STUDIES ON THE BIOGENESIS OF THE PYRROLIDINE RING OF NICOTINE IN THE TOBACCO PLANT

By

Burton L. Lamberts

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1958

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ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. Richard U. Byerrum, whose interest, encouragement, and guidance have greatly facilitated the completion of this problem, and to other members of the Department of Chemistry for helpful suggestions from time to time. The author is also indebted to Dr. L. J. Dewey of the Medical College of Virginia for his assistance at various times and for supplying the labelled dibromocotinine.

Finally the author wishes to thank the Department of Chemistry, the Atomic Energy Commission, and the National Institutes of Health for providing the funds necessary to support this work.

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Year 1958

Approved R U Byerrum

ABSTRACT

Glutamic acid-2-C^{14} was administered hydroponically to a high nicotine strain of tobacco, Nicotiana rustica var. humilis. The nicotine which was isolated after a two-week feeding period was radioactive.

A permanganate oxidation of the nicotine yielded nicotinic acid and potassium bicarbonate as products of pyrrolidine-ring cleavage. The potassium bicarbonate, representing positions 3, 4, and 5 of the pyrrolidine ring, was isolated as barium carbonate. Decarboxylation of the nicotinic acid permitted recovery of the carbon from position 2 of this ring as barium carbonate. The pyridine resulting from the decarboxylation was counted as the picrate. About 10 per cent of the carbon-14 was found with the pyridine ring. Half of the radioactivity of the pyrrolidine ring was located at position 2; the remainder was associated with positions 3, 4, and 5.

From the similar pattern of labelling obtained in a comparable study of nicotine with ornithine-2-C^{14}, it had been postulated that the radioactivity of the pyrrolidine ring was distributed equally between positions 2 and 5.

A procedure was developed to degrade nicotine, by which the carbon representing position 5 of the pyrrolidine ring can be recovered as barium carbonate. Nicotine was treated with bromine in acetic acid

and the resulting perbromide was reduced to cotinine. Hydrolysis of the cotinine with barium hydroxide yielded a product identified as 4-(3'-pyridyl)-4-methylaminobutyric acid. Since the sidechain of this compound tends to cyclize in neutral or acidic aqueous solutions, the compound was benzoylated in dilute sodium hydroxide. The product was isolated and identified as 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid. Esterification of this compound with diazomethane in ether, followed by an ammonolysis, yielded 4-(3'-pyridyl)-4-N-methylbenzoyl-aminobutyramide. A Hofmann degradation of this compound permitted recovery of the carbon from position 5 of the pyrrolidine ring as barium carbonate. The other product of this reaction, containing the remaining carbon skeleton of the nicotine molecule, was isolated and identified as a thiourea derivative of 3-(3'-pyridyl)-3-N-methylbenzoylamino-propylamine.

Nicotine, labelled by glutamic acid-2-C^{14}, was degraded by this procedure. The intermediate produced at each step of the degradation was isolated and counted. About half of the carbon-14 was found at position 5 of the pyrrolidine ring; the amine derivative contained the remaining radioactivity. A similar degradation was carried out on dibromocotinine (3,5-dibromo-5-(3'-pyridyl)-N-methyl-2-pyrrolidone), from nicotine labelled by ornithine-2-C^{14}. The distribution of radio-activity in the pyrrolidine ring was essentially the same as was found in the glutamic acid-2-C^{14} study.

The similarity in the pattern of nicotine-labelling by glutamic acid-2-C^{14} and ornithine-2-C^{14} supports the postulation of a symmetrical

intermediate along the pathway of synthesis of the pyrrolidine ring. Several possible intermediates, which may conceivably be formed from the intact amino acid carbon chain, have been discussed.

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INTRODUCTION

The significance of glutamic acid in the metabolic processes of living organisms has long been recognized. To a large extent, the metabolic activity of this amino acid is related to protein synthesis or to the reactions of the tricarboxylic acid cycle. However, animal and microorganism studies have shown that glutamic acid may also be converted to ornithine or to proline, without cleavage of the carbon chain.

Dakin (1), in 1913, observed the glucogenic effect of glutamic acid, ornithine, and proline on diabetic animals and suggested a metabolic relationship among these amino acids. Stetten and Schoenheimer (2) demonstrated, by isotopic labelling, the conversion of proline into glutamic acid and ornithine in the rat. In another study with mice, these workers noted the conversion of ornithine, labelled with deuterium, into glutamic acid and proline (3). Sallach, Koeppe, and Rose (4) found relatively high concentrations of carbon-14 in proline and arginine isolated from rats fed glutamic acid-C^{14}.

