the existence of a symmetrical

intermediate, perhaps Δ^1-pyrroline-5-C^{14} and Δ^1-pyrroline-2-C^{14}, which could result from the decarboxylation of dl-Δ^1-PC·HCl-5-C^{14}, followed by a shift of the double bond. This symmetrical intermediate could well be the ultimate precursor for biogenesis of the pyrrolidine ring of nicotine in tobacco plants.

Nicotine isolated from leaves, stems, and roots showed a ratio of specific activities 1:3:4, whereas the ratio of distribution of the nicotine (mg/g of dry matter) was 17:3:1, respectively. This result supports the previous finding that the roots of tobacco plants are the main site of nicotine biosynthesis and that the leaves serve as a depot for nicotine.

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INTRODUCTION

The metabolic interrelationship of ornithine, glutamic acid and proline has attracted the interest of biochemists and physiologists for many years. Some publications concerned with this problem have provided direct experimental evidence for the participation of Δ^1-pyrroline-5-carboxylic acid (Δ^1-PC-5) in the interconversions of glutamic acid or ornithine to proline. Most of these investigations were restricted to mammals (1) and microorganism (2). The experimental data identifying this metabolic route with higher plants are still meager.

The conversion of labeled proline in vivo to glutamic acid and ornithine in intact rats has been investigated by Stetten and Schoenheimer (3). Deuterio-l-proline-N^{15} was found to contribute a significant quantity of isotope to glutamic acid, which was isolated from various tissues of sacrificed rats. Ornithine, although to a lesser extent, also has been found to be labeled. The pattern of labeling in the isolated glutamic acid and ornithine corresponded to that of the precursor. These results indicated in toto incorporation of the carbon chain of the precursor into these metabolically related amino acids. In similar experiments adult mice were fed with deuterio-dl-ornithine (4), dl-ornithine-α-N^{15} and dl-ornithine-δ-N^{15} (5). The isolated glutamic acid, proline and arginine were found to be labeled. Proline predominantly derived its imino nitrogen from the α-amino nitrogen whereas glutamic acid derived its nitrogen from the δ-amino group of ornithine. Glutamic-γ-semialdehyde was postulated as a plausible intermediate in the glutamic acid-proline transformation. In the view of these results the Krebs' proposal that α-keto-δ-amino valeric acid is an intermediate in conversion of proline to glutamic acid (6), appeared to be less probable. The studies of Stetten et al. (3, 4, 5) yielded not only very persuasive evidence of

glutamic acid-proline interrelationship, but also indicated the overall reversibility of the involved transformations.

Taggart and Krakaur (7), utilizing in vitro studies with rabbit kidney homogenates, were able to demonstrate that from all naturally occurring amino acids only three, l-proline, l-glutamic acid and l-hydroxyproline, were rapidly oxidized via the tricarboxylic acid cycle. This oxidation under favorable conditions proceeded to completion. The products were carbon dioxide, water and ammonia. Incomplete oxidation resulted in accumulation of a carbonyl intermediate. Although they did not obtain conclusive proof that this carbonyl intermediate was glutamic -γ-semialdehyde, they obtained sufficient evidence which excluded α-keto-δ-aminovaleric acid as a possibility (6, 8). Glutamic acid has been identified as one of the intermediates of proline oxidation by the citric acid cycle.

Smith and Greenberg (9) prepared partially purified glutamic semialdehyde reductase from rat liver. This preparation catalyzed the conversion of synthetic dl-Δ^1-PC-5 (or glutamic-γ-semialdehyde) to proline. They suggested that reduction of glutamic semialdehyde is performed after a ring closure while this compound is still attached to the surface of the enzyme. Meister et al. (10) obtained a DPN* dependent enzyme from rat tissue mitochondria which catalyzed the reduction of Δ^1-PC-5 and Δ^1-pyrroline-2-carboxylic acid (Δ^1-PC-2) to proline and Δ^1-piperideine-2-carboxylic acid to pipecolic acid.

Strecker and Mela (11) also observed that liver mitochondria extracts in the presence of DPN and inorganic phosphate catalyzed the reduction of a synthetic preparation of Δ^1-PC-5 to proline. Recently Strecker (12) described the properties of an enzyme preparation from rat liver mitochondria catalyzing specifically the reaction: Δ^1-PC-5 $\rightarrow$ glutamic acid. The investigations conducted on the glutamic acid-proline

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*Diphosphopyridine nudestide (DPN).

transformation have produced sufficient evidence that it was not a single step process but that it included a series of enzymatic reactions. Strecker believes that although the overall result of the glutamic acid-proline transformation is apparently reversible, the individual reactions involved are essentially irreversible. Based on this assumption he proposed the following sequence of reactions:

Glutamic acid $\longrightarrow$ Δ^1-PC-5 $\longrightarrow$ proline $\longrightarrow$

$\longrightarrow$ Δ^1-PC-5 $\longrightarrow$ glutamic acid.

Similar interconversions in microorganism were either postulated or conclusively proved (13).

Vogel and Davis (14) used three mutants of Escherichia coli exhibiting different requirements for proline or proline precursor. A mutant 55-1, requiring proline for growth, accumulated Δ^1-PC-5 in its culture medium. This medium supported the growth of another autotroph, 55-25. The third mutant responded equally well to proline, glutamic acid or Δ^1-PC-5. Comparison of the behavior of these three mutants disclosed not only the specific metabolic blocks but also suggested again the proline-Δ^1-PC-5-glutamic acid relationship.

Fincham (15) reported the isolation and partial purification of ornithine transaminase from Neurospora crassa which catalyzed the reaction: ornithine + α-ketoglutarate $\rightleftharpoons$ glutamate + glutamic-γ-semialdehyde. Strecker (16) essentially reproduced the results of Vogel and Davis (14) with E. coli mutant 55-1 which was dependent on l-proline as a substrate. Furthermore, he observed that washed, resting cells of the E. coli mutant increased the synthesis of Δ^1-PC-5 from α-ketoglutarate if other substrates were added to the medium such as pyruvate, lactate and formate. Meister et al. (10) in continuation of their work with rat tissue mitochondria obtained the same enzymatic preparation from Neurospora crassa. A mutant of this fungus and a mutant of

Aerobacter aerogenes genetically blocked at the stage of reduction of Δ^1-PC-5 to proline could utilize for normal growth Δ^1-pyrroline-2-carboxylic acid (Δ^1-PC-2) as a substrate for proline synthesis. On this basis they suggested the possible existence of two different enzymes, one utilizing Δ^1-PC-5 and the other Δ^1-PC-2 as substrates. They also observed in the presence of DPNH a non-enzymatic reduction of Δ^1-PC-2, but not Δ^1-PC-5, to proline. Proline formed by enzymatic redution in both cases was found to be of the l-configuration.

Yura and Vogel (17) also isolated an enzyme system from N. crassa, which was DPN dependent and catalyzed the reduction of synthetic Δ^1-PC-5 to proline.

Vogel and Kopac (18), as a result of their studies with N. crassa, were able to extend the metabolic role of Δ^1-PC-5 to the glutamate-arginine-proline interrelationship. Using dl-ornithine-2-C^{14} as the precursor they found that exogenous ornithine readily contributed to proline synthesis, whereas the endogenous ornithine-arginine pathway, did not.

Detained discussions of the glutamic acid-proline interrelation have been compiled in reviews by Stetten (1) and Voegl (2).

By far less information has been derived from experiments with higher plants. There is not available any direct conclusive evidence that glutamic-γ-semialdehyde (or its cyclized analog, Δ^1-PC-5) participates in similar transformations in plant. However, by analogy with mammals and microorganisms, glutamic acid-proline interconversion is expected also to be linked by the same common intermediate. There is evidence that this analogy may not be generally valid in plants. Naylor and Tolbert (19) fed uniformly labeled glutamic acid to barley seedlings and found the isolated proline and ornithine were not labeled.

The structural similarity between proline and pyrrolidine ring containing compounds presupposes a priori the biogenetic relationship

in plants. Several naturally occurring compounds which contain a pyrrolidine ring are alkaloids, e.g. nicotine, stachydrine and hyoscyamine. Therefore, it is not surprising that most of the work involving a study of the metabolic interrelationship between glutamic acid-ornithine-pyrrolidine compounds, were performed with alkaloidogenic plants.

Morgan and Marion (20) succeeded in obtaining radioactive glutamic acid from seedlings of Medicago sativa L. Grimm after administering dl-ornithine-2-C^{14}. Proline and stachydrine were found to be non-radioactive. Robertson and Marion (21) confirmed this observation that lebeled ornithine or proline are not the real precursors of stachydrine. However, they found incorporation of hygrinic acid (1-methyl-2-pyrrolidine carboxylic acid) into stachydrine. On the basis of this evidence they concluded that hygrinic acid and the pyrrolidine ring of stachydrine were not derived from proline, but from smaller metabolic fragments.

Robinson (22) as far back as 1917, suggested that ornithine is a source of putrescine and consequently of pyrrolidine ring compounds in plants. However, James (23), in contrast to Robinson's suggestion, was unable to decarboxylate ornithine to putrescine by using slices of belladonna species as a source of the enzyme. When arginine and ornithine were fed to Atropa belladonna a significant increase in synthesis of hyoscyamine was observed. Aerobic oxidation of ornithine in the presence of belladonna tissue yielded α-keto-δ-aminovaleric acid. This is in good agreement with similar observation made by Krebs (6) with mammalian tissues. Klein and Linser administered proline, ornithine and glutamate to the plants, Stachys palustris, L., Stachys recta L. and Galeopsis ochroleuca Lan. (24) and tobacco plants (25) and observed an apparent increase in stachydrine and nicotine content, respectively. Leete and Marion (26) investigated the biosynthesis of the pyrrolidine ring in the tropine base of hyoscyamine. Ornithine-2-C^{14} was fed to Datura stramonium. Degradation of the hyoscyamine showed that the

radioactivity was located in both carbons adjacent to the nitrogen in the pyrrolidine ring. They suggested α-keto-δ-aminovaleric acid as a possible intermediate. Strangely enough, they failed to find any significant incorporation of ornithine-2-C^{14} into the pyrrolidine ring of stachydrine (27).

The supposition that there was a metabolic relationship, ornithine-glutamic acid-Δ^1-PC-5-proline-pyrrolidine ring, was most extensively and systematically employed in studies of the biosynthesis of nicotine in tobacco plants. Dewey et al. (28) isolated radioactive nicotine from tobacco plants, Nicotiana rustica, var. humilis after feeding ornithine-2-C^{14} to hydroponically grown intact plants. Almost all radioactivity was found in the pyrrolidine ring of nicotine. The pyridine ring contained only negligible radioactivity. Degradation of the nicotine revealed that approximately half of the radioactivity was located in carbon 2 of the pyrrolidine ring. The remaining half was assumed to be located in position 5.

Independently, Leete (20) administered the same precursor to intact Nicotiana tabacum L. and obtained similar results. In a subsequent publication (31) he showed that carbons 2 and 5 of the pyrrolidine ring were equally radioactive. Oxidation of nicotine with nitric acid resulted in the formation of nicotinic acid and a pyrazole derivative of nicotine. The latter compound possessed all the carbons of the original nicotine molecule except carbon 5 which was displaced by nitrogen. The radioactivity in carbon 2 was determined directly by decarboxylation of nicotinic acid and collection of carbon dioxide as barium carbonate. The radioactivity located in carbon 5 was established only by the difference between the original radioactivity found in nicotine and the pyrazole derivative, 3-nitro-5-(3'-pyridyl)-pyrazole.

An identical observation was made by Lamberts et al. (33). Dl-ornithine-2-C^{14} was administered hydroponically to three months

old intact tobacco plants, N. rustica, var. humilis. The radioactive nicotine was found to possess approximately an equal distribution of radioactivity between carbons 2 and 5 of the pyrrolidine ring. The significant contribution of this work was the development of a new degradative procedure leading specifically to isolation of carbon 5 of the pyrrolidine ring. Lamberts and Byerrum (34) also fed glutamic acid-2-C^{14} to Nicotiana rustica, var. humilis. Again, positions 2 and 5 of the pyrrolidine ring shared equally the total radioactivity found in the isolated nicotine.

An extension of Leete's work (29) included comparative data obtained from feeding experiments with dl-ornithine-2-C^{14}, putrescine-1, 4-C^{14} hydrochloride, l-proline-C^{14}, and dl-glutamic acid-2-C^{14} to intact Nicotiana tabacum L. (var. Maryland Mammoth). All of these precursors were found to contribute to the biosynthesis of pyrrolidine ring. The incorporation of ornithine, putrescine, proline and glutamic acid was 0.48, 0.12, 0.032 and 0.0078 per cent, respectively. As a working hypothesis it was suggested that nicotine is synthesized in a process involving reduced DPN and a symmetrical intermediate Δ^1-pyrroline (29).

All experimental data with higher plants accummulated to date strongly suggest the participation of Δ^1-PC-5 as an intermediate between glutamic acid and proline, but conclusive proof is still desired. One can hypothesize that the pyrrolidine ring of nicotine can be formed by the following sequence of metabolic reactions:

$$\Delta^1\text{-PC-5} \xrightarrow{\text{enzymatic decarboxylation}} \Delta^1\text{-pyrroline (a symmetrical intermediate) (30, 32)} \xrightarrow{\text{reduction}} \text{pyrrolidine ring.}$$

There is not as yet any direct proof of the existence of this metabolic route, but its acceptance as a working hypothesis is very plausible.

It was the main objective of this study to investigate the quantitative participation of synthetic dl-Δ^1-pyrroline-5-carboxylic acid-5-C^{14}

dl-Δ^1-PC-5-C^{14}) in the biogenesis of nicotine in tobacco plants.

Pursuing this objective, the following work was preformed:

1. Synthesis and isolation of dl-Δ^1-PC-5-C^{14} specifically labeled in position 5.
2. Administration of this precursor to tobacco plants under reasonably standard conditions and isolation of radioactive nicotine.
3. Degradation of isolated radioactive nicotine in order to determine the pattern of labeling.

Equal incorporation of radioactivity in positions 2 and 5 of the pyrrolidine ring of nicotine would indicate the incorporation of the whole intact ring of the precursor into the nicotine molecule. Furthermore, if this anticipated result occurred, it would provide evidence for the metabolic interrelationship ornithine-glutamic acid-Δ^1-PC-5--proline or pyrrolidine ring. Evidence would thus be obtained for the existence of the postulated symmetrical intermediate, Δ^1-pyrroline.

EXPERIMENTAL AND RESULTS

Synthesis of dl-Δ^1-Pyrroline-5-carboxylic Acid (dl-Δ^1-PC-5)

The formation of Δ^1-PC-5, as pointed out previously, has been demonstrated in some biological systems (1, 2, 3, 12, 15, 16).

This fact stimulated a whole series of investigations converging in the attempt to elucidate its participation in biosynthetic pathways. The determination of its absolute concentration in solutions has been difficult due to the presence of some undesirable contaminants. Investigators have tried to simplify this matter by making assumptions which allowed them to estimate the effective concentration: Vogel and Davis (14) applied a biological assay, Smith and Greenberg (9) used oxidation of DPNH as a criterion of concentration and Strecker (16) made an assumption that γ, γ-dicarbethoxy-γ-acetamidobutraldehyde is quantitatively hydrolyzed to Δ^1-PC-5. It has been known for some time that o-aminobenzaldehyde forms a stable yellow complex, dihydroquinazolinium (14), (35) by condensation with Δ^1-pyrroline ring containing compounds (35, 36). This reaction has been found useful for quantitative tests for pyrrolines and piperideines (37). The colorimetric procedures based on this reaction were not of great help for the quantitative determination of Δ^1-PC concentration because of the lack of a pure standard which is necessary for preparation of a calibration curve (16).

Vogel and Davis (14) first reported the synthesis of Δ^1-PC-5, but failed to isolate it from solution. The excessive polymerization encountered during the attempted separation was the main difficulty. The presence of Δ^1-PC-5 in the solution has been identified by its reduction to proline and by isolation and characterization of this compound as a picrate. Specific requirement of E. coli mutant (55-25) for Δ^1-PC-5

as a substrate for proline biosynthesis has also been used as a tool for its identification. Good and Mitchell (38) confirmed the results of the Vogel and Davis synthesis (14) by obtaining this compound through a different synthetic route. They synthesized glutamic-γ-semialdehyde diethyl acetal, which, when subjected to acid hydrolysis, produced a compound having the same chemical and biochemical behavior.

All the work related to this topic strongly suggested that glutamic-γ-semialdehyde exists in solution in equilibrium with its cyclized form, Δ^1-PC-5 (11, 14, 15, 16, 18, 38, 39). Very likely this equilibrium exists also in biological systems as a non-enzyme catalyzed reversible process.

A great part of this study has been devoted to the synthesis of Δ^1-PC-5-C^{14} and to its isolation. The original objective was to obtain this compound either in the pure form or in a useful, relatively stable derivative suitable for feeding to plants. This goal has been achieved by obtaining Δ^1-PC-5-C^{14} as an hydrochloride in a relatively high degree of purity. Simultaneously with this work, the identical problem was pursued by Strecker, who independently succeeded in isolation of Δ^1-PC-5 as the hydrochloride (39). Details pertaining to physical properties, chemical stability and preparation and characterization of some derivatives of Δ^1-PC·HCl-5 are presented in this publication.

Since Strecker's work appeared after the completion of the synthesis in this laboratory, his report was of little help in achieving the pursued objective. Prior to the attempted synthesis of dl-Δ^1-PC·HCl-5-C^{14} the following anticipated limitations were critically studied:

1. Availability of the suitable starting compound having C^{14} in a position which would allow the end product, dl-Δ^1-PC·HCl, to have a label specifically in position 5.
2. The feasibility of a semi-micro scale in the individual steps involved in synthesis.
3. The costs of the starting material and the yield of the final product.

In the first attempt, glutamic acid was used as a starting compound. The proposed sequence of reactions which was anticipated to give the dl-Δ^1-PC, is briefly indicated below.

D-glutamic acid $\xrightarrow{p\text{-}CH_3\text{-}C_6H_5SO_2Cl}$ N-p-toluenesulfonyl-d-glutamic acid $\longrightarrow$ $\xrightarrow{n\text{-butanol}}$ di-n-butyl-(N-p-toluenesulfonyl)-d-glutamate $\xrightarrow{\text{aqueous KOH,}}$ $\xrightarrow{1\text{ equivalent}}$ mixture of products $\xrightarrow{\text{separation}}$ mono-n-butyl-(N-p-toluene-sulfonyl)-d-glutamate $\xrightarrow{SOCl_2}$ mono-n-butyl-(N-p-toluenesulfonyl)-d-glutamyl chloride $\xrightarrow{\text{Rosenmund reduction}}$ mono-n-butyl-(N-p-toluenesulfonyl)-d-γ-glutamic aldehyde $\xrightarrow{\text{hydrolysis}}$ d-glutamic-γ-semialdehyde $\rightleftharpoons$ d-Δ^1-pyrroline-5-carboxylic acid.