Glutamic acid has also been shown by Tatum, Abelson, Vogel, and others (5) to be a precursor of proline and ornithine in the bacterium E. Coli. Studies of mutant strains of T. Utilis and N. Crassa have offered evidence of similar interconversions among these amino acids in fungi (6). Reviews by Stetten (7) and Vogel (7) of these

interrelations in animals and microorganisms provide many details that will not be covered in this study.

The information on this subject with regard to higher plants is, however, quite meager. Morgan and Marion (8) obtained radioactive glutamic acid from extracts of alfalfa seedlings fed carbon-14-labelled ornithine. When pyroxidine was added to the nutrient medium with the ornithine, radioactive proline was isolated. Naylor and Tolbert (9) administered glutamic acid, uniformly labelled with carbon-14 to barley seedlings. Although radioactive glutamine, γ-aminobutyric acid, aspartic acid, and various constituents of the tricarboxylic acid cycle were detected, labelled proline and ornithine were not reported; hence, were evidently not found in significant amounts.

Pathways of biogenesis of the alkaloid, nicotine, would very probably be influenced by such interconversions, were they to take place in the tobacco plant. Robinson (10) first proposed that the pyrrolidine ring of nicotine arises from ornithine. Trier (11), on the other hand, postulated the formation of both rings of the nicotine molecule from proline. Prior to the general availability of carbon-14 labelled compounds, there was no decisive experimental confirmation of either of these suggestions. Early studies of alkaloid biogenesis involved feeding supposed precursors to plants and comparing the alkaloid content of treated plants to that of controls. Klein and Linser (12), for example, observed an increase in the nicotine content of tobacco after solutions of proline, ornithine, and glutamic acid had been

injected into the plant stems. Petrosini (13) noted that proline, glycine, 3-indole acetic acid, tryptophan, alanine, aspartic acid, and glutamic acid promoted, in decreasing order of activity, the synthesis of nicotine in germinating seeds of tobacco cultured in the dark. Dawson (14) has reported an increased nicotine content in leaves of tobacco shoots cultured in aqueous solutions of l-proline, nicotinic acid, l-pyrrolidone-carboxylic acid, and d-glutamic acid. However, in subsequent experiments, Dawson (15) did not observe a synthesis of nicotine when proline and nicotinic acid were administered to initially nicotine-free tobacco leaves.

Similar studies were carried out with other alkaloids containing pyrrolidine rings. Klein and Linser (16) noted an apparent increase in stachydrine, an alfalfa alkaloid, when solutions of proline, ornithine dihydrochloride, or sodium glutamate were injected into the stems of Stachys palustris L., Stachys recta L., and Galeopsis ochroleuca Lan. James (17) showed significant increases in the hyoscyamine content of Atropa belladonna, following the feeding of ornithine or arginine to experimental plants.

However, variations of metabolism among the plants under study, due to individual differences and, possibly, to seasonal effects, could defeat attempts to reproduce experimental data by this method. Hence, it would be quite difficult to distinguish the real from the simply apparent precursor.

With the advent of carbon-14-labelled compounds, however, studies of alkaloid biogenesis have been placed on a firmer basis. Leete,

Spenser, and Marion (18), employing Datura stramonium, observed the incorporation of ornithine-2-C^{14} into the pyrrolidine ring of hyoscyamine. The activity appeared either to be distributed between carbons 1 and 5 of hyoscyamine (i.e., carbons 2 and 5 of the pyrrolidine ring) or to be located at one of these positions. Morgan and Marion (8) found no incorporation of ornithine-2-C^{14} into the pyrrolidine skeleton of stachydrine, but this was attributed to a lack of synthesis of the alkaloid under the particular conditions employed in their experiments.

Dewey and Byerrum (19) administered ornithine-2-C^{14} to three-month-old tobacco plants (N. rustica), and obtained radioactive nicotine. Essentially all of the radioactivity was found to be present in the pyrrolidine ring--half of this radioactivity was located at carbon 2, with the remainder associated with carbons 3, 4, and 5. An independent study by Leete (20), feeding ornithine, similarly labelled, to six-month old plants (Nicotiana tabacum L.) confirmed these observations.