The first two reactions have been described by McChesney and Swann (40), and Gurin and Clarke (41). This route of synthesis proved to be inapplicable on a semi-micro scale. It was discontinued at the stage of the partial hydrolysis of the diester to monoester. This step resulted in a mixture of products with approximately 50 per cent recovery of the original diester. White needles of a mono-n-butyl-(N-p-toluene-sulfonyl)-d-glutamate were obtained in the yield of 15-20 per cent (calculated on the basis of the diester), which melted at 106-107°C. The neutralization equivalent was found to be 345, which is 3 per cent less than the actual value calculated for the monoester.

The second attempted synthesis was a partial adaptation of the procedure proposed by du Vigneaud and Miller (42). This synthesis includes the following sequence of reactions:

l-glutamic acid $\xrightarrow{C_6H_5CH_2CO_2Cl,^{*}MgO}$ carbobenzoxy-l-glutamic acid $\xrightarrow{(CH_3CO)_2O}$ carbobenzoxy-l-glutamic anhydride $\xrightarrow{CH_3ONa}$ α-methylcarbobenzoxy-l-glutamate $\xrightarrow{PCl_5}$ α-methylcarbobenzoxy-l-glutamyl chloride $\xrightarrow{\text{Rosenmund reduction}}$ α-methylcarbobenzoxy-γ-l-glutamyl aldehyde $\xrightarrow{HCl}$ l-glutamyl-γ-semialdehyde $\longrightarrow$ l-Δ^1-pyrroline-5-carboxylic acid hydrochloride.

This procedure up to the stage of the α-methyl carbobenzoxy-l-glutamyl chloride appeared to be very promising. The reactions proceeded with relative ease, the individual yields were relatively high and dl-glutamic acid-2-C^{14} was available commercially. However, this method at the stage of Rosenmund's reduction of the acyl chloride was discontinued because of unsatisfactory results.

The synthesis of glutamic-γ-semialdehyde diethyl acetal was reported by Good and Mitchell (38). This procedure consisted of the condensation of acetamidocyanoacetate with β-chloropropionaldehyde diethyl acetal. This method of synthesis was not seriously considered because the starting radioactive compound was not commercially available and the reported yield was approximately 1 per cent calculated on the basis of acetamidocyanoacetate.

However, the subsequent commercial availability of diethyl aminomalonate-2-C^{14} and later diethyl acetamidomalonate-2-C^{14} made the procedure of Vogel and Davis (14) the most applicable of all the methods considered.

Since the starting quantity of the acetamidomalonate containing the desired radioactivity, 0.5 mC, weighed only 104.5 mg, it was

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*$C_6H_5CH_2CO_2Cl$ (carbobenzoxychloride) was purchased from Mann Research Laboratory, Inc., New York.

necessary to use some non-radioactive acetamidomalonate as a diluent. Therefore, the overall sequence of reactions includes the synthesis of diethylacetamidomalonate from diethylmalonate. Details concerning the synthesis of dl-Δ^1-PC-5-C^{14} and its precursors are as follows:

Diethyl malonate $\xrightarrow{\text{n-butyl nitrite}}$ diethyl isonitrosomalonate $\xrightarrow{\text{reduction;Ni, } H_2}$

diethyl aminomalonate $\xrightarrow{(CH_3CO_2)O, \text{ reflux}}$ diethyl acetamidomalonate

$\xrightarrow{\text{acrolein, } NaOC_2H_5}$ γ-γ-dicarbethoxy-γ-butyraldehyde $\xrightarrow{HCl, \text{ hydrolysis}}$

dl-glutamic-γ-semialdehyde $\rightleftharpoons$ dl-Δ^1-pyrroline-5-carboxylic

acid hydrochloride.

Synthesis of n-butyl nitrite

The procedure of Noyes (43) was followed for the synthesis of n-butyl nitrite. Fourteen and one-half cc of concentrated H_2SO_4 was diluted with 10 cc of water and cooled to $0^{\circ}C$, followed by addition of 39 g (48.6 ml) of n-butanol and reduction of the temperature again to $0^{\circ}C$. During the 10 min. period a homogenous solution resulted. The solution of sodium nitrite (38 g sodium nitrite dissolved in 150 ml of water) was introduced with the aid of a separatory funnel beneath the surface of the cooled solution. Vigorous stirring and cooling in an ice bath was continued for 20 minutes. On standing at room temperature three phases separated: n-butyl nitrite, a saturated solution of sodium sulfate and crystals of sodium sulfate. The top layer, n-butyl nitrite, was separated, dried over anhydrous sodium sulfate and distilled. The yield obtained was 75 per cent of theory; b.p. 77-78$^{\circ}C$.

Synthesis of diethyl isonitrosomalonate

The modified method of Redemann and Dunn (44), with partial application of the procedure described by Snyder and Smith (45) has been used.

Three and one-half g of sodium was dissolved in 50 ml of absolute ethanol and placed in a 100 ml round bottom three neck flask fitted with a mechanical stirrer, reflux condenser and dropping funnel with stopcock. To a clear solution of sodium ethoxide, 25 g of redistilled diethyl malonate was added. The flask was suspended in an ice bath and 17.5 g of redistilled n-butyl nitrite was added gradually with stirring. As the reaction proceeded the separated sodium diethylmalonate slowly disappeared. At the end of the reaction period a clear orange-yellow solution resulted. Solvents were evaporated by vacuum distillation. The oily brownish residue of the impure diethyl isonitrosomalonate was treated with crushed ice and 30 ml of 6N sulfuric acid. The oil which separated, isonitrosomalonate, was then extracted several times with 20 ml portions of diethyl ether. The ether solution was washed with a saturated solution of sodium bicarbonate and then dried over anhydrous sodium sulfate. Ether was removed by distillation. It is essential that the ether used for these extractions is free of peroxides. Isonitrosomalonate in the presence of peroxide forms an explosive mixture when heat is applied.

Diethyl isonitrosomalonate was further purified by vacuum distillation. It was distilled at 120-122^{o}C at 0.01 mm pressure. The yield obtained was 24.8 g, which is 73 per cent of theory. Pure diethyl isonitrosomalonate is a colorless oil at room temperature, but solidifies as a white crystalline mass when cooled below 0^{o}C.

Preparation of diethyl aminomalonate

Catalytic reduction of diethyl isonitrosomalonate results in the formation of diethyl aminomalonate. Levene and Schormueller (46)

reported the successful use of low pressure hydrogenation for this reduction. Some other modifications could be found in the literature (44, 45). In the preliminary test the Levene and Schormueller procedure was adopted (46), which was operated at 40 lbs pressure of hydrogen at 50°C. The yield obtained, calculated on the basis of isolated diethyl aminomalonate hydrochloride, did not exceed 50 per cent of theory. Some ammonia formation was observed. The reduction under these conditions obviously had proceeded too far. The reaction medium turned green, probably due to the formation of a complex between ammonia and nickel. The low pressure hydrogenation unit available in this laboratory was technically difficult to adjust for the use of the small quantities of reactants. Therefore, a rather simple hydrogenation unit was assembled which operated at room temperature and atmospheric pressure.* This apparatus was successfully used in all subsequent reductions of samples ranging from 50 mg to 10 g.

Several catalysts for the hydrogenation were tried: Palladium deposited on charcoal, Adams' catalyst, rhodium deposited on alumina and Raney's nickel. Adams' catalyst appeared to be the most efficient. However, since Adams' catalyst was expensive, Raney's nickel catalyst, prepared by the procedure of Mozingo (47), was used routinely and with good results, provided 30-50 per cent of catalyst was used per weight of diethyl isonitromalonate. Under these conditions the hydrogenation proceeded smoothly and in 6-8 hours was completed. The hydrogenation was discontinued when the uptake of hydrogen decreased to a small and constant level per unit of time. This point usually was reached when 80-86 per cent of the expected hydrogen volume was absorbed.

Reduction of diethyl isonitrosomalonate

Diethyl isonitrosomalonate and approximately 30-50 per cent of

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*For the design of the hydrogenation apparatus I am deeply obliged to Dr. Gordon L. Kilgour.

Raney's nickel catalyst was placed into a horizontal, cylindrical glass boat and covered with absolute ethanol. The boat had two outlets fitted with stopcocks. During the hydrogenation the boat was placed on a shaker and connected through a Tygon tube to the hydrogenation apparatus. The hydrogenation assembly consisted of three calibrated cylinders (500, 100, and 25 ml) joined together through a system of tubes and stopcocks, and the boat. The entire hydrogenation unit including the boat with its contents was flushed for a few minutes with hydrogen. Any of the three cylinders could be joined to the hydrogen train independently and serve as an immediate hydrogen supply. After the completion of the flushing, the proper cylinder, with respect to the size of the sample, was then selected by opening a stopcock and hydrogenation started. During the flushing period the catalyst was saturated with hydrogen. The reduction was proceeding at this stage. The initial reading taken from the cylinder did not account for this hydrogen uptake. If quantitative measurements were desired, a solution of the compound to be reduced was first introduced into a small tube and placed in a vertical position in the mouth of the boat outlet. After completion of the flushing the tube containing the compound was slid into the boat by rotating it in a 45° angle.

When the reduction was completed, the catalyst was filtered on a sintered glass funnel. The filtrate was evaporated to dryness. The oily residue of impure diethyl aminomalonate was used for the next reaction without any further purification.

Synthesis of diethyl acetamidomalonate

To improve the yield of the product it was found advantageous to modify the procedure of Snyder and Smith, (45) for synthesis of diethyl-acetamidomalonate. Dry impure diethyl aminomalonate was dissolved in absolute ethanol, treated with an excess of acetic anhydride (6 ml per 5 g of diethyl isonitrosomalonate) and refluxed for 3 hours.

The resulting, slightly greenish, solution was then evaporated to dryness in a flash evaporator at 50°C, treated with 20 ml of 50 per cent aqueous ethanol and evaporated again. This procedure was repeated until all remaining acetic anhydride was decomposed or evaporated. Some crystallization of diethyl acetamidomalonate resulted at this point. The semi-solid residue was dissolved in a small volume of 95 per cent ethanol with warming on the steam bath. Then water was added dropwise until cloudiness appeared. The solution was warmed again and left to crystallize, first at room temperature and then overnight in the refrigerator. After separation of the first yield of crystals the volume of the filtrate was diminished and crystallization allowed to occur again. Both samples were combined and recrystallized from water. Slow crystallization from hot water resulted in the formation of white, relatively large, cubical crystals which melted at 96-97°C. The yield on the basis of diethyl isonitrosomalonate was 67-80 per cent, depending on the size of sample used.

Synthesis of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde

Generally the method of Moe and Warner (48) was followed with some modification with regard to purification of the product. A representative procedure was as follows: 0.445 g of diethyl acetamidomalonate and a magnetic stirring bar were placed into a 50 ml oval standard-taper flask. The compound was dissolved in 4.5 ml of absolute ethanol and cooled to 0°C in an ice bath. The solution of sodium ethoxide in absolute ethanol, 0.5 ml, equivalent to 2.5 mg of sodium, was added as a catalyst. At this point a slight cloudiness resulted in the solution due to precipitation of the sodium salt of diethyl acetamidomalonate. Vigorous stirring was then started. A rubber stopper having two small holes was placed into the neck of the flask. A micropipette containing 0.35 ml of redistilled acrolein was inserted in the hole of the rubber stopper.

The micropipette was fitted with a syringe and microscrew to facilitate delivery of acrolein in 20 μl volumes at a time. The other small hole in the rubber stopper served only as a means of equalizing the inner pressure in the reaction vessel with atmospheric pressure during the delivery of acrolein. The addition of acrolein was completed in 75 minutes. As the reaction proceeded the color of the solution changed to light green. Disappearance of the sodium salt of diethyl acetamidomalonate was not an indication of the completion of the reaction. Stirring at a temperature 0-3°C was continued 1½ hour. An excess of glacial acetic acid, 0.05 ml, was added to the reaction mixture to neutralize the sodium ethoxide. After addition of the glacial acetic acid the color of the solution changed to orange-brown. At this point the ethanolic reaction mixture contained γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde (DCAABaldehyde), sodium acetate, excess of acrolein or its condensation products, possibly unreacted diethyl acetamidomalonate and acetic acid. Diethyl acetamidomalonate was the most objectional contaminant because in the next reaction it would yield glycine on hydrolysis, which could effect the labeling pattern of nicotine after being fed to the tobacco plants. Therefore, great care was exercised in purification of DCAABaldehyde.

Vogel and Davis (14) used phenylhydrazine for purification of DCAABaldehyde as the phenylhydrazone, followed by decomposition of the phenylhydrazone by addition of benzaldehyde to give benzaldehyde phenylhydrazone. The DCAABaldehyde was then extracted with water. This would be the method of choice if the purity of the product rather than the yield were considered. An attempt to use levulinic acid in preference to benzaldehyde for transderivation of DCAABaldehyde phenylhydrazone failed to give a suitable product (78). A neutralized ethanolic solution of DCAABaldehyde was evaporated to dryness. This was followed by the addition of 20 ml of absolute ethanol and the solution

was evaporated again. This process was repeated several times until the characteristic odor of acrolein disappeared. The remaining light-brown oil, the impure DCAABaldehyde, was expected at this point to be free of acrolein and acetic acid. However, when water was added to this oil, the solution became cloudy indicating the presence of some impurity. The cloudy solution could be cleared by addition of absolute ethanol in a great excess. This impurity was believed to be some condensation product of acrolein. To prove or disprove this assumption the following experiment was undertaken: to an ethanolic solution of sodium ethoxide, acrolein was gradually added in small volumes. Diethyl acetamidomalonate was omitted from reaction medium. The product obtained from this reaction was a slightly brown oil much like the oil obtained from the regular addition reaction of acrolein to diethyl acetamidomalonate. It was obvious that this oily product could be nothing else than some polymer of acrolein. The product was soluble in common organic solvents; such as, ether, hexane, methanol, ethanol, benzene, not appreciably soluble in acetone and insoluble in water. An attempted crystallization of this polymer from ethanol and water was unsuccessful. However, after evaporation of this solution an amorphous brownish solid separated, whith melted at 120-121°C. Further characterization of this compound was not undertaken.

It was found that purification of small samples of DCAABaldehyde could be achieved more readily utilizing chromatography on an alumina column. In a preliminary test the chromatographic method of Vogel and Davis (14) was used without any modification. According to this method the transfer of DCAABaldehyde could be achieved by dissolving it in a hexane-benzene mixture (55:45). However, the DCAABaldehyde was found not to be appreciably soluble in this solvent; e.g., for a quantitative transfer of approximately a 1 g sample of DCAABaldehyde on the alumina column it was necessary to use four 50 ml volumes of

solvent. The remaining white solid residue in the flask after the oil has been dissolved and transferred to the column was identified as sodium acetate. The collected hexane-benzene eluate contained some polymer, probably the "dimer" of acrolein as a detectable contaminant. When this eluate was treated with 6 N hydrochloric acid in a closed system, no evolution of carbon dioxide was observed, nor could any Δ^1-PC-5 be detected in the treated eluate. These findings led to the conclusion that diethyl acetamidomalonate or DCAABaldehyde were absent in the hexane-benzene eluate.

The elution of the column was continued with 200 ml of a benzene-diethyl ether mixture (50:50). A colorless, viscous oil remained in the flask after evaporation of the solvent from the eluate. The oil was treated with water and this treatment resulted in a mixture which had a cloudy appearance. This was an indication that the "dimer" of acrolein was present also in this fraction. Chromatography of the water mixture on a charcoal column of Norite A, resulted in the removal of the supposed acrolein "dimer" contaminant from the product. At this stage of the purification the only contaminant expected should be the unreacted diethyl acetamidomalonate. Before any conclusions could be made with regard to this possibility, it was necessary to test the absorptive capacity of the alumina column for diethyl acetamidomalonate.

The solution of 95.9 mg of diethyl acetamidomalonate in 200 ml of a hexane-benzene mixture (55:45) was chromatographed on an alumina column (16 cm long and 1 cm in diameter) and eluted with 200 ml of a benzene-ether mixture (50:50), followed by 200 ml of absolute ethanol and finally by 200 ml of 50 per cent ethanol. All eluates were collected, evaporated to dryness and weighed. The weights of solid residue from the individual elutions were 9.8, 3.7, 0.0, and 18.5 mg; respectively. The residue from the first and second elution was soluble in organic solvents, but insoluble in water. Both had a semi-solid appearance

and the first had also a repulsive odor. The fourth residue was a white solid. All three residues and 20 mg of diethyl acetamidomalonate, used as a control, were individually hydrolyzed with 6 N hydrochloric acid. During the hydrolysis the system was continuously flushed with nitrogen. Carbon dioxide was absorbed into a saturated solution of barium hydroxide. The first and second residue did not show any evidence of carbon dioxide evolution; the fourth fraction and the control produced 9.0 and 17.5 mg of barium carbonate, respectively. All hydrolysates were evaporated to dryness and dissolved in 1 ml of water. The resulting solutions were tested for the possible presence of glycine. Twenty aliquots of each hydrolyzed residue and the control were spotted on filter paper and sprayed with polychromatic indicator for amino acids (49). A positive test for glycine was obtained only from the hydrolysate of fraction four and from the control. The preceding experiments indicated that diethyl acetamidomalonate under the described conditions can be efficiently separated from DCAABaldehyde by chromatography on an alumina column.

Compiling the accumulated experience from preliminary tests, the purification of DCAABaldehyde was finally accomplished as follows:

Impure DCAABaldehyde was dried several hours in a vacuum desiccator over phosphorus pentoxide, then dissolved in anhydrous ether and refrigerated. Sodium acetate which settled out, was separated by filtration. The filtrate was evaporated to dryness and dissolved in water. The cloudy solution of DCAABaldehyde was passed through a charcoal column. This step effectively removed the acrolein "dimer." The eluate from the charcoal column was evaporated to dryness, dissolved in the smallest possible volume of water and placed in the refrigerator. Under these conditions unreacted diethyl acetamidomalonate crystallized out. The filtrate after separation of diethyl acetamidomalonate was evaporated again to dryness and purified through an alumina column as described previously.

Hydrolysis of γ,γ-dicarbethoxy-γ-acetamido-butyraldehyde

Vogel and Davis (14) reported that prolonged hydrolysis of DCAABaldehyde resulted in diminished yields of Δ^1-PC-5. A similar observation was made in this laboratory. Refluxing times up to 30 minutes at a temperature of 135°C did not result in a reduction of the product. Therefore, this condition was used routinely for hydrolysis. In two cases the carbon dioxide evolved from decarboxylation of DCAABaldehyde during the hydrolysis was swept with nitrogen into the saturated solution of barium hydroxide. The yields of barium carbonate obtained were 92 and 96 per cent of theory, which reflected a high degree of purity of DCAABaldehyde. In the following lines, for the sake of completeness, are presented the data for hydrolysis of a representative sample of DCAABaldehyde with a detailed description of all observations which seemed important.

Purified DCAABaldehyde obtained from 0.44 g of acetamidomalonate was introduced into a 50 ml round bottom flask and treated with 5 ml of 6 N hydrochloric acid. A faint pink color developed, which probably indicated the presence of minor quantity of some impurity. The same pink color developed when the control, the oily product obtained from a simulated addition reaction, was treated with 6 N hydrochloric acid. (The oily product, most likely a polymer of acrolein, used as a control, was prepared by addition of acrolein to absolute ethanol in the presence of a catalytic amount of sodium ethoxide, but in the absence of diethyl acetamidomalonate.) The flask was then suspended in a preheated oil bath at 135°C and refluxed 25 minutes. During the course of the hydrolysis the solution turned slightly brown, whereas the control turned dark brown. The hydrolysate was diluted with water and evaporated at 40°C to a small volume. Water was added and the mixture evaporated again. This process was repeated several times until the

characteristic odor of hydrochloric acid disappeared. It was observed that the repeated evaporation to complete dryness increased polymerization of the product. The brown oil, impure Δ^1-PC.HCl-5, remaining in the flask after the last evaporation, was dissolved again in water, 20 µl aliquots were taken for paper chromatography and the remaining solution was chromatographed on a charcoal column. The colorless water eluate was concentrated by evaporation to a small volume and 20 µl aliquots were again chromatographed. The remaining solution was lyophilized. A greenish, amorphous product was obtained which had the appearance of a solid foam. This product was purified once more through the charcoal column and the eluate lyophilized. The final product was a white amorphous solid. The final yield based on diethyl acetomidomalonate was 61 per cent of theory. A few mg of the product was dissolved in water and saved for paper chromatography. The results of chromatography will be discussed later.

Characterization of dl-Δ^1-pyrroline-5-carboxylic acid hydrochloride(dl-Δ^1-PC·HCl-5)

Several physical and chemical techniques were applied in an attempt to characterize the final product as follows:

1. Elemental analysis.
2. General physical properties.
3. Some considerations of stability.
4. Reaction with o'-aminobenzaldehyde.
5. Preparation of derivatives.
6. Paper chromatography and electrophoresis.
7. Preparation of a titration curve.
8. Modified Volhard titration.

1. An elemental analysis gave the following values for $C_5H_8NO_2Cl$ (149.58)

Found: C, 39.39; H, 5.49; N, 8.86; Cl, 22.68%

Calculated: C, 40.10; H, 5.35; N, 9.35; Cl, 23.70%

These values are in reasonable agreement with the calculated values although they still indicated the presence of some contaminants.

2. General physical properties. The final product was found to be extremely hygroscopic. Exposure to the atmosphere resulted in the gradual liquifaction of the solid product and subsequent to this a browning of the oily liquid. Browning, as will be pointed out later, was associated with polymerization of Δ^1-PC. To avoid exposure of the whole yield of Δ^1-PC hydrochloride to the atmosphere whenever it was manipulated, the product was divided into samples of 20 to 100 mg which were placed in small vials and kept in a desiccator over phosphorus pentoxide. Under these conditions the product was stable and the color remained white. When heated over 120°C it started to foam and turn brown and at 160°C it charred. Δ^1-PC·HCl-5 is soluble in water, methanol and ethanol, but insoluble in other common organic solvents.

3. Some considerations of stability. Generally compounds containing the pyrroline ring have a tendency to polymerize. The structure of pyrroline polymers has not been elucidated as yet. Schoepf (37) suggested that piperideine, a homolog of pyrroline, forms a trimer. Conclusions drawn from the literature pertaining to stability of Δ^1-PC·HCl-5 solutions are partially contradictory. Vogel and Davis (14) claimed a greater stability of refrigerated solutions than those exposed to room temperature. They also found diluted solutions more stable than concentrated. Strecker (39), on the other hand, found solutions kept at room temperature and those refrigerated at 3°C were equally stable, whereas those refrigerated at -15°C had a considerably diminished stability. His conclusions were based on colorimetric measurements of o-aminobenzaldehyde complex concentrations in individual solutions. Δ^1-PC·HCl-5 in the solid state, when kept over a desiccant, is stable. Acidic solutions are considerably more stable than neutral or basic solutions (9, 14, 39). It has been observed that

solutions kept for some time under any condition developed more intense polymer spots on chromatograms than those freshly prepared from the stored solid product. Aging solutions of Δ^1-PC·HCl-5 gradually developed a brown color, which seem to be an indication of progressive polymerization. Stored solutions when passed through a charcoal column were decolorized to a great extent. The polymer spot was much less intense on chromatograms of freshly decolorized solutions.

It was originally believed that polymerization of Δ^1-PC-5 might proceed through a free radical mechanism and that exposure to a strong light and air would accelerate this process. In order to test this assumption two identical solutions, containing 20 mg of Δ^1-PC·HCl-5 per ml of water, were prepared with the difference that one of these contained hydroquinone in 0.01 per cent concentration as an antioxidant. These solutions did not show any apparent difference upon paper chromatography whether they were kept for some time at room temperature under a nitrogen atmosphere, or whether they were exposed to ultraviolet or natural light. The same solutions were placed in the window and exposed to natural light for 13 months. Occasionally the aliquots were subjected to paper chromatography. The only conclusion which can be safely derived from these chromatograms was that there was a gradual increase in the quantity of the polymer, which after approximately 2 months remained constant. Paper chromatography of this solution after a 13 months period still disclosed two overlapping o-aminobenzaldehyde positive spots, although, these were much less intense than those produced by the original solution. A progressive browning with aging of the solution was also observed. On the basis of these results the free radical mechanism does not seem to be responsible for the polymerization of Δ^1-PC·HCl.

4. Reaction with o-aminobenzaldehyde.* Schoepf et al. (35, 36, 37)

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*Purchased from K. and K. Laboratories, Long Island City, N.Y.

discovered that o -aminobenzaldehyde will form a stable orange-yellow condensation product with compounds containing either the Δ^1-pyrroline or Δ^1-piperideine ring. These colored complexes provide very useful qualitative tests for these rings. It was suggested that the condensation product is a dihydroquinazolinium derivative (14, 35). Recently, this complex with Δ^1-PC-5 was isolated and characterized (39). A quantitative colorimetric method with o-aminobenzaldehyde has suffered until recently (39) from the lack of an adequate standard. The original calibration curve prepared by Strecker (16) was based on the assumption that DCAAB-aldehyde was quantitatively hydrolyzed to Δ^1-PC·HCl-5 (see curve V, Figure 1). In his later publication (39) the more accurate calibration curve appeared based on the relatively pure and stable Δ^1-PC·HCl-5 (see curve VI, Figure 1). Comparison of these two curves (V and VI) disclosed as much as 50 per cent difference for the concentrations in the range from 0 to 20 μg of Δ^1-PC·HCl-5 per ml. This fact clearly indicated that the hydrolysis was not a clear cut reaction and that it did not proceed quantitatively to the desired Δ^1-PC·HCl-5 as the sole product. For the sake of comparison and for the stability considerations four "calibration" curves have been prepared from the solutions of Δ^1-PC·HCl-5 obtained under different treatments: two curves were based on the assumption of the quantitative hydrolysis of DCAABaldehyde to Δ^1-PC·HCl-5 (curves I and II, Figure 1) and two based on the actual concentration of the isolated, relatively pure Δ^1-PC·HCl-5 (Curves III and IV, Figure 1). Curve I represents the plot of the absorbancy at 430 mμ against the theoretically expected yield of Δ^1-PC·HCl-5 in the hydrolysate after evaporation of the excess hydrochloric acid; curve II was prepared from solution I after purifying it with charcoal; curve III was prepared from a weighed quantity of solid Δ^1-PC·HCl-5 obtained after lyophilization of solution II; curve IV was prepared from the solution of relatively pure Δ^1-PC·HCl-5.

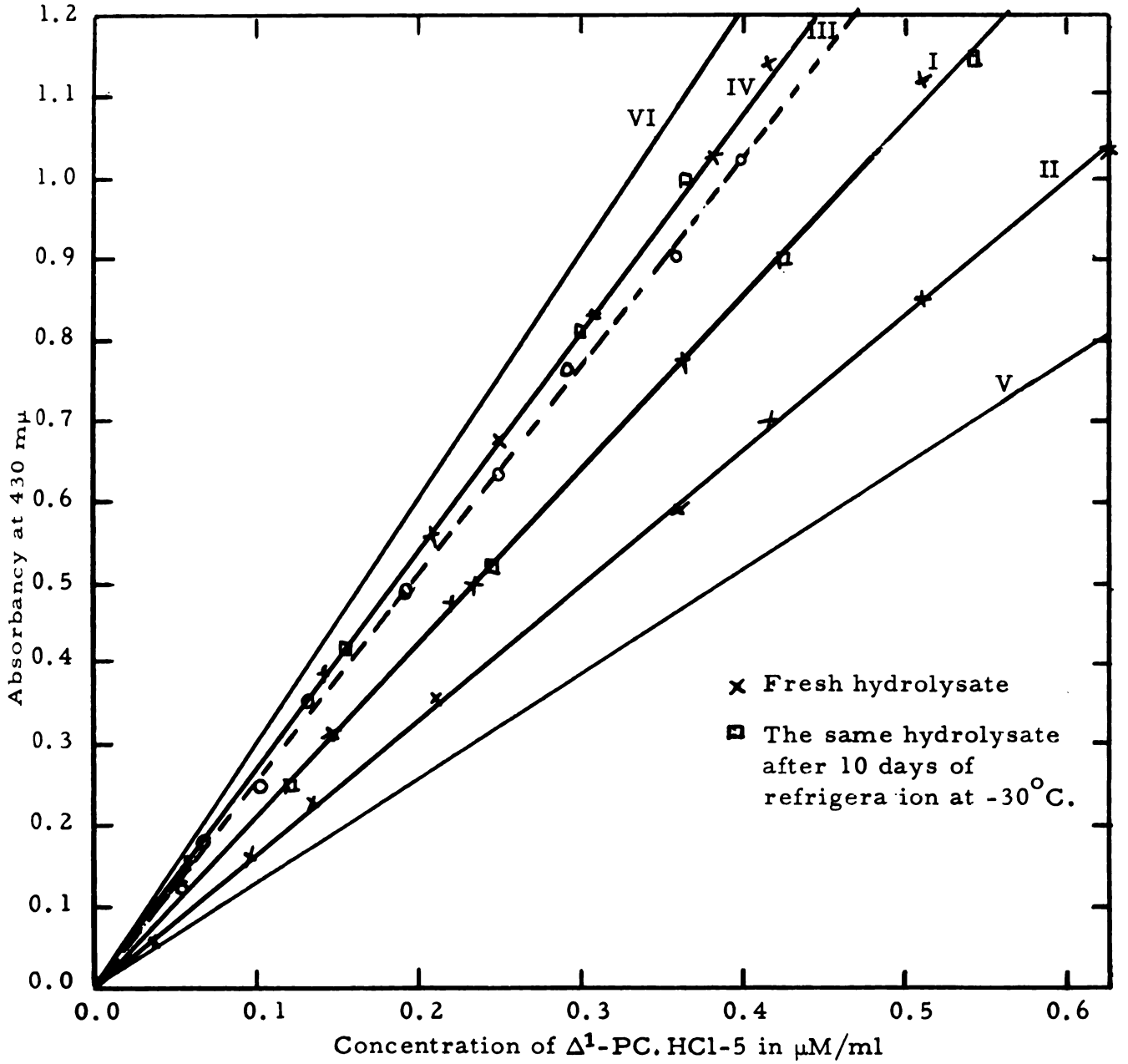

Figure I. Calibration curves for Δ^1-pyrroline-5-carboxylic acid after various treatments:

I Hydrolysate
II Hydrolysate run through charcoal
III Same as II, lyophilized and weighed
IV Pure Δ^1-PC. HCl-5
V, VI Strecker's curves

Some interesting conclusions can be drawn from the analysis of these curves. It has been learned from measurements of carbon dioxide evolution during hydrolysis that the hydrolysis proceeded to 96 per cent of theory. If one assumes that the products of DCAAB aldehyde hydrolysis were only carbon dioxide and Δ^1-PC·HCl-5, it was logical to expect that the "calibration" curves I and IV should be identical. This, however, was not the case. The difference displayed between curves I and IV indicated again that Δ^1-PC·HCl must be partially destroyed during hydrolysis. If purification through charcoal would involve only the removal of polymer, curves I and II should be also identical. The great difference exhibited between these two "calibration" curves indicated that besides the polymer also Δ^1-PC·HCl-5 was absorbed on charcoal. However, it has to be pointed out, that Δ^1-PC·HCl-5 can be eluted with water from the charcoal column more efficiently than one would conclude from curve II.

The hydrolysate (Curve I) and the solution of "pure" Δ^1-PC·HCl-5 (curve IV) were tested for changes in absorbancy after 10 and 18 days of cooling at 3°C, respectively. Surprisingly enough, there was no detectable change in reactivity with o-aminobenzaldehyde. These results are in good agreement with those of Strecker (39).

The overall differences among the curves (I-IV) can be attributed to polymerization of the Δ^1-PC·HCl-5 and to the shift of equilibrium between Δ^1-PC·HCl-5 and glutamic-γ-semialdehyde.

All colorimetric measurements were made at 430 mμ with a Beckmann DU spectrophotometer. Approximately 0.2 per cent solutions of o-aminobenzaldehyde in 95 per cent ethanol were used for developing the colored complex. This technique, when utilized for the determination of the concentration of remaining Δ^1-PC·HCl-5 in nutrient solutions recovered after completion of the feeding experiments, proved to be inapplicable because of interference by the relatively high content of inorganic salts and the polymer.

5. Preparation of derivatives. Δ^1-PC·HCl-5 was catalytically reduced to proline with hydrogen at the room temperature and atmospheric pressure. Generally the procedure of Vogel and Davis (14) was followed. Several experiments were performed under modified conditions with regard to catalysts and to the medium used. Two catalysts, Raney nickel and Adams' catalyst were employed for the sake of comparison. There was no difference with respect to the yield of the end product when either of these two catalysts was used. The reaction medium, when Raney nickel was used as catalyst, was 95 per cent ethanol. An increase in pressure was observed in the hydrogenation system when Raney nickel catalyst was used in an acetic acid medium. Some unidentified gaseous substance was formed at a constant rate during the reduction. The solution turned green due to the production of Ni^{++} ion as indicated by the formation of the characteristic colored complex with dimethylglyoxime reagent.

A typical reduction of Δ^1-PC·HCl-5 was performed as follows: 53.7 mg of purified Δ^1-PC·HCl-5 was dissolved in 2 ml of 80 per cent acetic acid. Approximately 20 µl of this solution was used for paper chromatography. The solution was transferred to a $1\frac{1}{2}$ inch long glass tube of 4 ml volume. Adams' catalyst, approximately 30 mg, was not mixed directly with the solution of Δ^1-PC·HCl-5, but it was added separately into the hydrogenation boat and covered with 8 ml of 80 per cent acetic acid. Then the tube, containing the solution of Δ^1-PC·HCl-5, was placed vertically into the mouth of the hydrogenation boat in order to prevent mixing of the compound to be reduced with catalyst before it was saturated with hydrogen. After completion of a 5 minute flushing period with hydrogen, the tube containing the Δ^1-PC hydrochloride solution was tipped into the boat and hydrogenation started. The reduction was completed in 50 minutes. The actual uptake of hydrogen, 8.5 ml at a temperature of 27°C, corresponded to 92 per cent of

theory in the reduction of Δ^1-PC·HCl-5 to proline. After completion of the hydrogenation the catalyst was removed by filtration. Several 10 μl volumes of the filtrate were placed on filter paper (Whatman No. 1) and sprayed with o -aminobenzaldehyde (0.25 per cent solution in 95 per cent ethanol), ninhydrin (0.25 per cent solution in 95 per cent ethanol) and isatin (0.2 per cent in acetic acid). No color developed with o-aminobenzaldehyde, indicating the completion of the reduction. Spots treated with isatin spray developed a blue color, characteristic of the pyrrolidine ring. The ninhydrin spray resulted in a gray color and not a yellow one as anticipated for pure proline. This color was apparently a mixture of colors produced by the reaction of ninhydrin with proline and polymer, which is also present.

Twenty μl volumes of the filtrate, and of the original solution of Δ^1-PC hydrochloride before its reduction, were subjected to ascending chromatography. The results of this chromatography experiment will be described later.

The residual filtrate containing the proline hydrochloride was evaporated to dryness and treated with 3 ml of a saturated solution of picric acid in methanol. No precipitate of the picrate appeared at this point because of its solubility in methanol. Therefore, the solution was evaporated to dryness, dissolved in 3 ml of glacial acid with warming and left to crystallize at room temperature. Yellow needles of dl-proline picrate were filtered and dried in a desiccator. The yield obtained was 105.6 mg (66 per cent of theory). The melting point determined in a sealed tube was 136-137°C (reported 135-136°C (14)).

Aqueous solutions of Δ^1-PC·HCl-5 presumably are in equilibrium with glutamic-γ-semialdehyde as mentioned previously. In order to test the existence of this equilibrium an attempt was made to prepare an aldehyde derivative: the phenylhydrazone and sodium bisulfite addition product (50). Neither of these derivatives could be obtained. Strecker (39) obtained the 2,4-dinitrophenylhydrazone from freshly

prepared solutions and the bisulfite addition product from solutions stored for some time at -15°C.

6. Paper chromatography and electrophoresis. In preliminary tests, 50 per cent aqueous ethanol, 50 per cent aqueous propanol and a mixture of acetic acid:water:ethanol (20:30:50) were used as the moving phase in ascending paper chromatography. The best resolution was obtained with 50 per cent aqueous ethanol which subsequently was employed routinely as the solvent in chromatography of Δ^1-PC·HCl-5 and closely related compounds. The indicators used were solutions of ninhydrin, o-aminobenzaldehyde, and isatin in concentrations already indicated. Occasionally a 2 per cent potassium permanganate solution was used also as an indicator for Δ^1-PC·HCl-5.

Developed chromatograms showed three ninhydrin positive spots with the following Rf values: 0.44-0.45 (grey blue) (I); 0.59-0.61 (blue) (II); 0.68-0.70 (pink with bluish center) (III), respectively. When o-aminobenzaldehyde reagent was applied to identical chromatograms two yellow spots were formed corresponding to spots II and III obtained with the ninhydrin reagent. Spot II was faint yellow and spot III was intense yellow. These two spots were also instantly oxidized with permanganate spray as could be seen by the formation of two white areas in the pink permanganate background. Vogel and Davis (14) reported only one o-aminobenzaldehyde positive spot with an Rf value of 0.76 in 50 per cent aqueous ethanol.