Considering the ornithine-proline-glutamate relationship in the metabolic functions of mammals and microorganisms, the early studies of alkaloid precursors, and the labelled ornithine experiments, it was decided to administer glutamic acid-2-C^{14} to tobacco plants. The investigation was conducted with the following objectives in mind: 1) to test the capacity of glutamic acid to serve as a precursor of the pyrrolidine ring of nicotine and, 2) in the event glutamic acid functioned as a precursor, to establish the sites of radioactivity in the pyrrolidine ring through degradative studies; 3) to compare the labelling effects of ornithine-2-C^{14} and glutamic acid-2-C^{14} by

carrying out similar degradative studies of nicotine from the labelled ornithine-feeding experiments. A similar pattern of labelling by each of these amino acid precursors would suggest the formation of one or more common intermediates in the pathway of nicotine biogenesis.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

Preparation of Plants

The tobacco plants used in this study were of a high nicotine strain, Nicotiana rustica, var. humilis. The seeds were planted in a greenhouse, in flats containing Vermiculite.[1] After two or three weeks the plants were transplanted, being spaced at intervals of two to three inches. Every day the plants were watered and twice a week they received a nutrient solution of the following composition: 1 mg. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4, 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$ in four liters of tap water. After two to three months, depending somewhat on seasonal conditions, the plants had attained the desired height of five to seven inches. The plants were then removed from the flats, rinsed briefly with tap water to loosen the Vermiculite clinging to the roots, and the roots were trimmed to within about two to four mm. from the plant stems. This technique, described by Dewey (21), was designed to force the plants to develop new root systems. Dawson (22) has shown that nicotine is synthesized in the roots of tobacco plants; hence, improving the health and vigor of root systems should promote the synthesis of nicotine and, accordingly, the incorporation of carbon-14 into the nicotine molecule. Following a rinse with distilled water, each plant was placed in a 125-ml. Erlenmeyer flask in a nutrient medium. This solution was prepared by diluting, with two parts of water, one part

[1] A commercially available heat expanded mica.

of a stock solution of the following composition: $Ca(NO_3)\cdot 4H_2O$, 2.61 g.; $MgSO_4.7H_2O$, 756 mg; KCl, 500 mg.; KH_2PO_4, 500 mg.; $(NH_4)_2SO_4$, 500 mg.; $FeCl_3.6H_2O$, 5.6 mg.; water, added to a volume of two liters. A thin cotton plug was inserted into the mouth of each flask to retard evaporation of the solution and to lend support to the plant.

After a two-week period, under conditions of natural lighting, the plants had usually developed extensive root systems. In early stages of this development, plants would occasionally wilt and die, particularly during experiments conducted in the hot summer months. Normally, growth of new roots was not attended by appreciable growth of other parts of the plants.

To prepare the plants for hydroponic administration of the glutamic acid-2-C^{14}, the plants were transferred to clean 125-ml. Erlenmeyer flasks. To each flask was added 50 ml. of a nutrient medium which was prepared as follows: one part of the stock solution was diluted with two parts of a fungicide solution (to be described later), and the resulting solution was aerated for 10 or 15 minutes. Five-tenths ml. of an aureomycin solution (50 mg. per 50 ml. of solution) was also added to the flasks.

In some of the initial experiments, a comparison was being made with one of Dewey's ornithine-feeding experiments (21), hence the roots of the plants were not shielded from light. Later, however, the flasks were covered with shields of black construction paper to promote root growth; this practice was followed from the beginning of the root-growing period until the plants were eventually harvested.

The plants were kept in a hood during the period of administration of the glutamic acid-2-C^{14}. A nutrient medium (stock solution diluted with two parts of water) was supplied as required by the plants. Artificial lighting was provided twelve hours daily during this period by two 36-inch, 30-watt fluorescent tubes and by a 100-watt incandescent bulb, placed approximately fourteen inches above the plants. The light intensity thus supplied the plants was about 200 foot-candles.

Uptake of Glutamic Acid

Since Dewey (21) has shown that ornithine may be administered to tobacco plants hydroponically, it was decided to follow the same procedure with glutamic acid. Before feeding the glutamic acid-2-C^{14}, however, an uptake study was undertaken with non-radioactive glutamic acid.

In the first uptake experiment, 52.5 ml. of solution was added to each of twelve 125-ml. Erlenmeyer flasks. Each flask contained 50 ml. of nutrient solution (16.6 ml. stock solution diluted with two parts of water), 0.5 mg. of aureomycin, and four mg. of glutamic acid, adjusted to a final volume of 52.5 ml. with water.

Four tobacco plants were removed from a flat and the roots soaked briefly with tap water. Following careful removal of the Vermiculite, the roots were rinsed with distilled water and the plants transferred to four of the flasks. Six root fragments were introduced into each of four other flasks to determine the extent of glutamic acid loss to plant root microorganisms. The remaining flasks served as controls.

The flasks were placed in the hood and artificial light was supplied for a twelve-hour period each day. Oxygen was administered at the beginning and end of each lighting-period. After forty-eight hours, the plants were removed from the flasks, the roots were rinsed with the nutrient solution and the washings were added to the flask contents. Due to loss of solution by plant uptake and evaporation, most of the solution volumes had dropped below 52.5 ml. The flask volumes were restored with nutrient solution and the contents of each flask filtered separately through Whatman 2 paper. A ten-ml. aliquot was then removed from each flask and diluted to 100-ml., in preparation for the glutamic acid analysis.