The correct interpretation of the chromatograms has been found a difficult task and despite numerous attempts complete characterization of individual spots is still desired. Among the impurities in the solutions of Δ^1-PC·HCl-5 which might logically be expected were polymer, glycine, glutamic acid and proline. One dimensional paper chromatography did not result in a clear resolution of these compounds. The individual Rf values of these compounds would be as follows:

polymer, 0.44; glycine, 0.55; ninhydrin positive spot II, 0.58-0.60; glutamic acid, 0.64; ninhydrin positive spot III, 0.68-0.70; proline, 0.72. Two dimensional paper chromatography showed the presence of glutamic acid as the only contaminating amino acid present. This finding was confirmed by subjecting the solution to paper electrophoresis at pH 8.6, in veronal buffer (0.1 M). Strecker (39) also reported about 1 per cent of glutamic acid as the impurity found in his synthetic dl-Δ^1-PC·HCl-5 preparation. Smith and Greenberg (9) on the other hand found proline and glycine as the impurities in their synthetic preparation of dl-Δ^1-PC·HCl-5.

An attempt was also made to characterize chemically the three ninhydrin positive spots on chromatograms. For this purpose a solution containing 16 mg of dl-Δ^1-PC·HCl-5 was placed on Whatman No. 1 filter paper in a horizontal line, 10 cm long. The developed chromatogram was cut into strips corresponding to the ninhydrin positive spots I, II and III. The individual zones were eluted with water, evaporated to dryness and weighed. The recoveries from zones I, II, and III, were 1.2, 1.4 and 11.4 mg, respectively. The overall recovery of material was approximately 88 per cent. The material obtained from zones I, II and III, was dissolved in 0.06, 0.07 and 0.57 ml of water, respectively, so that each of the resulting solutions had approximately equal concentrations, 20 mg per ml. All three solutions were rechromatographed on paper with the following results: the solution from zone I, produced only one spot with an Rf value of 0.45. This spot corresponded to the polymer. The Rf value was the same as was found for ninhydrin spot I before elution. The solutions from zones II and III were not the solutions of single compounds, as one would expect. When rechromatographed, these solutions were resolved again into three ninhydrin positive spots, like the original solution of dl-Δ^1-PC·HCl-5, with Rf values of 0.44, 0.59 and 0.68. These Rf values corresponded to the three ninhydrin spots of the original chromatogram. It should be pointed out that

ninhydrin spot II from zones II and III was very faint. Ninhydrin spot III from zone III was the only spot giving a color with o-aminobenzaldehyde and potassium permanganate.

On the basis of this evidence the three ninhydrin positive spots, I, II and III, were tentatively identified as the polymer of dl-Δ^1-PC·HCl-5, glutamic-γ-semialdehyde (pure or overlapping with another form of polymer) and dl-Δ^1-PC·HCl-5, respectively. The glutamic acid spot overlapped with spot II and III and could not be distinguished by one-dimensional paper chromatography.

Further evidence that ninhydrin spot I was the polymer, was obtained from paper chromatography of the following four solutions:

1) Hydrolysate of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde after evaporating the excess hydrochloric acid.
2) The same hydrolysate as above, chromatographed on a Norite A column and eluted with water.
3) The solution, obtained from elution of the charcoal column, with acetic acid: 95 per cent ethanol (20:80) mixture after the column has been eluted previously with water, as indicated above.
4) A freshly prepared solution of relatively pure, solid dl-Δ^1-PC·HCl-5.

Solution 1, the hydrolysate of DCAABaldehyde, produced with ninhydrin a heavy polymer spot of Rf value 0.44. Solution 2, the same hydrolysate, passed through charcoal, disclosed a very faint polymer spot. Solution 3, the acetic acid: ethanol (20:80), eluate of the charcoal, free from the bulk of dl-Δ^1-PC·HCl-5, which was previously eluted with water, showed a very intense polymer spot and only weakly developed spots of "glutamic-γ-semialdehyde" and dl-Δ^1-PC·HCl-5. This again demonstrated that charcoal column absorbed beside the polymer also dl-Δ^1-PC·HCl-5. Charcoal appears to function to some extent like a

cation exchange column. E.g., when 5 ml of 0.1 N solution of silver nitrate was chromatographed on the charcoal column the silver ion was absorbed almost quantitatively.

Chromatographic studies with radioactive preparations of dl-Δ^1-pyrroline-5-carboxylic acid-5-C^{14} hydrochloride (dl-Δ^1-PC·HCl-5-C^{14}) were in excellent agreement with those already discussed for non-radioactive preparations. Furthermore, chromatography of radioactive dl-Δ^1-PC·HCl-5-C^{14} preparation proved to be a valuable supplementary tool for measuring the distribution of the radioactivity in individual spots resulting from chromatography. Again, the polymer spot from freshly prepared solutions or from solutions purified with charcoal, was negligible. Chromatograms from the stored solutions showed progressively increasing count in areas corresponding to the polymer spot. The "glutamic-γ-semialdehyde" and dl-Δ^1-PC·HCl-5-C^{14} spots were fused together because of close Rf values and could not be resolved by scanning the chromatograms.* The latter two spots always appeared like a single peak on the recording paper. Nevertheless, dl-Δ^1-PC·HCl-5-C^{14} spot possessed by far more activity than "glutamic-γ-semialdehyde" spot. A radiogram of the same chromatogram showed much more distinct resolution of these two spots. Again the dl-Δ^1-PC·HCl-5-C^{14} spot was much darker than any of the other two spots.

Catalytically reduced dl-Δ^1-PC·HCl-5 disclosed also three ninhydrin positive spots, I, II and III, with Rf values of 0.45, 0.62 and 0.70, respectively, and two isatin positive spots, II and III, with Rf values of 0.62 and 0.70, respectively. Spot II, sprayed with isatin indicator, developed only very faintly. The reactivity of spot II with isatin was an unexpected observation and undoubtedly indicated presence

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*The scanning was performed with a Nuclear Chicago Model 1620 A strip counter (Nuclear Instruments and Chemical Corporation, Chicago 10, Ill.) with an Esterline-Angus automatic recorder (Esterline-Angus Company, Inc., Indianapolis, Ind.).

of the pyrrolidine ring. Spot III, as determined against the chromatographed standard solution, corresponded to proline, Ninhydrin positive spots, I and II represented the unreducible contaminants present in the dl-Δ^1-PC.HCl-5 solution, namely the polymer and "glutamic-γ-semialdehyde" or possibly two distinct polymers.

A few electrophoretic determinations were also performed on the radioactive dl-Δ^1-PC·HCl-5 solutions. A "Servall" electrophoretic apparatus (Ivan Sorvall, Inc., New York, N.Y.) was used. It was operated for four to eight hours at the constant current of 15 mA. Veronal buffer, 0.1 M, at a pH 8.6, was used as the solvent. In a characteristic four hour run the dl-Δ^1-PC·HCl solution was resolved into three spots, I, II and III, distinguishable with ninhydrin indicator. The migration distance for these three spots was 4.0, 6.2, and 7.7 cm from the origin, respectively. Spot I and II could not be characterized sufficiently to identify the individual compounds. Spot III was definitely identified as glutamic acid. The scanning technique of the radioactive strips and the corresponding radiograms showed that radioactivity was located predominantly in spot II. This also was the only spot reactive with o-aminobenzaldehyde spray. The individual spots, I, II and III, are tentatively identified therefore as polymer, dl-Δ^1-PC·HCl-5 and glutamic acid, respectively.

7. Preparation of titration curves. An attempt has been made to use titration as a criterion of purity of dl-Δ^1-PC·HCl-5. These experiments did not yield data which could be used for this end. Although the shape of titration curves was surprisingly reproducible, the molecular weights calculated on the basis of titration equivalents varied between 135 and 166. (Correct molecular weight for dl-Δ^1-PC·HCl-5 is 149.58.) Calculation of molecular weights from titration curves of a few freshly prepared solutions of dl-Δ^1-PC·HCl-5 approached closely the theoretical value (Figure II). The equivalent weight calculated

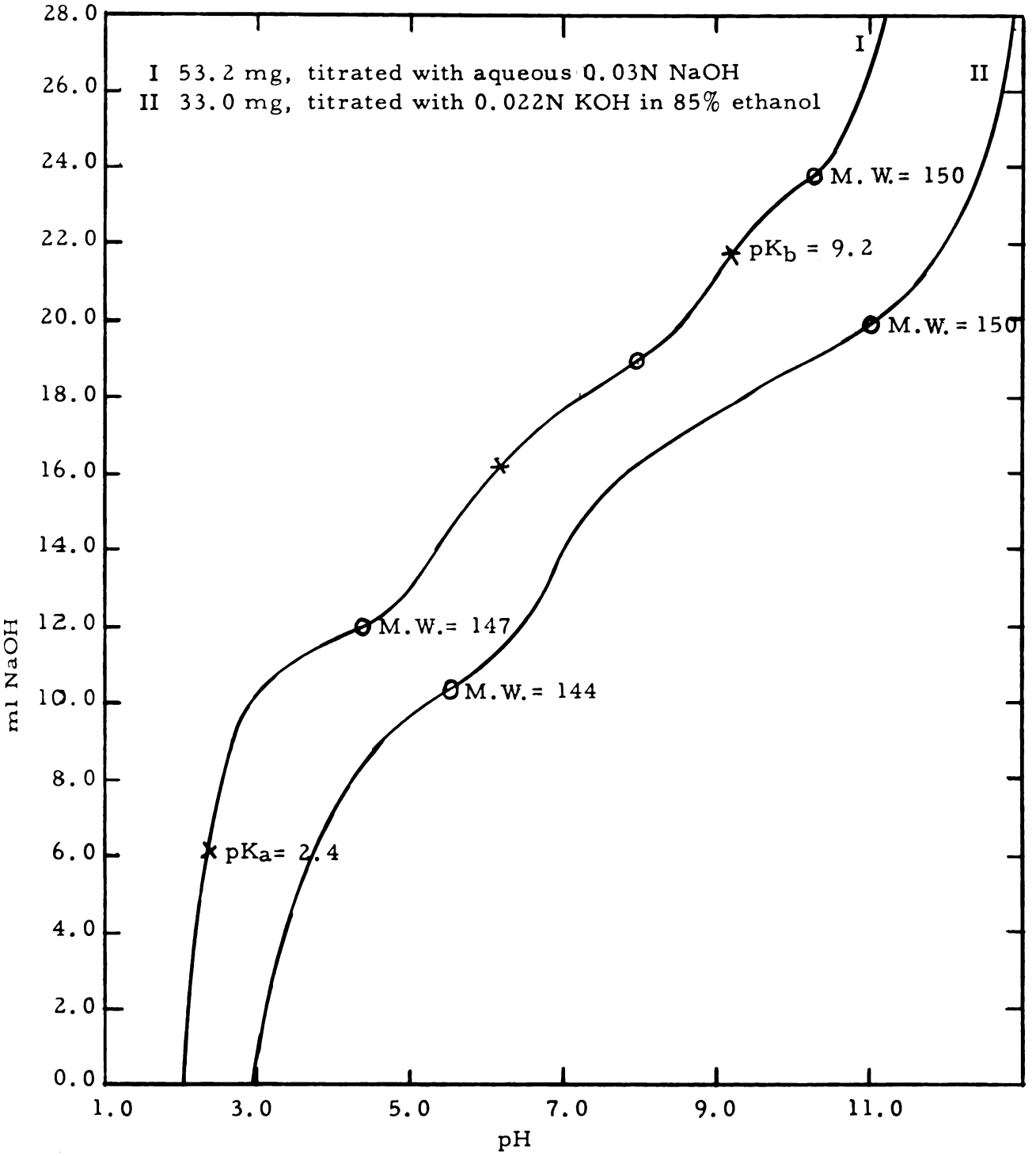

Figure II. Titration curve of DL-Δ^1-pyrroline-5-carboxylic acid hydrochloride.

from titration curves of aged samples of dl-Δ^1-PC·HCl-5 were generally higher than the theoretical equivalent weight. Whether this change was due to increased polymerization, due to a shift of equilibrium toward glutamic-γ-semialdehyde, or due to oxidation of the semialdehyde to glutamic acid, could not be determined. The apparent pK values of aqueous solutions also varied slightly, (pKa 2.4-2.7;pKb 9.2-9.5), from fresh to aged solutions, but generally were reproducible.

A reasonable interpretation of the titration curves, despite considerable effort, could not be achieved. Very confusing was the fact that all curves exhibited three inflection points, at pH 4.25-4.40, 7.65-8.0 and 10.3-10.5, although only two inflection points were expected. Whether the three inflection points represent differences in ionization characteristic of the polymer, glutamic-γ-semialdehyde and dl-Δ^1-PC·HCl-5, present simultaneously in the solution, is not known.

8. Modified Vohlard titration. Caldwell and Moyer's (51) modification of the standard Vohlard's method has been used for the determination of chloride ion concentration in dl-Δ^1-PC·HCl-5 solutions. The average molecular weight calculated from the data obtained by employing this procedure was 155 which is approximately 3 per cent higher than the theoretical value.

Synthesis of dl-Δ^1-pyrroline-5-carboxylic Acid-5-C^{14} hydrochloride (dl-Δ^1-PC·HCl-5-C^{14})*

For the purpose of providing a reference for use later in this study a brief resumé of dl-Δ^1-PC·HCl-5-C^{14} synthesis is presented here.

Two separate preparations of radioactive dl-Δ^1-PC·HCl-5-C^{14} were made and were fed separately to the tobacco plants. The first was

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*For the sake of brevity dl-Δ^1-PC·HCl-5-C^{14} will be occasionally referred to as a precursor.

synthesized from 105.4 mg of diethyl acetamidomalonate-2-C^{14} containing 0.5 millicuries (mC) of radioactivity diluted with 385.0 mg of non-radioactive acetamidomalonate. The synthesis and the purification followed exactly the procedures outlined in detail previously for non-radioactive material. The only exception was the use of a stoichiometric quantity of acrolein, namely 0.15 ml, in the addition reaction to acetamidomalonate. Consequently, the yield of the product was reduced and approximately 30 per cent, 147.8 mg, of acetamidomalonate was recovered from the reaction mixture. The total yield of dl-Δ^1-PC·HCl-5-C^{14} was 156 mg which is 66 per cent of the theoretical yield, based on acetamidomalonate. The entire yield of dl-Δ^1-PC·HCl-5-C^{14} was dissolved in 7.8 ml of water, so that the resulting stock solution contained 20 mg of dl-Δ^1-PC·HCl-5-C^{14} per ml. This solution was kept in the freezer at a temperature of -15°C. Portions were used for the preparation of the solution to be fed to the plants, which had a concentration of 2 mg per ml and 1.1×10^6 counts per minute (c.p.m.) per ml. This count corresponded to a specific activity of 8.2×10^7 c.p.m./mmole of the precursor.

A second batch of radioactive dl-Δ^1-PC·HCl-5-C^{14} was synthesized from 668.4 mg of acetamidomalonate-2-C^{14}, having a total radioactivity of 2.53×10^9 c.p.m. This was a mixture of 309.8 mg of non-radioactive acetamidomalonate with 210.8 mg (2.20×10^9 c.p.m.) and 147.8 mg (0.33×10^9 c.p.m., recovered from previous synthesis) of acetamidomalonate-2-C^{14}. The total yield of 279 mg of dl-Δ^1-PC·HCl-5-C^{14} (61 per cent of the theoretical yield), obtained from this synthesis, was dissolved in 10 ml water. Approximately 2 ml portions of this solution were transferred into five previously weighed vials and lyophilized. After lyophilization and drying in a vacuum desiccator over phosphorus pentoxide, the vials were fitted with screw caps (to prevent the absorption of moisture during the weighing) and weighed again. The vials, containing dl-Δ^1-PC·HCl-5-C^{14} in the solid state, were then stored in a desiccator over calcium chloride and refrigerated at -15°C. This

technique was used to secure the highest possible stability of the dl-Δ^1-PC·HCl-5-C^{14}. The feeding solution (with few exceptions) was prepared immediately before use. Its final concentration was adjusted so that it contained 1 mg of the precursor per ml, having 2.14×10^6 c.p.m. per ml. The specific activity of this precursor was calculated to be 2.93×10^8 c.p.m./mmole.

The efficiency of the counter determined by the use of the synthetic dl-Δ^1-PC·HCl-5-C^{14} was found to be 37 per cent.

Preparation of the Plants

Nicotiana rustica, var. humilis, a strain of tobacco plant having a high nicotine content, was employed in this study as the experimental organism for the study of nicotine biosynthesis. The tobacco plants were grown in the greenhouse in flats containing Vermiculite.* Cultivation of the tobacco plants followed the technique described by Lamberts (52). When plants reached a height of 6-8 inches (the stage of growth preceding flowering), they were transferred into the laboratory and grown for several weeks in a nutrient medium. Dawson (53) showed that the roots of tobacco plants are the main site of nicotine synthesis. In a later publication (54) he reported that the rate of nicotine synthesis was proportional to the rate of growth of the tobacco roots. Taking advantage of this observation, it was obvious that significant incorporation of the precursor into nicotine could be realized only from plants with a vigorous, newly developing root system. The validity of this supposition had been confirmed by the studies preceding this work (32, 52). Consequently, applying Dewey's technique, the plants brought from the greenhouse were forced to develop a new root system. The old roots were clipped

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*A commercially available heat-expanded mica.

approximately 1 cm from the root base, rinsed with distilled water and placed into a 125 ml Erlenmeyer flask containing about 30 ml of aerated or oxygenated nutrient solution (55). This solution was obtained by diluting the stock nutrient solution with water in the ratio 1:2, respectively. (The stock solution consisted of 2.610 mg of $Ca(NO_3)_2 \cdot 4H_2O$; 756 mg of $MgSO_4 \cdot 7H_2O$; 500 mg of KH_2PO_4; 500 mg of KCl; 500 mg of $(NH_4)_2SO_4$ and 5.6 mg of $FeCl_3 \cdot 6H_2O$, dissolved in 2 l of distilled water.) A cotton plug, inserted into the mouth of the flask, served as a support for the plants and as a mean for prevention of evaporation and possible contamination with undesired microorganisms from the air. Lamberts and Byerrum (34) found that shielding of the flask promoted the root growth and the nicotine synthesis. Therefore, the flasks containing plants were wrapped in shields cut from black construction paper, and only after this treatment were exposed to natural light in the laboratory. Occasionally, on rainy days or during the winter season, the plants were subjected to artificial illumination. A few ml of aerated or oxygenated distilled water were added to each flask every day. Under favorable conditions the first new roots appeared on the fourth day. A sufficient root system, suitable for feeding of the radioactive precursor, usually developed in 10-18 days. The growing conditions were intentionally kept as constant as technically possible. However, seasonal effects could not be completely avoided. As will be later shown (Table I) seasonal effects were probably responsible for the decreased incorporation of the radioactive precursor into nicotine. This decrease was most apparent during the hot summer months. On the other hand, survival of plants, after transferring them from the greenhouse into the laboratory, was most critical in winter. Many of the plants during the winter developed a stem rot in the laboratory. It was believed, at first, that this disease was due to some infection brought from the greenhouse. Later it was found that the development of the stem rot was actually the

Table I--Summary of Feeding Experiments.

Experiment Number	Number of Plants Fed	Date of Feeding	Radioactivity Fed Cpmx10^{-6}	Radioactivity Recovered Percent	Dry Matter g/plant	Nicotine dipicrate Cpm/mM x 10^{-4}	Nicotine dipicrate mg/plant	C^{14} * Incorporation Percent
1	13	Nov. 25	1.10	17.0	0.405	2.43	7.6	0.027
2	12	Jan. 6	1.00	38.8[1]	0.512	4.31	12.8	0.081
3	8	Feb. 3	1.60	20.1	0.650	2.70	25.4	0.069
4	11	Feb. 9	2.06	19.3	0.734	5.68	24.3	0.108
5	5	Feb. 12	1.55	18.3	0.726	3.63	22.6	0.085
	5	Feb. 12	1.03	18.9	0.678	2.05	19.1	0.092
6	5	Feb. 27	1.56	20.5	0.714	4.08	21.9	0.093
	5	Feb. 27	1.04	21.6	0.690	3.24	21.2	0.106
	5	Feb. 27	0.52	19.4	0.705	1.33	23.1	0.096
7	9	June 12	4.29	17.1	1.320	7.61	39.0	0.111
	10	June 12	2.14	18.7	1.322	4.43	37.5	0.138
	6	June 12	1.07	17.1	1.298	1.79	38.9	0.105
8	31	June 19	2.05	15.4	1.030	1.53	41.7	0.051
9	4	June 19	3.07	11.7	1.200	1.89	36.0	0.073
10	31	July 7	2.05	17.8	1.280	0.79	51.9	0.032
11	27	July 8	2.05	17.3	1.500	0.44	56.8	0.020
	7	July 8	2.05	18.3	1.771	0.34	65.9	0.018
	5	July 8	2.05	28.9	0.942	1.40	20.7	0.023
12	20	July 19	2.05	21.5	1.137	0.33	36.5	0.010
13	5	Aug. 5	1.05	59.5[1]	0.820	0.41	26.3	0.017
	5	Aug. 5	1.05	27.6	0.900	0.37	27.6	0.016
	5	Aug. 5	1.05	32.7	0.810	0.52	24.0	0.019
14	10	Aug. 10	2.00	22.1	0.784	0.94	14.7	0.022
15	10	Aug. 28	2.00	28.5	0.879	0.54	23.3	0.010
16	15	Aug. 28	2.52	17.1	0.767	1.30	17.8	0.015
17	15	Sept. 16	2.90	42.6[1]	0.820	1.84	20.5	0.021
	16	Sept. 16	2.90	63.0[2]	0.860	1.89	21.1	0.022
	8	Sept. 17	3.39	78.5[2]	0.813	0.59	22.2	0.006
	7	Sept. 18	2.90	58.8[2]	0.823	0.31	21.1	0.004

*See Appendix for calculation. [1]Recoveries after completion of "dry" feeding. [2]Three subsequent recoveries of the original feeding solution. For details see Table IV.

secondary pathological symptom, resulting most likely from diminished resistance of the plants due to insufficient photosynthesis. This disease could be prevented by bringing the plants from the greenhouse at least one week before the intended hydroponic cultivation and exposing them under laboratory conditions (while the plants were still in flats) to strong illumination with incandescent and fluorescent light.

Sporadically, during the summer months (July and August), the growth of microorganisms in a few flasks developed to such an extent, that the nutrient medium displayed a cloudy appearance. Generally, the nutrient media remained clear even in the summer.

Feeding of Plants

Plants with newly developed healthy roots were carefully removed from flasks. The roots were then washed in distilled water and dried on soft paper tissue. The plants finally were transferred into new 125 ml Erlenmeyer flasks containing 1 or 2 ml of the feeding solution. This solution was prepared either by dissolving the solid dl-Δ^1-PC·HCl-5-C^{14} in a small volume of water, or by diluting the radioactive stock solution, followed by the adjustment of pH to 5.6 with 0.2 N sodium hydroxide and finally increasing the volume to obtain the desired concentration. An automatic pipette was used for the transfer of this solution into the flasks. Ten µl aliquots were deposited on aluminum planchets, dried and counted, in order to determine the radioactivity fed per plant. Simultaneously 10-20 µl aliquots were used for paper chromatography.

The general feeding procedure used, unless stated otherwise, was as follows: The total quantity, 1 to 4 mg of the precursor, dl-Δ^1-PC·HCl-5-C^{14}, was administered to the roots of the intact plants in a single 1 or 2 ml dose. The roots of the plants were manipulated on the bottom of the flask, so that they were in optimal contact with the feeding solution. The flask was then fitted with a cotton plug, shielded, as

mentioned before, and exposed in the hood to artificial light of 200 foot-candles intensity. This light was supplied by two 36-inch 30-watt fluorescent tubes and two 100-watt incandescent bulbs, placed approximately 14 inches above the plants. Under this intensity of light, the 1-2 ml of feeding solution was usually absorbed in $1\frac{1}{2}$ - $2\frac{1}{2}$ hours to apparent dryness, as far as could be observed visually. This feeding was followed by the absorption of two to three 2 ml volumes of water to assure the highest possible intake of the precursor.* To each flask was then added 30 ml of inorganic nutrient solution having the composition previously indicated. During the two summer months (July and August) 0.5 mg of aureomycin was administered to each plant. The period of illumination was 12 hours per day. The plants were then left to grow under these conditions for 7 days before they were harvested. The above described feeding procedure is a slight modification of the technique developed by Henderson et al. (56).

Preliminary Feeding Experiments with the Non-radioactive dl-Δ^1-pyrroline-5-carboxylic acid hydrochloride (dl-Δ^1-PC·HCl-5)

The tolerance of the tobacco plant roots to the concentration of the precursor and to the pH of the feeding solution was tested prior to administration of the radioactive precursor.

In an attempt to study this point, three groups of 7 plants, grown under identical conditions, were fed a 1 ml solution, containing 1, 2 and 4 mg of dl-Δ^1-PC·HCl-5, with approximate osmotic pressures of 0.5, 1, and 2 atmospheres, respectively. The pH of the solution was adjusted to 5.6, because this was the pH of the nutrient solution. The mode of feeding employed was the same as previously described.

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*This part of the feeding procedure in the further text will be referred to as the "dry" feeding period.

After completion of the "dry" feeding period the flasks were rinsed with 5 ml of water. An aliquot of this solution was tested for the quantity of the unabsorbed precursor. Spectrophotometric measurements showed that 20-40 per cent of the administered precursor was not absorbed by the roots. It was obvious at this point that the plants exhibited differences in absorption of dl-Δ^1-PC·HCl-5. In later feedings with the radioactive precursor, this observation was confirmed. The details on the uptake of the radioactive precursor are apparent from Table I. During the "dry" feeding period,which was completed approximately in 6 hours, no ill effects was observed in the plants. On this basis it was concluded that concentrations of dl-Δ^1-PC·HCl-5 up to 4 mg per ml and at the pH 5.6, were in no way toxic to the plants.

In similar experiments the response of the plants toward changes in pH of the solution was tested. To three groups, each containing 5 plants grown under identical conditions, was administered 1 ml of the feeding solution containing 2 mg of dl-Δ^1-PC·HCl-5, at pH 2.2 (the aqueous solution of the solid dl-Δ^1-PC·HCl-5), 5.6 and 7.2, respectively. Simultaneously, another group of 5 plants was used as a control. Each plant of this later group was treated with 1 ml of water instead of the feeding solution. The first group of the plants, fed at the pH 2.2, started to wilt after about 2 hours. The second and third groups fed at pH 5.6 and 7.2, behaved normally with no observable ill effects. The recovered dl-Δ^1-PC·HCl-5 after the "dry" feeding period, determined spectrophotometrically, averaged 88, 35 and 18 per cent of the administered precursor, respectively. (The pH of the recovered solutions was found to be 2.4, 7.2 and 7.4, respectively.) Taking these recoveries at their face value, one could conclude that the uptake of dl-Δ^1-PC·HCl-5 was greater at pH 7.2 than at pH 5.6. Chromatograms of the solutions at these two pH levels, 5.6 and 7.2, showed different reactivity toward o-aminobenzaldehyde. The feeding solution at pH 7.2 produced a less

intense spot with o-aminobenzaldehyde than at pH 5.6. It is believed that these differences are due to increased polymerization of dl-Δ^1-PC·HCl-5 in neutral or basic medium.

On the basis of these two preliminary feeding experiments it was decided that in the studies, in which dl-Δ^1-PC·HCl-5-C^{14} was to be administered, to use feeding solutions of the precursor at pH 5.6 and at concentrations lower than 4 mg per ml.

Isolation and Purification of Nicotine

The feeding period of the plants was terminated on the eighth day after the administration of the radioactive dl-Δ^1-PC·HCl-5-C^{14}. The plants were removed from the flasks and the roots thoroughly rinsed with distilled water. The combined washings of the roots and flasks were used for determination of residual radioactivity after completion of the feeding. The plants were then sacrificed by cutting them into small pieces and drying them under infrared heat lamps. The temperature was regulated not to exceed 80°C. The dried plant tissue was pulverized in a mortar, weighed, mixed with 20 per cent by weight of calcium oxide and steam distilled in a micro-Kjeldahl apparatus. Larger samples, exceeding 6 g of dry pulverized tissue, were distilled from a 500 ml Kjeldahl flask utilizing steam directly from the steam line. The distillate was collected in a flask containing 1-3 ml of 6 N hydrochloric acid. The distillation was discontinued when the distillate showed no precipitate with silicotungstic acid (57). (This compound is a very sensitive reagent for the quantitative detection of the alkaloids. It forms a white stable precipitate with nearly all alkaloids.) The distillate was then evaporated in a flash evaporator to a small volume. The resulting solution of the impure nicotine hydrochloride was treated with 6 N sodium hydroxide until basic to litmus and the nicotine purified by an azeotropic distillation with water through a Widmer column (58).

The distillate collected in a small volume of 6 N hydrochloric acid was again evaporated to dryness. The solid, white, hygroscopic nicotine hydrochloride was dissolved in a small volume of methanol and precipitated by addition of an equal volume of a saturated methanolic solution of picric acid. The yellow amorphous precipitate of nicotine dipicrate, after standing 2-3 hours at room temperature, was filtered off, washed with a small volume of methanol and recrystallized from a hot saturated water solution. Slow cooling of the saturated aqueous solution of nicotine dipicrate resulted in well developed crystals in the form of long yellow needles. This product melted at 226°C*; recorded m.p. 218°C.

Determination of Radioactivity

All measurements of radioactivity, except those in which paper strips were scanned for radioactivity, were made with two instruments: A Nuclear-Chicago Model 192X Ultrascaler (Nuclear Instruments and Chemical Corporation, Chicago 10, Ill.) with a Tracelab Model SC-16 proportional gas flow counter (Tracelab, Inc., Boston 10, Mass.); and a Nuclear-Chicago Model 192 A with Model D-47 proportional gas flow counter (Nuclear Instruments and Chemical Corporation, Chicago 10, Ill.). The proportional counting gas consisted of 90 per cent argon and 10 per cent methane. (Tracelab, Inc., Boston 10, Mass.) The counting system was 40 per cent efficient as determined with a standard preparation of sodium carbonate-C^{14} supplied by the National Bureau of Standards. All samples to be counted were plated on aluminum planchets having 2.83 sq. cm of area. In order to secure as much uniformity as possible in counting, all compounds, except barium carbonate, were thoroughly ground in a mortar before plating on the planchets. The material to be

*Capillary melting point tubes were used for determination of all melting points throughout this study. The stem correction was neglected.

plated was uniformly distributed on the planchets by tapping, packing and smoothing the surface with a spatula. Precipitated barium carbonate was plated directly by filtering with suction the colloidal suspension through a special stainless steel funnel, having a filtration area of 2.83 sq. cm. The resulting thin cake was transferred to a planchet, dried in an oven at 105°C for a minimum of one hour, weighed and counted. The radioactivity of all counted compounds were corrected for self-absorption.*

Feeding Experiments with dl-Δ^1-pyrroline-5-carboxylic Acid-5-C^{14} hydrochloride (dl-Δ^1-PC·HCl-5-C^{14})

Approximately 17 feeding experiments were performed (Table I) in order to collect enough radioactive nicotine for degradation. A few of the experiments were also designed to supply some additional information with regards to the factors influencing the biosynthesis of nicotine. The results and observations drawn from the feeding experiments can be summarized as follows:

1. The radioactivity of the isolated nicotine was found to be (in the limits of experimental error) directly proportional to the radioactivity of dl-Δ^1-PC·HCl-5-C^{14} fed to the plants (Table I, experiments No. 5, 6 and 7). The quantity of the precursor fed per plant ranged from 0.5 to 4.0 mg with radioactivities from 0.52×10^6 to 4.29×10^6 c.p.m. These concentrations, as has been pointed out previously, did not evoke any ill effects in the plants.

2. The uptake of the active precursor through the "dry" feeding period was found to be 40-60 per cent. (The lower recovery of a non-radioactive dl-Δ^1-PC·HCl-5, 20-40 per cent, determined spectrophotometrically, as mentioned previously, most likely indicated polymerization.)

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*See Appendix for calculation.

If however, the plants after "dry" period of feeding were supplemented with nutrient solution and left 7 days in contact with the remainder of the unabsorbed radioactive precursor, the recoverable radioactivity still averaged about 25 per cent of the originally administered quantity (Table I). More detailed data on the process of absorption of dl-Δ^1-PC·HCl-5-C^{14} by the plants are presented in Table II. Two groups of the plants, A and B, each containing 3 plants, were fed 2.0×10^6 c.p.m. per plant, respectively. After completion of the "dry" feeding period, the recovery of unabsorbed radioactive material in group A was 38.5 per cent and for group B 59.7 per cent. The rate of absorption of the remaining radioactive precursor in the solution was considerably decreased.

The chemical composition of the remaining radioactive material in the solution could not be conclusively established. Scanning of chromatograms of the residual solutions showed the one peak of radioactivity which corresponded to the original feeding solution. The only noticeable difference was the much broader base under the peak than would be expected on a chromatogram of the precursor. This can be associated either with a high concentration of inorganic salts or due to overlap of the polymer salt with dl-Δ^1-PC·HCl-5-C^{14} spot. Concentrated residual solutions were positive to ninhydrin reagent, but only very faintly positive with o-aminobenzaldehyde.

It was considered possible that one of the optical isomers of dl-Δ^1-PC·HCl-5-C^{14} could be absorbed by the roots in a much slower rate than its optical antipode. This assumption is highly hypothetical, because with dl-glutamic acid-2-C^{14} and dl-ornithine-2-C^{14}, fed to the plants under similar conditions, this behavior was not experienced. The determination of the optical activity of the mixed residual solutions of the precursor was not attempted because of the anticipated confusion due to the presence of the antibiotic, aureomycin, and a large excess of inorganic salts.

Table II--Absorption of the Remaining Radioactivity by Tobacco Plants After Completion of "Dry" Feeding Period.[1]

Absorption Time Hours	Nutrient Solution Added ml	Remaining Radioactivity A[2] %	Remaining Radioactivity B[3] %
19	10	38.5	59.7
29	15	28.2	35.1
48	25	27.0	30.3
21	10	25.1	28.6
23	10	20.7	20.2
18	10	18.5	----
11	5	17.0	18.0

[1]The absorption of 1 mg of the precursor followed by the absorption of three 2 ml volumes of water.

[2]3 plants, fed 2.06×10^6 c.p.m/ml (1 mg of dl-Δ^1-PC-5-C^{14})

[3]3 plants, fed 4.12×10^6 c.p.m./2 ml (2 mg of dl-Δ^1-PC-5-C^{14})

Another possibility which could serve to explain the decreased rate of absorption is the possible existence of a quasi-equilibrium between the polymer and its precursor. The polymer once formed is most likely relatively stable and consequently would not rapidly be converted to free dl-Δ^1-PC·HCl-5-C^{14} to restore the equilibrium upset by the absorption of this compound.

Finally, it is plausible to assume, that the polymer itself is absorbed by the roots after the bulk of Δ^1-PC·HCl-5-C^{14} has been absorbed during the "dry" feeding period. It is known that biological polymers are absorbed through cell membranes at a much slower rate than their corresponding monomers. Assuming that the polymer of dl-Δ^1-PC·HCl-5-C^{14} is a dimer or trimer, it is only logical to expect its absorption at a slow but measurable rate.

3. The radioactive precursor absorbed after the completion of the "dry" feeding period does not appear to contribute to nicotine biosynthesis (Table III and IV, compare experiments I and II). Thirty-one tobacco plants were fed 2.9×10^6 c.p.m. per plant. Fifteen of these plants were allowed to complete the usual feeding period of 7 days. At the end of this period 37 per cent of the radioactive material was still recoverable in the residual nutrient solution. The roots of the remaining 16 plants at the end of the "dry" feeding period, approximately 6 hours, were thoroughly rinsed and transferred into new flasks containing nutrient solution. The combined washings of the roots and rinsed solution from the flasks (pH 8.0) were evaporated to dryness by lyophilization. The residual radioactive material was dissolved in distilled water, the pH brought up to 5.6 and the final volume adjusted so that it approximated the concentration of radioactive material in the original solution. Aliquots of this solution were plated and counted. The radioactivity of this solution was found to be 3.39×10^6 c.p.m. per ml which represents approximately 60 per cent of the original radioactive material used for

Table III--Partial Evaluation of the Effect of Microorganisms on Absorption of DL-Δ^1-pyrroline-5-carboxylic Acid-5-C^{14} and Its Incorporation into Nicotine.

Number of Plants Fed	Conditions After Completion of "dry" Feeding Period.	Nicotine Dipicrate C.p.m./mmole x 10^{-4}	Nicotine Dipicrate mg/plant	Dry Matter g/plant	Per cent of Recovered Radioactivity
5	Roots were washed in order to recover unabsorbed radioactivity, then plants were transferred into 30 ml of nutrient solution.	0.41	26.3	0.820	59.5
5	Thirty ml of nutrient solution added.	0.37	27.6	0.900	27.7
5	Thirty ml of nutrient solution and 0.5 mg of aureomycin added	0.52	24.0	0.810	32.7

*The absorption of the precursor (dl-Δ^1-PC-5-C^{14}) administered in 1-2 ml solution, was followed by the absorption of three 2 ml volumes of water.

Table IV--Comparison of the Incorporation of DL-Δ^1-Pyrroline-5-carboxylic acid-5-C^{14} with the Incorporation of the Radioactive Residue Obtained Through Two Successive Recoveries of the Originally Administered Feeding Solution.