Glutamic acid was determined by the method of Gordon (23). A standard curve of glutamic acid vs. optical density was prepared from known quantities of glutamic acid, and covered the range of concentrations encountered in this study. Zero to 10 ml. aliquots of a 10 γ/ml. solution of glutamic acid were added to seven 100-ml. volumetric flasks. The nutrient solution of inorganic salts and aureomycin, of the concentration described above but minus the glutamic acid, was diluted 1:20 with water and 10-ml. aliquots of the diluted solution were added to each volumetric flask. Then 2.3 ml. of a freshly prepared solution of sodium hypochlorite (prepared by bubbling chlorine through 0.5 M sodium hydroxide and diluting 1:100 with 0.1 M sodium hydroxide) was added to each flask to oxidize the glutamic acid. Exactly ten minutes after each sodium hypochlorite addition, 0.8 ml. portions of an oxidizable

green dye[2] solution were added to the flasks to consume the excess sodium hypochlorite. The dye faded to a yellow-orange color upon oxidation. Exactly ten minutes after each addition of the dye solution, each flask received a ten-ml. aliquot of a sulfate buffer[3] to stabilize the colors, and the flask volumes were adjusted to 100 ml. with water. The optical density of each flask was determined with a Beckman Model B Spectrophotometer, at 630 mu, at 10-minute intervals after each buffer addition.

To analyze the solutions from the uptake experiments, a 10-ml. aliquot of each diluted sample (as described above) was added to a 100-ml. volumetric flask. The subsequent steps were those followed with the known quantities of glutamic acid; the concentration of each sample was determined from the standard curve of concentration vs. optical density.

The experimental results indicated that, though essentially all of the glutamic acid had been absorbed by the plants in the 48-hour period, there was also an average loss of 38 per cent of the glutamic acid in the flasks containing root fragments. The cloudy appearance of these solutions suggested that the loss was due to fungi; hence, a solution of fungicide was prepared. The fungicide[4] was only slightly soluble in water--less than one part fungicide per 100,000 parts of water.

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[2]Fast Green FCF (The Matheson Co., E. Rutherford, N. J.).

[3]150 ml. of 10 M H_2SO_4 and 245 ml. of NaOH, diluted to one liter.

[4]A commercial brand, "Ubavit Neu" (Schering-Kahlbaum A. G. Berlin).

In the second uptake experiment, twelve 125-ml. Erlenmeyer flasks were set up as before except that the nutrient solution was composed of one volume of stock solution, diluted with two volumes of fungicide solution. In other respects, the experiment was conducted in the same manner as the first experiment. A new standard curve was prepared to compensate for possible discrepancies arising from the use of the fungicide.

The results of this experiment showed that 95 per cent of the glutamic acid had been absorbed by the plants, with an average loss of 4 per cent of the glutamic acid in the root-fragment flasks. The solutions of these flasks were clear and appeared to be free of fungal growth. Hence, with the subsequent administration of glutamic acid-2-C^{14}, in the later experiments, both fungicide and aureomycin were added to the nutrient solutions.

Isolation and Purification of Nicotine

At the end of each administration period, the plants were removed from the flasks, the roots were rinsed with distilled water and blotted on cheesecloth. The plants were cut into small pieces and dried under infrared heat lamps. Temperature was regulated to facilitate rapid drying without heating the plant material above 70 to 80° C. The dried material was thoroughly ground in a mortar, mixed with 20 per cent by weight of calcium hydroxide, and steam distilled in a Kjeldahl flask. Distillation was continued until the distillate no longer gave a white precipitate with silicotungstic acid, a reagent used to detect the

presence of alkaloids. The distillate was collected in a small quantity (usually about 5 ml.) of 6N HCl, and concentrated to 30-40 ml. with a flash evaporator. Nicotine was purified by two successive azeotropic distillations with water, using a Widmer column as described by Smith (24). The distillation was effected from an alkaline medium and the distillate collected in 6N HCl. The acidic solution was concentrated to dryness under reduced pressure, and the resulting hydrochloride dissolved in a small amount of water and methanol. Nicotine dipicrate was precipitated upon addition of an excess of methanol saturated with picric acid. After a short period of cooling, the dipicrate was separated by filtration with a sintered glass filter, washed with methanol, and recrystallized from hot water. (M.p., 219-221; Recorded value 218°).[5]

Determination of Radioactivity

All measurements of radioactivity were made with a Nuclear-Chicago Model 192X scaler (Nuclear Instruments and Chemical Corporation, Chicago 10, Illinois) and a Tracerlab Model SC-16 proportional flow counter (Tracerlab, Inc., Boston 10, Massachusetts). The counting gas, purchased from Tracerlab, Inc., consisted of 90 per cent argon with 10 per cent methane. The counting system was 40 per cent efficient, as determined by a National Bureau of Standards $Na_2C^{14}O_3$ sample.