Experiment Number	Number of Plants	Radioactivity			Nicotine dipicrate			C^{14} *
		Fed	Recovered	Absorbed	Mg per Plant	mg/g of Dry Tissue	c.p.m./mM x 10^{-4}	Incorporated
		c.p.m./plant x 10^{-6}		%				%
I*	15	2.90	1.16	57.2	20.5	25.0	1.84	0.021
II*	16	2.90	1.07	36.9	21.1	24.6	1.89	0.022
III*	8	3.39	0.72	21.3	22.2	26.0	0.59	0.006
IV*	7	2.90	1.19	41.1	21.1	25.7	0.31	0.004

*Exp. I. Complete Feeding of dl-Δ^1-PC-5-C^{14}.

Exp. II. After "dry" feeding the remaining precursor was recovered and fed again to 8 new plants in Exp. III.

Exp. III. After "dry" feeding of the recovered residual precursor from Exp. II, the remaining radioactive material was recovered again and fed to 7 new plants in Exp. IV.

Exp. IV. Complete feeding of the recovered residue from Exp. III.

+See Appendix for calculation.

feeding (Table III). One ml aliquots of this recovered solution were then fed to a new group of 8 plants which were then subjected to the same procedure as described above. The radioactivity of the solution recovered from the second feeding was adjusted exactly to 2.9×10^6 c.p.m. per ml and fed again to a group of 7 plants. These plants were left in contact with the residual radioactive material until the end of the complete feeding period of 7 days. The data from this experiment are presented in Table IV. It was interesting to note that whether the absorption of the precursor was 37 per cent or 57.2 per cent, the incorporation into the nicotine was the same (Table IV, compare experiments I and II). Repeated feeding with recovered solutions (Table IV, compare experiments III and IV) showed the expected gradual decrease in incorporation of the precursor as has been discussed previously. A more comprehensive understanding of the results summarized in Table IV would require the separate degradation of the nicotine collected from the individual feeding experiments to discover the pattern of labeling for each individual sample of nicotine. Such an investigation was beyond the scope of this study. It was, however, assumed that the pattern of labeling was identical in all fractions of the collected nicotine.

3. A small portion of the remaining precursor in the nutrient solution appeared to be consumed by microorganisms. To test this possibility, three groups of 5 plants, each fed dl-Δ^1-PC·HCl-5-C^{14} having 1.05×10^6 c.p.m. per plant, were compared in a single experiment. The result and the experimental conditions are shown in Table III. Since only the precursor absorbed in the "dry" period of feeding (Table IV) seemed to be incorporated into nicotine the utilization of the remaining precursor in the feeding solution was apparently of no importance. On the basis of the above experiment it was apparent that the use of antibiotic was unnecessary. However, to avoid during the hot summer months (July and August) any microbial interference with uptake of the precursor by the roots, the antibiotic was added.

4. The radioactive precursor did not show any stimulating or depressive effect on nicotine biosynthesis. In a single experiment 30 plants of the same age and approximately the same size, were selected. Twenty of these plants were fed the radioactive precursor at two different concentration levels, 1 and 2 mg of dl-Δ^1-PC·HCl-5-C^{14}, respectively. The results of this experiment are shown in Table V.

5. The maximum specific activities of the nicotine (counted as nicotine dipicrate), isolated separately from the leaves, stems and roots showed a progressive increase. The average ratio of these activities in three separate experiments was found to be as follows: leaves:stems: roots = 1.0:3.1:4.2 (Tables VI and VII). These data confirmed again the established fact, that nicotine is, indeed, synthesized in roots (53, 54, 59) from which it is transported and deposited in leaves as a relatively stable end product of metabolism (60). Leete (29) found the reverse order for increasing activity of nicotine in leaves, stems and roots. Since he harvested the plants 9 weeks after administration of the precursor, his result is not surprising.

6. The distribution of the nicotine in different tissues of the tobacco plant appears to be function of age. Nicotine biosynthesis apparently reaches a maximum and then decreases with progressing age. This is, to some extent, experimentally documented in Table VII (Compare also Table VI). The distribution in the various tissues of the plants as presented in Table VII, Column I, was comparable with that found by Patton (61).

7. The incorporation of radioactivity from dl-Δ^1-PC·HCl-5-C^{14} into nicotine in a large number of feeding experiments performed over a period of a year are summarized in Table I. The growing and feeding conditions of the plants throughout this study were kept constant as much as it was technically possible. (The deviation from constant conditions were mainly due to the seasonal effects and to a minor extent due to age and size of the plants.)

Table V--Effect of DL-Δ^1-Pyrroline-5-carboxylic Acid (Precursor) on the Production of Nicotine in Nicotiana rustica, var. humilis.

Number of Plants Fed	Precursor Fed mg	Dry Matter g/plant	Nicotine dipicrate	
			mg/plant	mg/g of Dry Matter
10	0	1.365	44.1	32.3
10	2	1.322	37.5	28.3
10	4	1.320	39.0	29.5

Table VI--Distribution of Radioactive Nicotine in Different Tissues of Tobacco Plants After Completion of Feeding.*

Experiment Number	Number of Plants Used	Plant Tissue	Dry Matter		Nicotine dipicrate		
			g	%	mg	%	c.p.m./mmole x 10^{-4}
I	31	Leaves	17.67	55.34	1,159	89.45	1.26
		Stems	11.57	36.30	125	9.64	3.78
		Roots	2.68	8.40	8	0.91	5.32
		Total	31.92	100.00	1,292	100.00	1.53
II	31	Leaves	20.86	52.54	1,375	85.45	0.60
		Stems	15.55	39.17	215	13.42	1.90
		Roots	3.29	8.29	19	1.13	2.27
		Total	39.70	100.00	1,609	100.00	0.79
III	27	Leaves	21.28	52.45	1,346	87.74	0.34
		Stems	16.42	40.48	173	11.28	1.09
		Roots	2.88	7.07	15	0.98	1.56
		Total	40.58	100.00	1,534	100.00	0.44

*Each plant fed 2 mg (2.0×10^6 c.p.m./mmole) of DL-Δ^1-pyrroline-5-carboxylic acid-5-C^{14}.

Table VII--Distribution of Nicotine in Nicotiana rustica var. humilis at Two Different Ages.

Plant Tissue	Nicotine Content			
	I*	II*		
	%	%	%	%
Leaves	63.60	89.45	85.45	87.74
Stems	28.30	9.64	13.42	11.28
Roots	7.90	0.91	1.13	0.98

*I Young tobacco plants, 5 weeks old.
II Plants 9-10 weeks old. Data taken from Table VI.

One would expect that the tobacco plants at the same age and growing conditions would exhibit some uniformity in the rate of nicotine synthesis. However, closer examination of the data in Table I shows that the percentage of the incorporation and the maximum specific activities and the yields of nicotine (nicotine dipicrate) are not mutually related. It is also apparent from Table I that in summer months the incorporation of the precursor into the nicotine dropped markedly.

Degradation of Radioactive Nicotine

It has been established from previous studies that ornithine-2-C^{14} (28, 33), glutamic acid-2-C^{14} (29, 34), proline-C^{14} (29) (uniformly labeled) and putrescine-1,4-C^{14} (29) when used as precursors for nicotine biosynthesis contributed their radioactivity almost entirely to the pyrrolidine ring of nicotine. The results of degradation showed that the radioactivity of isolated nicotine was located in carbons 2 and 5 of the pyrrolidine ring. Considering known metabolic relationships between the compounds ornithine, glutamic acid, Δ^1-PC-5, and proline, it was anticipated by analogy that Δ^1-PC-5 could be an intermediate for the biosynthesis of the pyrrolidine ring of nicotine. To prove this hypothesis it was also necessary to degrade nicotine, isolated from plants fed Δ^1-PC-5-C^{14} and determine the pattern of labeling.

The degradation of nicotine pursued in this study followed established procedures with only minor modifications. The degradation consisted of the following major steps:

1. Oxidation of nicotine with neutral potassium permanganate, leading to the isolation of two products: nicotinic acid and carbon dioxide (from carbons 3, 4 and 5 of pyrrolidine ring and possibly the carbon from the methyl group of nicotine).

2. Decarboxylation of nicotinic acid, resulting in the isolation of carbon 2 of the pyrrolidine ring as barium carbonate and the pyridine ring of nicotinic acid as pyridine picrate.

3. Demethylation of nicotine and isolation of the methyl group of nicotine as methyltriethylammonium iodide.

4. Degradation of radioactive nicotine with isolation of carbon 5 of the pyrrolidine ring.

1. Oxidation of nicotine with potassium permanganate. Nicotine subjected to the action of the neutral potassium permanganate solution is oxidized to two major products: nicotinic acid and carbon dioxide. This method of degradation was originally described by Laiblin (62). It is obvious that the yields of the main products and the nature of the side-products of permanganate oxidation will depend on the conditions under which it is conducted. Laiblin reported the following products: nicotinic acid (80 per cent yield); potassium carbonate (approximately 2 moles per mole of nicotine); methylamine, ammonia and other unidentified compounds. The permanganate oxidation routinely employed in this laboratory (52) produced the following products and their approximate yields: nicotinic acid, (40-50 per cent purified by sublimation and recrystallized from 95 per cent ethanol, Tables VIII, IX, and X); potassium carbonate (30-45 per cent calculated on the basis of 4 carbons, Tables VIII, IX, and X); ammonia (90 per cent isolated as ammonium chloride, Table IX, calculated on the assumption that only nitrogen of the pyrrolidine ring is reduced to ammonia); and other unidentified products.

Seven permanganate oxidations were performed in this study: four with radioactive (Tables VIII, IX) and two with inactive nicotine and one with radioactive 4-(3'-pyridyl)-4-methylaminobutyric acid (Table X). The yields of the major products did not differ significantly (Compare Tables VIII, IX, and X).

Table VIII--Degradation of Radioactive Nicotine with Isolation of Carbon 2 of Pyrrolidine Ring and the Methyl Carbon of Nicotine.

Compound	Radioactivity		Yield	
	c.p.m/mmole x 10^{-4}	%	mg	%
Nicotine dipicrate	1.20	100.0	--	--
	1.28*	--	--	--
Nicotinic acid	0.50	39.0	165.5	50.0
	0.51*	39.6*	91.8*	97.6*
Barium carbonate (from nicotine oxidation)[1]	0.54	42.2	715.7	45.0
Barium carbonate (from decarboxylation of nicotinic acid)[2]	0.35	27.3	122.4	108.6
	0.37	28.9	109.4	101.0
Pyridine picrate[2]	0.04	3.1	81.2	46.4
	0.03	2.4	91.0	54.0
Methyltriethylammonium iodide	0.36	28.4	11.0	43.1

*Results refer to barium carbonate obtained from combustion.

[1] Calculation based on the assumption that four moles of barium carbonate are formed per mole of nicotine.

[2] Results refer to two separate experiments.

Table IX--Degradation of Radioactive Nicotine with Isolation of Carbon 2 of Pyrrolidine Ring.

Compound	Radioactivity		Yield	
	c.p.m./mmole x 10^{-4}	%	mg	%
Nicotine dipicrate	2.05	100.0	--	--
	2.06*	--	51.5*	98.1*
Nicotinic acid	0.89	50.0	114.5	40.0
	0.85*	41.2*	64.7*	97.4*
Barium carbonate (from nicotine oxidation)[1]	0.54	26.2	387.9	56.0
Barium carbonate (from decarboxylation of nicotinic acid)[1]	0.56	27.1	92.2	103.1
Pyridine picrate	0.03	1.5	76.0	54.7
Ammonium chloride[2]	--	--	124.6	90.7

*Results refer to barium carbonate obtained by combustion.

[1] Calculations based on the assumption that four moles of barium carbonate are formed per mole of nicotine.

[2] Calculations based on the assumption that only nitrogen of the pyrrolidine ring was reduced to ammonia.

Table X--Degradation of Radioactive 4-(3'-pyridyl)-4-methylaminobutyric Acid (MAPBAcid) with Isolation of Carbon 2 of the Pyrrolidine Ring and Methyl Carbon of Nicotine.

Compound	Radioactivity		Yield	
	c.p.m./mmole x 10^{-4}	%	mg	%
MAPBAcid+	0.687	100.0	--	--
Barium carbonate[1]	0.390	57.3	421.9	30.8
Nicotinic acid	0.230	33.3	106.2	50.0
	0.226*	32.8*	80.5*	94.7*
Barium carbonate (from catalytic decarboxylation of nicotinic acid)	0.198	28.7	33.3	103.4
Barium carbonate (from pyrolytic decarboxylation of nicotinic acid)[2]	0.181	26.4	24.5	33.1
Pyridine picrate	0.020	3.0	16.8	14.5
Methyltriethylammonium iodide	0.200	29.0	63.6	61.1

+Obtained from degradation of nicotine. See Table XI.

*Results refer to barium carbonate obtained from combustion of nicotinic acid.

[1] Obtained from permanganate oxidation of MAPBAcid. Calculations based on assumption that all four carbons in the side chain of MAPBAcid were oxidized to carbon dioxide.

[2] Decarboxylation performed with heating in a sand bath.

In an attempt to account for the presence of methylamine among the products of the permanganate oxidation, the following experiment was performed: 406 mg of nicotine (1,550 mg of nicotine dipicrate, 2.05×10^4 c.p.m./mmole) was oxidized with neutral permanganate under standard conditions (52). During the oxidation the system was continuously flushed with nitrogen. The gases were passed through a receiving flask containing 1 N hydrochloric acid. After completion of the oxidation (approximately 10 hours on the steam bath) the hydrochloric acid solution in the receiving flask was tested for the presence of ammonia with Nessler's reagent and for methylamine with 5-nitrosalicylaldehyde-nickel chloride reagent (63). Only Nessler's reagent gave a positive test. The filtrate and washings of manganase dioxide were combined and evaporated to dryness. The combined filtrate and the washings had a pH of 9.7. After evaporation to dryness and resolution in water they had a pH 10.6. Under this condition the bases ammonia and methylamine would be free and would be lost during the evaporation. Therefore, the vapor was condensed and collected in 1 N hydrochloric acid. This solution after diminishing the volume gave a positive test for ammonia but not for methylamine. Ammonium chloride obtained from the combined hydrochloric acid solutions represented 90.7 per cent of the theoretical yield (assuming that only the nitrogen of the pyrrolidine ring was reduced to ammonia) (Table IX). The methyl group of the methylamine formed during the permanganate oxidation therefore was apparently oxidized to carbon dioxide whereas the amine nitrogen was reduced to ammonia.

Further proof that neutral permanganate oxidizes methylamine to ammonia and carbon dioxide was demonstrated by oxidizing the solution of methylamine itself under similar conditions.

The yield of barium carbonate from the pyrrolidine ring (and eventually from the N-methyl group), calculated on the basis that either 3 or 4 carbons of the molecule yielded barium carbonate, was 56 and 42 per cent, and the incorporation of the radioactivity into these

carbons was 26.7 and 35.6 per cent, respectively. No attempt was made to study the permanganate oxidation any closer in order to explain the fate of the missing 44 per cent (based on 3 carbons) or 58 per cent (based on 4 carbons) of carbon from pyrrolidine ring. Most likely during the permanganate oxidation some two and three carbon compounds, neutral or acidic, are formed as by-products of this oxidation. In view of this experimental evidence the results pertaining to barium carbonate obtained from oxidation of the pyrrolidine ring of nicotine were judged to have little quantitative significance. They can serve only as a means of approximating the radioactivity residing in carbons 3, 4 and 5 of the pyrrolidine ring. Laiblin's (62) conditions used for permanganate oxidation of nicotine were much milder than those routinely used in this laboratory. Therefore, it is not surprising that he found some methylamine among the by-products. Potassium carbonate obtained from the oxidized pyrrolidine ring also did not exceed 50 per cent of theoretical yield.

In order to test the stability of nicotinic acid itself during the permanganate oxidation, 115 mg of nicotinic acid was oxidized by 0.5 g of potassium permanganate under conditions identical to those used routinely for oxidation of nicotine. At the end of the oxidation the excess of permanganate was destroyed by hydrogen peroxide. A test for ammonia in the filtrate was positive. The yield of barium carbonate obtained was 44 mg which quantity corresponded to 4.7 per cent of nicotinic acid, if completely oxidized. The dry residue after evaporation of the filtrate disclosed the same characteristic odor which had been noticed with nicotine oxidation. This impurity, as far as it could be traced by odor, sublimed partially with nicotinic acid, but it could be removed by crystallization from ethanol. These results indicated that prolonged heating of the nicotine mixture would also partially consume nicotinic acid.

The pH of the filtrate after completion of the oxidation was 9.7 which indicated that carbon dioxide produced during the oxidation was present in the reaction mixture almost entirely in the form of carbonate.

The dry residue remaining after evaporation of the filtrate consisted of two main products: potassium nicotinate and carbonate. Potassium nicotinate was first extracted with absolute ethanol leaving potassium carbonate behind as an insoluble residue. The carbonate residue was tested with the Koenig reagent (64) for the presence of nicotinate. The test was found to be negative, suggesting the complete extraction of nicotinate.

Potassium carbonate was then decomposed in the closed system with 6 N hydrochloric acid and the evolved carbon dioxide was swept with the stream of nitrogen into carbonate free, 0.25 N solution of sodium hydroxide. Barium carbonate from this solution was precipitated by addition of a few ml of barium chloride. The resulting barium carbonate precipitate was filtered, dried, plated and counted as described previously.

The alcoholic solution of potassium nicotinate was evaporated to dryness and dissolved in a small volume of water. The pH of this solution was adjusted to the isoelectric point of nicotinic acid (pH 3.0-3.3) (65) and free nicotinic acid was extracted with diethyl ether in a liquid-liquid extractor for 48 hours (66). The ether extract was evaporated to dryness and the dry solid residue of the crude nicotinic acid was purified by two sublimations (m.p. 225°C), followed by recrystallization from a minimum volume of absolute ethanol. This product melted at 236°C, reported m.p. 235-237°C. A mixed melting point with recrystallized commercial niacin was also 236°C. The final yield of the purified nicotinic acid was 40 per cent of the theoretical yield and the maximum specific activity was 0.50×10^4 c.p.m./mmole.

The residue after extraction of nicotinic acid with ether showed only negligible traces of inorganic salts.

2. Decarboxylation of nicotinic acid. Radioactive nicotinic acid was decarboxylated either pyrolytically or catalytically. The pyrolytic method, originally described by Huber (67) and Weidel (68) with the

modifications of Dewey (32), was used routinely. The catalytic method (69) was applied occasionally as confirmatory support of the result obtained by pyrolysis (Table X). The average weight of the sample of nicotinic acid used for pyrolysis was approximately 65 mg. Since pyrolysis was performed in a nitrogen atmosphere, only two major products were obtained: pyridine (collected in a saturated methanolic solution of picric acid and isolated as pyridine picrate) and carbon dioxide (trapped as calcium carbonate in the reaction flask). After completion of pyrolysis calcium carbonate was decomposed in the closed system of the addition of 5-10 ml of 6 N hydrochloric acid and flushed for 1 hour with the stream of nitrogen into two receiving tubes containing carbonate free, 0.25 N aqueous sodium hydrochloride. Occasionally carbon dioxide was absorbed in a saturated solution of barium hydroxide. The barium carbonate in the first instance was precipitated upon addition of 0.5 ml of 0.5 N barium chloride, filtered, dried, plated and counted as previously described.