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[5]All melting-point values recorded in this thesis were obtained by means of capillary melting-point tubes. The melting points are uncorrected.

The materials to be counted were plated on aluminum planchets, 2.83 sq. cm. in area. Large crystals were pulverized in a small mortar before transfer to planchets. The surface of each plate was made as smooth and uniform as possible before counting. Barium carbonate samples were plated from a slurry in 95 per cent ethanol, as described by Calvin (25). Activities were corrected to "infinite thinness" by reference to appropriate self-absorption curves. (See Appendix for calculations.)

Preliminary Biosynthetic Studies with Glutamic Acid-2-C^{14}

Administration of Radioactive Glutamic Acid

In the first experiment with glutamic acid-2-C^{14}, the radioactive compound was diluted with non-radioactive glutamic acid. As in the uptake study, each plant received four mg. of glutamic acid. However, since Dewey (21) reported an increased synthesis of carbon-14 labelled nicotine when ornithine-2-C^{14} was supplied at two different times during the administration period, it seemed desirable to follow this procedure with the glutamic acid.

Forty plants were prepared, as described previously. Each plant then received two mg. of the glutamic acid, having a calculated radioactivity of 2.5×10^5 cpm. Five days later another two mg. of the glutamic acid was added to each flask. The plants were harvested nine days later, and the nicotine was isolated as the dipicrate. However, the nicotine dipicrate contained no significant radioactivity. Since this result could mean that the reservoir of radioactive glutamic acid,

presumably present in each plant and available for nicotine synthesis, had been inordinately diluted by addition of non-radioactive glutamic acid, it was decided to omit the latter in the following experiment.

In the second experiment, each of forty plants received 0.35 mg. of glutamic acid-2-C^{14}, having a calculated radioactivity of 8×10^5 cpm. Five days later, another 0.35 mg. was given to each plant; again, after nine days, the plants were harvested. The nicotine dipicrate from this harvest showed a specific activity of 4.36×10^3cpm/mM. Since this experiment was modelled after an experiment of Dewey (21), which involved ornithine-2-C^{14} of equivalent radioactivity and molar quantity to the glutamic acid-2-C^{14}, it was hoped that a valid comparison could be made between glutamic acid and ornithine as precursors of nicotine. When this experiment was repeated with twenty plants the specific activity of the isolated nicotine dipicrate was 2.74×10^3cpm/mM. The discrepancy between these results is believed to have been due to seasonal conditions.

Later in the course of this study it was discovered that the counting instrument employed in the present studies was twice as efficient as the instrument Dewey had used in his experimental work. Hence, the necessary correction was made when the ornithine-2-C^{14} and the glutamic acid-2-C^{14} experiments were compared. This comparison is shown in Table I.

From the foregoing experiments, it is evident that glutamic acid is incorporated into nicotine, but, when ornithine and glutamate were fed in equivalent quantities and radioactivities, the nicotine from the ornithine-fed plants was considerably more radioactive. However,

TABLE I

INCORPORATION OF RADIOACTIVITY INTO NICOTINE BY ORNITHINE-2-C^{14} AND GLUTAMIC ACID-2-C^{14}

Amino Acid Administered	Experiment	Max. Spec. Activity of Nicotine d.p. (reported)	Max. Spec. Activity of Nicotine d.p. (corrected)*	Ratio of Max. Spec. Activity of Ornithine: Glutamic acid
Ornithine-2-C^{14}	---	1.21×10^5	6.05×10^5	---
Glutamic Acid-2-C^{14}	2	4.36×10^3	1.09×10^4	56:1
Glutamic Acid-2-C^{14}	3	2.74×10^3	6.85×10^3	88:1
				72:1 (average)

*Assuming each instrument to be 100 per cent efficient.

before degradative studies of nicotine could be undertaken to establish the focal points of radioactivity in the molecule, it was necessary to increase the supply of the carbon-14 labelled nicotine.

In a fourth experiment, each of fifteen plants received a total of 5.16 mg. of the glutamic acid-2-C^{14} (calculated radioactivity, 1.17×10^7 cpm) over the two-week experimental period. The nicotine dipicrate (360 mg.) had a maximum specific activity of 1.40×10^4 cpm/mM. Sufficient nicotine was thus available for a preliminary degradation, following a dilution of the radioactive with non-radioactive nicotine.