A yellow amorphous precipitate of pyridine picrate was separated from the excess of picric acid solution by filtration. The separated precipitate was washed with a small volume of methanol, transferred into a calibrated centrifuge tube and covered with a small volume (approximately 1.0-2.0 ml) of 95 per cent ethanol. A cotton plug with protruding glass stirring rod was placed in the top of the tube. The tube was suspended in a water bath and the temperature of the water bath was brought slowly to 75°C. The alcoholic suspension of pyridine picrate was stirred until dissolved. If, at the water bath temperature of 75°C, the pyridine picrate did not dissolve completely, a small volume of 95 per cent ethanol was added again to complete the solution. Pyridine picrate was then left to crystallize slowly from a hot water bath. The yellow, well developed, needles of the recrystallized product melted at 167-168°C. The yield of pyridine picrate averaged approximately

50 per cent. The maximal specific activity of pyridine picrate was approximately 3.0×10^2 c.p.m/mmole, which represents about 3 per cent of the total nicotine radioactivity.

The catalytic decarboxylation of nicotinic acid was performed by the procedure described by Leete (69). This method of decarboxylation was employed only in two cases. The sample of nicotinic acid, 20 mg, was mixed with 37.0 mg of copper chromite catalyst (70), placed into a small round-bottom, standard-taper flask (with side arm), covered with 5 ml of redistilled quinoline and suspended in a silicone oil* bath. The flask was fitted with a condenser which was joined at the top to a receiving tube containing a solution of 0.25 N sodium hydroxide for the absorption of carbon dioxide. The oil bath temperature was brought up slowly to 230°C. The contents of the flask were refluxed for 1 hour with continuous flushing of the system with nitrogen. The evolved carbon dioxide was swept in a nitrogen stream into the receiving tubes.

The results of several pyrolytic decarboxylations of the radioactive nicotinic acid are presented in Tables VIII, IX, and X. These results showed that approximately 30 per cent of the radioactivity was located in carbon 2 (carboxy group of nicotinic acid) of the pyrrolidine ring of nicotine. The pyridine ring of the nicotine was found to contain only negligible radioactivity. The major shortcoming of the pyrolytic decarboxylation was the fact that the yield of barium carbonate systematically exceeded 100 per cent. The average of ten determinations was 105.0 per cent. In an attempt to explain this discrepancy the following factors were considered:

a. An external source of carbon dioxide could arise from contaminated nitrogen, from calcium carbonate present as an impurity in calcium oxide or from additional oxidation of some other carbons of nicotinic acid, which could be

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*Silicone oil was purchased from the Dow Corning Corporation, Midland, Michigan.

caused by some unknown oxidant present in calcium oxide.

b. The co-precipitation of barium hydroxide with barium carbonate could occur or barium carbonate may have been incompletely dried.

c. The presence of impurities in the radioactive nicotinic acid may occur or possibly water of crystallization existed.

The following experimental evidence is presented which seems to eliminate most of the factors considered to be responsible for this discrepancy.

A sample of 250 mg of calcium oxide was "pyrolyzed" with omission of nicotinic acid. A tube with ascarite was inserted between the nitrogen supply and reaction vessel. Any carbon dioxide present in the nitrogen supply would be absorbed by ascarite. No evidence indicating formation of barium carbonate could be observed in the receiving tubes containing the saturated solution of barium hydroxide. After "pyrolysis" of calcium oxide, 10 ml of 6 N hydrochloric acid were added to the reaction flask in order to decompose any calcium carbonate present. The closed system was flushed continuously with nitrogen. The evidence for carbon dioxide evolution was again negative. When nitrogen from the cylinder was directly bubbled through the saturated solution of barium hydroxide for 12 hours, no formation of barium carbonate was observed.

These experiments indicated that calcium oxide and the nitrogen supply could not contain carbon dioxide.

The conventional pyrolysis of nicotinic acid conducted at 350-400°C in a flask covered with copper oxide fillings, was always accompanied by the evolution of some unidentified fumes. The residue in the reaction flask turned brownish, indicating some charring of the organic compound. Because of the absence of oxygen this observation is only suggestive of some "destructive distillation." To expect that "destructive distillation"

of nicotinic acid under these experimental conditions could be the additional source of carbon dioxide formation, is improbable. If, however, minute quantities of oxygen were present in nitrogen gas or if some nitrates and sulfates present as impurities in the calcium oxide could serve as the oxydizing agents, the formation of additional carbon dioxide from nicotinic acid during pyrolysis would occur.

If the pyrolysis of nicotinic acid was conducted in a sand bath instead of copper oxide fillings at a temperature of 350°C, no charring was observed and the decarboxylation was incomplete (See Table X). When the same source of radioactive nicotinic acid was subjected to catalytic decarboxylation, the isolated barium carbonate was approximately 9 per cent more radioactive than that obtained from pyrolytic decarboxylation (Table X). However, this difference in radioactivity had apparently no significance because the yield of barium carbonate from catalytic decarboxylation also exceeded 100 per cent (Table X). To check whether these results were due to some experimental error, a similar decarboxylation was repeated with 41.2 mg of the non-radioactive commercial nicotinic acid. The yield of barium carbonate was found to be 107.1 per cent. This evidence excluded the possibility that calcium oxide used in pyrolysis was responsible for the higher yield of barium carbonate, than could be expected theoretically. The comparison of specific activities and the yields of barium carbonate (Tables VIII, IX, and X) for both catalytic and pyrolytic decarboxylations of nicotinic acid indicated that the reaction most likely proceeded quantitatively. Therefore, it appears justified to increase the radioactivity of barium carbonate by the factor which will compensate for the yield exceeding 100 per cent. However, this correction is not included in Tables VIII, IX, and X.

The co-precipitation of barium hydroxide with barium carbonate was also eliminated as a possible factor. When isolated barium carbonate was decomposed in the closed system and the evolved carbon dioxide absorbed in ascarite the recovery was 99.2 per cent.

Barium carbonate in all experiments in this study was dried a minimum of 1 hour in an oven at a temperature of 105°C and kept several hours in a desiccator over calcium chloride before weighing or counting. This treatment excluded possible errors in weight due to insufficient drying.

The presence of some impurities in nicotinic acid is also improbable, because the melting point of the commercial nicotinic acid and the mixed melting point with radioactive nicotinic acid was found identical, 236°C. The presence of water of crystallization for nicotinic acid has not been reported.

On the basis of the above presented experimental evidence one may question whether the higher yields of barium carbonate obtained from decarboxylation by either method may have any quantitative significance. Ten out of eleven decarboxylations of nicotinic acid gave yields of barium carbonate exceeding 100 per cent. On the other hand, none of the fifteen combustions (performed by the method of Van Slyke et al.(71)), of various intermediate compounds involved in the degradation of nicotine, gave yields of barium carbonate over 100 per cent. However, the yields, in general, approached this limit quite closely (Tables VIII-XI).

Considering the remarkable reproducibility of these results one has to accept the possibility that the higher yields of barium carbonate obtained was the quantitative reality, although the reason underlying it was not known at the conclusion of this work.

3. Demethylation of the radioactive nicotine and MAPBAcid. In order to determine whether any radioactivity is localized in methyl group of nicotine, three demethylation reactions were performed, two using nicotine dipicrate and one with MAPBAcid. The procedure of Pregl (72) modified by Brown and Byerrum (73) was employed. Nicotine dipicrate was used directly for demethylation without prior conversion to nicotine. The results of these experiments are shown in Table XII. Methyl iodide

Table XI--Summary of the Degradation Leading to the Isolation of Carbon 5 of the Pyrrolidine Ring of Nicotine Isolated from Plants Fed DL-Δ^1-pyrroline-5-carboxylic acid-5-C^{14}.

Compound	Max. Specific Activity c.p.m./mmole		Yield of $BaCO_3$ %
	I[1]	II[2]	
Nicotine dipicrate	0.689	--	--
MAPBAcid	0.681	0.687	95.7
N-benzoyl MAPBAcid	0.672	0.681	92.1
N-benzoyl MAPBAmide	0.645	0.674	98.7
Thiourea derivative	0.434	0.468	92.1
Barium carbonate[3]	0.183	--	71.9

[1]Calculations based on the direct counting of individual compounds. Specific activities corrected for self-absorption.

[2]Calculations based on the counting of barium carbonate obtained by combustion of the individual compounds.

[3]Barium carbonate from decarboxylation of N-benzoyl MAPBAcid.

Table XII--Radioactivity in the Methyl Group of Nicotine from Plants Fed DL-Δ^1-Pyrroline-5-carboxylic acid-5-C^{14}.

Experiment Number	Compound	Radioactivity c.p.m./mmole x 10^{-4}	%	Yield* mg	%
I	Nicotine dipicrate	1.280	28.4	11.0	43.1
II	Nicotine dipicrate	0.345	29.1	23.1	56.7
III	MAPBAcid[1]	0.687	29.0	63.6	61.1

*Yield of methyltriethylammonium iodide.

[1]MAPBAcid obtained from degradation of nicotine. See Table XI.

evolved during the demethylation reaction was trapped in an ethanolic solution of redistilled triethylamine. The resulting methyltriethylammonium iodide was isolated, dried, plated and counted.

The two nicotine dipicrate samples used for demethylation (Experiments I and II, Table XII) were obtained from two different feeding experiments, whereas the sample of nicotine dipicrate and MAPBAcid (Experiments I and II, Table XII) were from the same feeding experiment. Nicotine isolated from tobacco plants fed dl-Δ^1-PC·HCl-5-C^{14} was found to possess 29.0 per cent of its total activity in the methyl group. The results presented in Table XII indicate that incorporation of radioactivity into the methyl group of nicotine was most likely identical for all feeding experiments performed in this study.

4. Degradation of Radioactive Nicotine with Isolation of Carbon 5 of the Pyrrolidine Ring. Leete and Siegfried (31) accounted for the radioactivity in carbon 5 of the pyrrolidine ring by the difference between the radioactivity of nicotine and that of 3-nitro-5-(3'-pyridyl)-pyrazole obtained as a by-product of the nicotine oxidation with nitric acid. Lamberts and Byerrum (34) devised a method for degradation of nicotine leading directly to isolation of carbon 5 of the pyrrolidine ring as barium carbonate. This procedure with minor modifications was employed in this study. The following series of reactions constituted this degradative procedure:

I. Nicotine dipicrate $\xrightarrow[\text{solution into HCl}]{\text{Distillation of basic}}$ Nicotine hydrochloride

II. Nicotine.HCl $\xrightarrow{Br_2 \text{ in } CH_3COOH}$ Dibromocotinine*

*Dibromocotinine (3, 5-dibromo-5-(3'-pyridyl)-N-methyl-2-pyrrolidone perbromide).

III. Dibromocotinine $\xrightarrow{Zn+\ HCl,\ \text{reduction}}$ Cotinine*

IV. Cotinine $\xrightarrow{Ba(OH)_2,\ \text{hydrolysis}}$ MAPBAcid*

V. MAPBAcid $\xrightarrow{C_6H_5COCl}$ N-benzoyl MAPBAcid*

VI. N-benzoyl MAPBAcid $\xrightarrow[2.\ NH_3\ \text{in}\ CH_3OH]{1.\ CH_2N_2}$ N-benzoyl MAPBAmide*

VII. N-benzoyl MAPBAmide $\xrightarrow{Br_2\ \text{in NaOH}}$ N-benzoyl MAPPAmine*

VIII. N-benzoyl MAPPAmine $\xrightarrow{C_6H_5NCS}$ Urethane*

The starting quantity of radioactive nicotine dipicrate was 6,748 mg (equivalent to 1,763 mg of nicotine) with a maximal specific activity of 1.5×10^4 c.p.m/mmole. Nicotine was recovered by the azeotropic distillation of the basic aqueous solution of nicotine dipicrate (58). The resulting solution of radioactive nicotine was mixed with 1,939 mg of non-radioactive nicotine and treated with 20 ml of 6 N hydrochloric acid. An aliquot of this solution, corresponding to 39 mg of nicotine, was used for the determination of the starting specific activity. Nicotine dipicrate prepared from this solution possessed 6.87×10^3 c.p.m./mmole, the maximal specific activity. (The calculated specific activity was 7.36×10^3 c.p.m/mmole).

The remaining solution of nicotine hydrochloride was evaporated to dryness, covered with 20 ml of 80 per cent acetic acid, cooled in an ice

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*Cotinine (5-(3'-pyridyl)-N-methyl-2-pyrrolidone)
MAPBAcid (4-(3'-pyridyl)-4-methylaminobutyric acid)
N-benzoyl-MAPBAcid (4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid)
N-benzoyl MAPBAmide (4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide)
N-benzoyl MAPPAmine (3-(3'-pyridyl)-3-N-methylbenzoylaminopropylamine)
Urethane (3-(3'-pyridyl)-3-N-methylbenzoylaminopropyl phenylthiocyanate)

bath and treated with 8 ml of bromine dissolved in 20 ml of 80 per cent acetic acid. The resulting dark-brown oil of dibromocotinine perbromide (3, 5-dibromo-5-(3'-pyridyl)-N-methyl-2-pyrrolidone perbromide) was separated, treated again with 50 ml of 80 per cent acetic acid and 50 ml of water and dissolved with heating in a water bath. Dibromocotinine perbromide crystallized from this solution on cooling in the form of orange-yellow needles, m.p. 143-145°C. The yield obtained was 7,834 mg (58.5 per cent based on nicotine).

The perbromide was dissolved in 45 ml of 2 N hydrochloric acid, cooled in an ice bath and reduced with zinc dust. After the completion of the reaction the unreacted zinc was removed by filtration. The filtrate was made strongly alkaline so that the initially precipitated zinc hydroxide redissolved as a zincate. The crude product, cotinine, (5-(3'-pyridyl)-N-methyl-2-pyrrolidone), was extracted with six 50 ml portions of chloroform. This solvent was evaporated and the remaining oil, crude cotinine, was purified by distillation under reduced pressure. Cotinine distills at 170-175°C at 1 mm pressure. (It was found later with non-radioactive cotinine, that the purification can be achieved more conveniently by precipitation of cotinine as the perchlorate (74), with subsequent decomposition of the perchlorate and the extraction of the free base with chloroform.) Pure cotinine is a nearly colorless oil. The total yield of pure cotinine was 2,077 mg (89.6 per cent calculated on the basis of the perbromide). Cotinine was treated with 15 ml of water and 10 g of $Ba(OH)_2.8H_2O$ and the resulting mixture was refluxed 24 hours on the oil bath. Cotinine, a δ-lactam, was hydrolyzed in this step to 4-(3'-pyridyl)-4-methylaminobutyric acid (MAPBAcid)(75, 76). The mixture was evaporated to dryness. The unreacted cotinine was extracted with acetone and precipitated as a perchlorate from ether solutions. This product melted at 218-223°C with decomposition (74). The weight of the recovered cotinine perchlorate was 747 mg, equivalent to 475.6 mg (22.8 per cent) of cotinine used for hydrolysis. The solid residue after the extraction of the unreacted

cotinine was dissolved in 100 ml of water, suspended in an ice bath and treated with dry ice. The precipitated barium carbonate was removed by filtration. The filtrate was evaporated by lyophilization, because it was observed that when the evaporation was conducted at room temperature, considerable lactamization of MAPBAcid occurred. This confirmed a similar observation made by McKennis et al. (75). The glassy residue of MAPBAcid was dissolved in methanol, saturated again with carbon dioxide by addition of dry ice and the traces of precipitated barium carbonate removed by filtration. The methanolic solution of MAPBAcid was lyophilized again. The white amorphous residue was covered with a small volume of ice cold 90 per cent ethanol (absolute ethanol facilitated lactamization at room temperature). Without achieving complete solution, the alcoholic suspension was treated with an excess of acetone. A white precipitate of MAPBAcid monohydrate appeared instantly. This product was found to be sufficiently pure, m.p. 126°C. McKennis et al. (75) reported the m.p. 132°C for the air dried product recrystallized from methanol. An attempt to recrystallize MAPBAcid from absolute ethanol and acetone at 60°C as suggested by Lamberts and Byerrum (34), resulted in approximately a 50 per cent loss of the product due to the lactamization. The yield of MAPBAcid was 1,316 mg (52.6 per cent, calculated on the basis of cotinine). The maximal specific activity of the MAPBAcid was found to be 0.681 c.p.m./mmole.

The next step involved in the degradation procedure was the benzoylation of MAPBAcid under the conditions of Schotten-Baumann reaction. Lamberts and Byerrum (34) found it necessary to block the N-methyl group of MAPBAcid to prevent the cyclization of cotinine. Essentially the procedure of McKennis et al. (76), was followed in the initial steps of benzoylation. The isolation of the benzoylated product was modified which improved the overall yield.

To the solution of 957 mg of MAPBAcid in 4.4 ml of 1 N sodium hydroxide was added over the period of 1 hour, dropwise and simultaneously,

26 ml of 2.5 N sodium hydroxide and 3.68 ml of benzoyl chloride. Care was taken that the pH of reaction mixture did not drop below 9. During the course of the reaction the contents of the flask were cooled and stirred. After completion of the reaction the pH of the solution was adjusted to 3.5 with dilute hydrochloric acid and the precipitated benzoic acid was filtered. The remaining benzoic acid in the solution was extracted from the filtrate with diethylether. (The benzoylated MAPBAcid is almost insoluble in ether.) The extracted filtrate was then evaporated to dryness, dissolved in water and the pH adjusted to 6.6 with ammonium hydroxide. At this point some 4-(3'-pyridyl)-4-N-methylbenzoylamino-butyric acid (N-benzoyl MAPBAcid) separated out when the solution was refrigerated. A more convenient method was to evaporate the solution to dryness and to extract the inorganic salts with a small volume of water. The undissolved residue was N-benzoyl MAPBAcid. This extraction was repeated three times which sufficed to remove quantitatively the inorganic salts from the product. All three yields from three successive extractions (554 mg, m.p. 173°C; 200 mg, m.p. 171°C; and 20 mg, m.p. 165°C), were combined and dissolved in methanol. The remaining negligible quantity of undissolved inorganic salts was removed by filtration. The volume of the filtrate was diminished by evaporation until crystallization occurred. At this point water was added and the resulting cloudiness was discharged by heating the solution on the steam bath. Methanol was evaporated under reduced pressure while N-benzoyl MAPBAcid simultaneously crystallized from the solution in the form of white needles. This product melted at 175-176°C. The final yield was 688 mg (51.2 per cent based on the MAPBAcid) and it had a maximal specific activity of 0.672×10^4 c.p.m./mmole.

Dry, crystalline N-benzoyl MAPBAcid, 600.5 mg, was covered with two 25 ml portions of an ethereal solution of diazomethane. The esterification (77) was completed in about 10 hours. The ether and excess

of diazomethane were evaporated in the hood. The impure ester, a brownish oil, was subjected to ammonolysis without further purification by treating it with 30 ml of a saturated methanolic solution of ammonia in a closed vessel for a period of 100 hours at room temperature. The methanolic solution of amide was evaporated to dryness. The glassy impure amide was dissolved in methanol, partially decolorized with Norit A and evaporated to dryness. Finally the amide was crystallized from a solution of chloroform and ether. The yield of 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide, (N-benzoyl MAPBAmide), was 473 mg (78.9 per cent based on N-benzoyl MAPBAcid). This product melted at 145°C and had a maximal specific activity of 0.645×10^4 c.p.m./mmole.

In the following step N-benzoyl MAPBAmide was decarboxylated by application of Hofmann's reaction. This reaction was performed on two separate samples of N-benzoyl MAPBAmide, 149.9 mg (I) and 201.0 mg (II). The amide was dissolved in sodium hypobromite, prepared by dissolving 0.035 and 0.040 ml of bromine in 12 and 15 ml of 0.25 N sodium hydroxide, respectively. The reaction flask was suspended in a water bath, warmed to 70-80°C, and kept at this temperature for 20-30 minutes to complete the reaction. The products were carbon dioxide (trapped in the solution as sodium carbonate) and 3-(3'-pyridyl)-3-N-methylbenzoylaminopropylamine(N-benzoyl MAPPAmine). Sodium carbonate was decomposed by addition of a slight excess of hydrochloric acid, (2.0 and 2.5 ml of 2 N hydrochloric acid, respectively), and the evolved carbon dioxide (representing carbon 5 of the pyrrolidine ring of nicotine) was swept for 2-3 hours in a stream of nitrogen into receiving tubes and isolated as barium carbonate. The yields and maximal specific activities of barium carbonate from both samples of N-benzoyl MAPBAmide (I and II) was: 71.4 mg (71.0 per cent), 0.184×10^4 c.p.m./mmole and 96.3 mg (71.9 per cent), 0.183×10^4 c.p.m./mmole, respectively. These results showed that approximately 30 per cent of the overall radioactivity of the nicotine was located in carbon 5 of the pyrrolidine ring.

The second product, N-benzoyl MAPPAmine was extracted with chloroform from the neutralized reaction medium. The extraction was repeated until no positive test with the silicotungstic acid reagent was obtained (57). The chloroform solution was evaporated and the oily residue dissolved in 3 ml of methanol. N-Benzoyl MAPPAmine was converted to the thiourea derivative by addition of 0.06-0.10 ml of phenyl isothiocyanate. A large excess of this reagent should be avoided because it prevents the crystallization of the thiourea. Isolation of the thiourea derivative from the sample II of N-benzoyl MAPBAmide failed. Most likely the excess of hydrochloric acid and prolonged exposure to this acidic condition (the system was swept with nitrogen overnight) caused the hydrolysis of N-benzoyl group and the resulting amine did not crystallize under the same conditions that the benzoylated amine did. The yield of thiourea derivative (m.p. 203-204^{o}C) obtained from sample I was 67.0 mg (33.0 per cent based on N-benzoyl MAPBAmide). The maximal specific activity of this product was 0.474×10^{4} c.p.m./mmole which is equivalent to 67.9 per cent of the total radioactivity of nicotine. The thiourea derivative possesses all the carbons of the nicotine molecule except carbon 5 of the pyrrolidine ring.

The data from the nicotine degradation leading to the isolation of carbon 5 of pyrrolidine ring are presented in Tables XI and XIII.

Table XIII--Distribution of Radioactivity in 4-(3'-pyridyl)-4-methylaminobutyric Acid (MAPBAcid).*

Compound	Maximum Specific Activity	
	c.p.m./mmole x 10^{-4}	%
MAPBAcid	0.687	100.0
Nicotinic acid+	0.230	33.3
Barium carbonate (from decarboxylation)	0.198	28.7
Pyridine picrate	0.020	3.0
Barium carbonate (from carbon 5 of pyrrolidine ring of nitocine)	0.183	26.6
Methyltriethylammonium iodide	0.200	29.0
Recovery	0.601	87.3

*Intermediate compound obtained from degradation of radioactive nicotine. See Table XI.

+Nicotinic acid obtained from permanganate oxidation of MAPBAcid.

DISCUSSION

On the basis of the evidence accumulated throughout this study it seems to be justified to consider Δ^1-PC-5 as a physiological precursor of the pyrrolidine ring of nicotine. This supposition can be substantiated by the following considerations:

1. The radioactivity incorporated into the nicotine molecule after feeding Δ^1-PC-5-C^{14} to tobacco plants was found to be associated almost exclusively with the pyrrolidine ring.

2. Positions 2 and 5 of the pyrrolidine ring of nicotine were found to be equally labeled. This fact, a priori, indicated that the pyrrolidine ring of the Δ^1-PC-5-C^{14} fed to the plants was incorporated into nicotine in toto, without disruption of the ring. Alternatively, the ring might have been cleaved, but the carbon chain remained intact. This pattern of labeling is also characteristic of glutamic acid-2-C^{14} (29, 34) and ornithine-2-C^{14} (28, 33). The only significant difference in the pattern of labeling between Δ^1-PC-5-C^{14} found in this study and glutamic acid-2-C^{14} and ornithine-2-C^{14} found in previous experiments, was a considerable incorporation of radioactivity (approximately 29 per cent) into the N-methyl group of nicotine (Table XII).

3. The quantitative incorporation of Δ^1-PC-5-C^{14} into nicotine approximated that of ornithine-2-C^{14}, when both were fed to plants under comparable conditions. (Compare Table I with the results of Dewey (32)).

The occurrence of Δ^1-PC-5 as a normal metabolite in higher plants was not as yet proven. Consequently, its physiological participation between glutamic acid and proline (or pyrolidine ring containing compounds), as it has been found to be the case of mammals (1), and microorganism (2), was not established. If, Δ^1-PC-5 is tentatively

considered a natural precursor of pyrrolidine ring of nicotine, its decarboxylation to Δ^1-pyrroline, followed by reduction to form the pyrrolidine ring must be accepted also as a logical consequence. This reasoning led Dewey (32) and Leete (31) to postulate the existence of a symmetrical intermediate in the pathway of biosynthesis of the pyrrolidine ring of nicotine. Several symmetrical intermediates like Δ^1-pyrroline, pyrrolidine, succinaldehyde (succinic acid) and 2, 5-dihydroxy pyrrolidine were considered. The latter two compounds are entirely hypothetical precursors, with no experimental support, therefore, discussion pertaining to their possible participation in pyrrolidine ring biosynthesis will be neglected.

An intermediate, which seemed to be plausible in the formation of pyrrolidine ring of nicotine, was Δ^1-pyrroline. This compound can arise either from oxidative deamination of the symmetrical diamine, putrescine, (1, 4-diaminobutane) or from decarboxylation of Δ^1-PC-5. Both of these compounds on the other hand, can arise from ornithine. Putrescine, could be formed by decarboxylation of ornithine, whereas Δ^1-PC-5 could arise by δ-deamination of ornithine to form glutamic-γ-semialdehyde, followed by its spontaneous cyclization to Δ^1-PC. If one accepts Δ^1-pyrroline as an intermediate precursor in the biosynthesis of the pyrrolidine ring of nicotine, the question simultaneously arises: which of these two compounds, putrescine and Δ^1-PC-5 is the natural source of Δ^1-pyrroline? If both of these compounds are involved in the conversion of ornithine to Δ^1-pyrroline in tobacco plants, it would be very convincing evidence for considering ornithine as the most likely precursor so far investigated for the formation of the pyrrolidine ring of nicotine in the tobacco plants. Therefore, both of these compounds, putrescine and Δ^1-PC-5, deserve separate consideration as possible metabolic precursors of the pyrrolidine ring of nicotine.

Putrescine is generally the product of bacterial decomposition of proteins. There is, however, some evidence that diamines like

putrescine (1, 4-diaminobutane) and cadaverine (1, 5-diaminopentane) occur as normal metabolites in microorganizms, mammals and plants. Jakoby and Frederiks (80), observed that a strain of Pseudomonas fluorescens contain a diamine oxidase system catalyzing the oxidation of putrescine to 4-aminobutyraldehyde, which cyclizes to Δ^1-pyrroline. Tabor et al. (81) also observed that diamines, like putrescine and cadaverine, in the presence of diamine oxidase preparation from kidney tissue, are oxidized to the corresponding aminoaldehydes, which subsequently cyclize to Δ^1-pyrroline and Δ^1-piperideine, respectively. Cromwell (82) obtained an enzyme preparation from Atropa belladonna L., which oxidized putrescine to 4-aminobutyraldehyde and ammonia. He also was able to isolate a small amount of putrescine from the tissue of A. belladonna L. and Datura stramonium L. Furthermore, he observed an increase in hyoscyamine biosynthesis in D. stramonium L., after injection of putrescine. Cromwell's findings indicated that putrescine could be a natural metabolite in some alkaloid producing plants. He postulated that succinaldehyde or 4-aminobutyraldehyde was the precursor for the pyrrolidine ring of the tropine base of the hyoscyamine molecule. However, in consideration of the fact that carbon-14 labeled succinate did not contribute any significant radioactivity to the pyrrolidine ring of nicotine (83), succinaldehyde is of dubious importance as a precursor.

Contrary to Cromwell's postulation, Diaper et al. (84), reported that hyoscyamine, isolated from D. stramonium L. after administration of putrescine-1, 4-C^{14}, was not radioactive. On the other hand, Leete et al. (26), found that ornithine-2-C^{14} was a precursor for the pyrrolidine ring of hyoscyamine. James (23) observed a significant increase in hyoscyamine synthesis in A. belladonna after feeding arginine and ornithine. However, using a tissue preparation of A. belladonna he did not observe any decarboxylation of ornithine to putrescine.

Considering the fact that putrescine did not result from decarboxylation of ornithine in A. belladonna and that it did not contribute any radioactivity to hyoscyamine in D. stramonium, it would appear to be an improbable precursor for the pyrrolidine ring of this alkaloid. In this particular case, glutamic-γ-semialdehyde and 2-keto-5-aminovaleric acid (23) appear to be more probable intermediates arising from the metabolism of ornithine and leading to the biosynthesis of the pyrrolidine ring of hyoscyamine.

Mann and Smithies (85) have investigated the formation of heterocyclic rings in vitro from the diamines, putrescine, cadaverine and lysine (2,6-diaminohexanoic acid) in the presence of a diamine oxidase preparation from pea seedings. The products of this oxidation were found to be Δ^1-pyrroline, Δ^1-piperideine and Δ^1-2-piperideine carboxylic acid. Leete (29) compared the incorporation of radioactivity into nicotine after feeding ornithine-2-C^{14} and putrescine-1,4-C^{14} to Nicotiana tabacum L., var. Maryland Mammoth. Both of these compounds were incorporated very efficiently into the nicotine molecule, 0.48 and 0.12 per cent, respectively, and the labeling patterns were identical. This result suggests that putrescine was converted to Δ^1-pyrroline before its incorporation into nicotine. Δ^1-Pyrroline appears to be an ultimate metabolic precursor whether it originates from oxidative deamination of putrescine or from decarboxylation of Δ^1-PC-5. Until further evidence is made available, no decision can be made as to whether both putrescine and Δ^1-PC-5, or only one of these compounds is a natural precursor in nicotine biosynthesis.

Leete (29) in the same experiment mentioned before in this discussion, also fed uniformly labeled proline-C^{14} to tobacco plants. Its incorporation into nicotine was found to be 0.032 per cent and the radioactivity was associated with the pyrrolidine ring only. This result also placed proline among the possible intermediates of pyrrolidine ring

biosynthesis. Whether proline is a direct precursor via pyrrolidine or an indirect precursor, undergoing pronounced metabolic changes to a more immediate precursor, is not known. There is not as yet any experimental evidence available indicating that pyrrolidine is present in plant tissues as a normal metabolite. However, proline could be enzymatically oxidized between the nitrogen and α-carbon from either side. The products resulting from this oxidation would be 2-keto-5-aminovaleric acid or glutamic-γ-semialdehyde. The formation of both of these compounds from proline was observed in animals and microorganisms (6, 7, 8, 79).

The first oxidation product of proline-2-C^{14}, 2-keto-5-aminovaleric acid-2-C^{14}, cyclizes to yield Δ^1-pyrroline-2-carboxylic acid-2-C^{14} (Δ^1-PC-2). This compound after decarboxylation would most likely yield Δ^1-pyrroline-2-C^{14} as a single compound without formation of Δ^1-pyrroline-5-C^{14} as another isotopic isomer (29). Consequently, if this is the case the total radioactivity should be incorporated into position 2 of the pyrrolidine ring of nicotine. This would be in opposition to the result obtained from feeding ornithine-2-C^{14}, glutamic acid-2-C^{14} and Δ^1-pyrroline-5-carboxylic acid-5-C^{14} (Δ^1-PC-5-C^{14}).

The second possible product of proline-2-C^{14} oxidation, glutamic-γ-semialdehyde, would spontaneously cyclize to Δ^1-PC-5-C^{14}. This compound on decarboxylation probably would yield a mesomeric anion resulting in a 50:50 mixture of Δ^1-pyrroline-5-C^{14} and Δ^1-pyrroline-2-C^{14}. If Δ^1-pyrroline is the ultimate precursor, the symmetrical intermediate, a mixture of Δ^1-pyrroline-2-C^{14} and Δ^1-pyrroline-5-C^{14} would give equal incorporation of radioactivity into position 2 and 5 of the pyrrolidine ring of nicotine. This pattern of labaling would be consistent with the results obtained in this work, and with the results obtained in previous works in which ornithine-2-C^{14} (28-33), glutamic acid-2-C^{14} (29, 34), putrescine-1, 4-C^{14} (29) and uniformly labeled proline-C^{14} (29) were fed.

In view of the results described, Δ^1-PC-5 appears to be a more probable precursor of pyrrolidine ring of nicotine than Δ^1-PC-2.

Further evidence that Δ^1-pyrroline can function as an ultimate precursor for pyrrolidine ring of alkaloids was obtained by Clarke and Mann (86) by demonstrating a model synthesis of alkaloids. Δ^1-Pyrroline (or putrescine) in the presence of α-keto acids and the enzyme, amine oxidase, condenses with simultaneous decarboxylation of the α-keto acid. Robinson (22), as far back as 1917 proposed this as a working hypothesis for biosynthesis of pyrrolidine rings in alkaloids.

It is not known as yet how the pyrroline ring or its methylated derivative condenses with the pyridine ring to form nornicotine, or nicotine, but the preceding type of condensation could be considered as one possibility. E.g., nornicotine could result from the condensation of Δ^1-pyrroline with 2-keto-nicotinic acid (2-pyridone-3-carboxylic acid) in the presence of an enzyme system in tobacco plant roots. Dawson et al. (66, 87) found that the pyridine ring of nicotinic acid is a precursor for pyridine ring of nicotine, but not the carboxy group. This indicated, a priori, that decarboxylation must have taken place before the condensation occurred.

The experimental evidence drawn from the present study and from elsewhere, supports the belief that all precursors of the pyrrolidine ring of nicotine--glutamic acid, ornithine, Δ^1-PC-5, proline and putrescine--are channeled to one common intermediate, Δ^1-pyrroline, which appears to be the ultimate precursor for the pyrrolidine ring in the nicotine molecule. Before the validity of this assumption can be established, the following investigation seems to be necessary:

1. Experimental proof that the labeling pattern in nicotine is different after administration of Δ^1-PC-5-C^{14} and Δ^1-PC-2-C^{14} (or 2-keto-5-aminovaleric acid) would be necessary.
2. A demonstration should be made that Δ^1-PC-5-C^{14} on decarboxylation produces a mesomeric anion, whereas Δ^1-PC-2-C^{14} does not.

It has been pointed out previously that nicotine isolated from tobacco plants after administration of dl-Δ^1-PC-5-C^{14} contained approximately 29 per cent of its total radioactivity in the methyl group (Table XII). Because of very meager information available on metabolism of dl-Δ^1-PC-5 in general, it is difficult to propose a sound working hypothesis, which could account for this incorporation.

The methyl group of nicotine, conceivably could arise from one or two carbon fragments resulting from metabolism of dl-Δ^1-PC-5. Metabolic units, which deserve consideration are: formaldehyde, glycine, glyoxalate and glycolate. All of these compounds were identified as very efficient precursors for the methyl group of nicotine and methyl group of some other plant compounds (73, 88).

At the present time the author has no plausible explanation for this observation on the labeling pattern of nicotine from tobacco plants fed dl-Δ^1-PC-5-C^{14}. Before any valid conclusion can be reached, further research is required.

SUMMARY

1. Dl-Δ^1-pyrroline-5-carboxylic acid-5-C^{14} was synthesized, isolated as the hydrochloride and fed to tobacco plants.

2. The isolated nicotine was found to be radioactive. The incorporation of the radioactivity approximated that obtained from feeding ornithine-2-C^{14} under similar conditions.

3. The labeling pattern of isolated nicotine was determined by appropriate degradations.

4. A degradation of radioactive nicotine demonstrated that most of the radioactivity was associated with pyrrolidine ring of nicotine. An equal amount of radioactivity resided in position 2 and 5 of the pyrrolidine ring, 30 per cent in each position, and approximately 29 per cent was located in the methyl group of nicotine. The pyridine ring of nicotine was found to contain only legligible radioactivity (approximately 3 per cent).

5. Equal distribution of the radioactivity in positions 2 and 5 of the pyrrolidine ring presupposes the existence of a symmetrical intermediate (most likely Δ^1-pyrroline) along the pathway of incorporation into the pyrrolidine ring. The pattern of labeling (with the exception of the methyl group), is similar to that found after the administration of glutamic acid-2-C^{14} and ornithine-2-C^{14}. This fact reflected the metabolic relationship between these compounds.

6. The distribution of nicotine in three different tissues; leaves, stems, and roots, of Nicotiana rustica, as well as the differences in radioactivity of nicotine isolated from these three tissues was determined.

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APPENDIX

1. The formula used in correcting the observed count to zero sample thickness was:

$$A_m = \frac{C_o \cdot M}{W \cdot b}$$

where A_m = maximum specific activity (counts/minute/millimole)

C_o = observed count (counts/minute)

M = molecular weight of compound

W = weight of sample counted

b = fraction of maximum activity at the sample thickness used (T)--obtained from self-absorption curve

Sample calculation:

Nicotine dipicrate--C_o = 178 c.p.m., W = 54.9

M = 620, T = 19.4 mg/cm^2, b = 0.31

$$A_m = \frac{178 \times 620}{54.9 \times 0.31} = 6.49 \times 10^3 \text{ c.p.m./mmole.}$$

2. Calculation of the percentage of the incorporation of the precursor into nicotine:

$$\text{Per cent incorporation} = \frac{\text{Total radioactivity isolated}}{\text{Total radioactivity fed}} \times 100 =$$

$$= \frac{\text{C.p.m./X mg of nicotine dipicrate}}{\text{C.p.m.fed/Y plants}} \times 100$$