The preliminary degradation of this radioactive nicotine did demonstrate a similar pattern of labelling of the pyrrolidine ring by glutamic acid and ornithine, as will be described later in this study. Hence, a further series of degradations of the nicotine seemed desirable, although this would require about seven to eight times the amount of

nicotine used in the preliminary study. However, the foregoing method of acquiring the radioactive nicotine for the degradative studies seemed impractical because of the expense of the radioactive glutamic acid and its apparently low degree of incorporation into nicotine. Therefore, the possibility of increasing still further the synthesis of carbon-14-labelled nicotine by the plants was studied.

Shields of black construction paper were fitted to the Erlenmeyer flasks to protect the roots from light. Following this modification, the radioactivity of the nicotine was increased several fold over that of the previous experiments; hence, shields were employed in all of the subsequent plant-feeding experiments. The author believes this increased incorporation of carbon-14 into nicotine to be due partly to the over-all improvement in health of plants upon shielding the roots from light; particularly, however, it is believed due to the development of healthier and more extensive root systems than were observed in the previous experiments.

As mentioned before, however, the radioactivity of the nicotine isolated from plants fed glutamic acid-2-C^{14} in this manner may vary considerably with any given experiment. This is believed to be a seasonal effect; at any rate, in conjunction with the "safety in numbers" maxim, it seemed practical to treat a large group of plants with a comparatively small amount of the radioactive compound per plant to obtain the necessary labelled nicotine for the degradations.

Hence, as the growing plants reached the desired size, they were utilized for the synthesis of radioactive nicotine. Approximately one

hundred forty plants were harvested, in six groups, besides the plants of the previously described experiments. The method of handling these plants was essentially the same as outlined in the previous experiments except that, with each group, a total of one mg. of the glutamic acid was administered per plant.

Although the direct purpose of these plant-growing operations was the accumulation of a supply of radioactive nicotine, nevertheless, certain comparative experiments were thereby rendered feasible. For example, it was noted that the radioactivity of the nicotine, isolated in experiments 3 and 4, was roughly proportionate to the amount of glutamic acid-2-C^{14} administered to the plants. These experiments, however, were conducted during the summer and autumn months respectively; hence, an attempt was made to clarify this effect by feeding two groups of plants simultaneously, to eliminate the influence of seasonal change. Six plants were fed a total of one mg. of glutamic acid-2-C^{14} per plant. Another group of six plants, similar to the first group with respect to the size and age of the plants, received a total of three mg. of the glutamic acid-2-C^{14} per plant. After the usual two-week administration period, the plants of both groups were harvested, and the nicotine isolated as the dipicrate. In this comparison, however, the difference in the amounts of nicotine isolated from each group of plants should be considered. The second group seemed to contain slightly more nicotine than was found in the first group. Assuming that each group synthesizes nicotine at approximately the same rate after the glutamic acid-2-C^{14} administration, and that the isolated nicotine dipicrate is a measure

of the total nicotine, the excess nicotine contained in the total reservoir of the second group of plants may be considered to function as a diluent. Upon application of this dilution factor to equate the total nicotine content of each group, the radioactivity of the nicotine was found to be approximately proportionate to the amounts of radioactive glutamic acid administered--at least up to three mg. of glutamic acid per plant. This result would indicate that the glutamic acid pool in the plant is much larger than the quantity of glutamic acid administered.

As mentioned previously, Dewey (21) reported an increased synthesis of nicotine in tobacco plants that had been forced to grow new roots, following removal of the original roots. Another comparative experiment was set up to assess this effect under the conditions employed throughout the present study. The roots of the first group of ten plants (Experiment 10, Table II) were removed, thus forcing the plants to grow new roots. The second group (Experiment 11, Table II) contained the same number of plants, of approximately the same average height; however, these plants were simply removed from the flats with the original roots left intact. Each plant received a total of one mg. of glutamic acid-2-C^{14} (calculated activity, 2.84×10^6cpm), administered, as before, in two portions. The plants were harvested after the usual two-week period. The nicotine from the first group showed about twice the radioactivity found in the nicotine from the second group of plants when a conversion factor was applied in an attempt to equate the nicotine content of each group, as described in the previous experiment.

Even though relatively few plants were involved in this experiment, the results support Dewey's observations.

Comparative data of the various harvests is provided in Table II.

Degradation of Radioactive Nicotine

1) Oxidation of Nicotine with Potassium Permanganate. Dewey and Byerrum (19) have shown that ornithine-2-C^{14} is incorporated into the pyrrolidine ring of nicotine. Half of the original radioactivity was located at carbon 2, with the remaining activity associated with carbons 3, 4, and 5 of the pyrrolidine ring. Since glutamic acid and ornithine may follow a similar route of incorporation into nicotine, it was decided to repeat this degradation with some of the radioactive nicotine isolated in the previous studies after the glutamic acid administrations.

This method of degradation, originally described by Laiblin (27), consists in the oxidation of nicotine, by neutral potassium permanganate, to nicotinic acid and potassium bicarbonate. The potassium bicarbonate, resulting from oxidation of carbons 3, 4, and 5 of the pyrrolidine ring, may be decomposed with acid and the liberated carbon dioxide collected as barium carbonate by bubbling it through barium hydroxide.

Initially, two grams of radioactive nicotine dipicrate, prepared by diluting the radioactive with non-radioactive material, were dissolved in a minimal quantity of hot water, recrystallized, and the resulting homogeneous sample of nicotine dipicrate was counted. The nicotine (0.39 g.) from 1.5 g. of the recrystallized sample was recovered by azeotropic distillation with water, as previously described,

TABLE II

EFFECTS OF LIGHT SHIELDS AND THE QUANTITY OF GLUTAMIC ACID ADMINISTERED ON THE INCORPORATION OF RADIOACTIVITY INTO NICOTINE BY GLUTAMIC ACID-2-C^{14}

Experiment	Number of Plants	Mg. Glut. Acid Fed Per Plant	Cpm Fed Per Plant	Grams Dry Wt. of Plants	Light Shields		Mg. Nicotine Dipicrate	Max. Spec. Activity of Nicotine d.p.
					Used	Omitted		
1	40	4.0	$5x10^5$	25.2		x	378	---
2	37	0.7	$1.6x10^6$	27.0		x	967	$4.36x10^3$
3	20	0.7	$1.6x10^6$	12.6		x	452	$2.74x10^3$
4	15	5.16	$1.17x10^7$	9.5		x	360	$1.40x10^4$
5	44	1.0	$2.84x10^6$	30.3	x		566	$2.20x10^4$
6	18	1.0	$2.84x10^6$	----	x		385	$2.71x10^4$
7*	76	1.0	$2.84x10^6$	47.0	x		1335	$2.84x10^4$
8	6	1.0	$2.84x10^6$	4.0	x		130	$1.59x10^4$
9	6	3.0	$8.52x10^6$	4.7	x		172	$3.68x10^4$
10	10	1.0	$2.84x10^6$	9.2	x		228	$9.90x10^3$
11	10	1.0	$2.84x10^6$	12.1	x		403	$2.80x10^3$

*A combination of four harvests of 16, 14, 19, and 27 plants respectively. The radioactive glutamic acid was administered to all of the plants during a four-week period.

except that no hydrochloric acid was added to the receiving vessel. The 400 ml. of distillate was transferred to a liter Erlenmeyer flask and was treated with 80 ml. of an aqueous solution of potassium permanganate (2.34 g., i.e., calculated as six times the weight of nicotine).

The potassium permanganate solution was added in five-ml. quantities at three-to-five-minute intervals; with each addition, the flask contents were thoroughly mixed. Initially, the oxidation was quite rapid, as evidenced by the disappearance of the purple permanganate color and the appearance of brown manganese dioxide. However, the rate of oxidation decreased with each addition of the reagent; after the final addition of the permanganate solution, the mixture was placed on a steam bath and was heated for about ten hours. to complete the oxidation and to promote coagulation of the manganese dioxide. At the end of this time, the flask was permitted to cool, the manganese dioxide was removed by filtration and was washed thoroughly with hot water. The washings and filtrate were combined to give a nearly colorless solution which was concentrated to dryness with a flash evaporator.

The residue, consisting of the potassium salt of nicotinic acid and of potassium bicarbonate, was dissolved in about 50 ml. of water. Acidification of this solution with dilute nitric acid released carbon dioxide which was swept into a saturated solution of barium hydroxide by a slow stream of nitrogen. The resulting barium carbonate was isolated, washed with hot water, and dried for one hour at 110° C. The barium carbonate was plated, dried in a vacuum desiccator, and counted.

Self-absorption corrections were made from a self-absorption curve, prepared for barium carbonate (36). After the solution of nicotinic acid was treated with dilute ammonium hydroxide until just alkaline to litmus, it was concentrated to dryness. Water was added to dissolve the residue, and the solution was again concentrated to dryness. The residue was dissolved in 10-15 ml. of water and 0.1 M silver nitrate was added in excess to form the insoluble silver salt of nicotinic acid. The salt was separated on a sintered glass filter and washed with distilled water. After slurrying the solid in about 50 ml. of hot water, hydrogen sulfide was introduced to decompose the silver salt. The silver sulfide was removed by filtration, the filtrate decolorized with a small amount of charcoal, and the solution concentrated to dryness under reduced pressure. The solid residue was transferred to a sublimation apparatus which was placed in a mineral oil bath and evacuated with a vacuum pump. The bath was slowly heated to 150° and was maintained at this temperature until no more sublimate appeared to form; this procedure was repeated four times to purify the nicotinic acid. With each sublimation, the amount of brownish residue remaining in the tube was diminished; with the final sublimation, only a trace of impurity was observed. About 120 mg. of the nicotinic acid was collected. (m.p., 230°; recorded value, 232°). A comparison of the infrared spectrum of the experimental preparation with that of commercial nicotinic acid confirmed the identification and purity of the product. The nicotinic acid was plated, dried in a vacuum desiccator, and counted.

2) Decarboxylation of Radioactive Nicotinic Acid. The nicotinic acid obtained from the oxidation of nicotine was divided into two 45-mg. portions, which were decarboxylated in separate operations. Following the procedure described by Dewey (21), the nicotinic acid was mixed with an excess of calcium oxide and transferred to a small flask, which was part of the apparatus employed in the demethylation of nicotine (26). A delivery tube was connected to the sidearm of the flask, and a stream of nitrogen was passed through the flask and delivery tube into a small centrifuge tube containing a solution of methanol saturated with picric acid. The flask, immersed in a copper oxide bath, was slowly heated with a Bunsen burner. As droplets of pyridine began to condense on the walls of the delivery tube, the temperature was raised to 300-350°, and maintained at this level for 15 to 20 minutes. The apparatus was permitted to cool and the delivery tube was rinsed several times, with methanol saturated with picric acid, into the centrifuge tube. After the solution had been cooled in the refrigerator for about an hour, the precipitate was separated with a sintered glass filter and washed with methanol. The pyridine picrate samples from the two decarboxylations were combined and recrystallized from hot water. Since further purification of this sample was believed necessary, 10 mg. of recrystallized inactive pyridine picrate was mixed with 10 mg. of the above recrystallized sample and the mixture was recrystallized from hot water. The pyridine picrate was plated from a slurry in 95 per cent ethanol, dried in a vacuum desiccator, and counted. Anal.[6] Per cent calculated for

[6]The microanalyses reported in this thesis were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan.

$C_{11}H_8N_4O_7$: C, 42.86; H, 2.62; N, 18.18; per cent found: C, 42.91; H, 2.75; N, 18.14. M.p., 163-164°; m.p. recorded, 165°.

Upon decarboxylating the nicotinic acid in an excess of calcium oxide, calcium carbonate was formed. Before recovering the carbon dioxide, a delivery tube was directed from the flask into a saturated solution of barium hydroxide. A glass Y-tube, attached to the side arm of the flask by tygon tubing, was also connected to a dropping funnel and to a nitrogen tank. Dilute nitric acid, introduced into the Y-tube through the dropping funnel, was forced into the flask by a stream of nitrogen. The liberated carbon dioxide was swept into the barium hydroxide by the nitrogen. The barium carbonate was isolated and plated, as described previously.

Results

The data obtained upon counting the samples of nicotine and its degradation products, described above, are presented in Table III. The results of this degradation reveal a labelling-pattern for the nicotine which is very similar to that reported by Dewey and Byerrum (19) for nicotine from tobacco fed ornithine-2-C^{14}.

The oxidation of nicotine by the neutral potassium permanganate yields nicotinic acid, potassium bicarbonate, and methylamine. Through cleavage of the pyrrolidine ring, carbon 2 is converted to the carboxyl carbon of nicotinic acid; carbons 3, 4, and 5 are oxidized to potassium bicarbonate and the N-methyl grouping is converted to methylamine. It is not definitely known whether or not any of the methyl carbon is

TABLE III

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER THE ADMINISTRATION OF GLUTAMIC ACID-2-C^{14}, WITH ISOLATION OF CARBON 2 OF THE PYRROLIDINE RING

Compound	Maximum Specific Activity (cpm/mMole x 10^{-3})
(1) Nicotine dipicrate	2.7
(2) Barium carbonate* (from nicotine oxidation)	1.1
(3) Nicotinic acid	1.3
(4) Barium carbonate (from decarboxylation)	1.2
(5) Pyridine picrate	0.2

*Calculations based on the assumption that three moles of barium carbonate are formed per mole of nicotine.

converted to bicarbonate. Laiblin (27), however, has isolated methylamine from the oxidation mixture, and reports a strong odor of methylamine throughout the oxidation procedure. In the present study, as in the previous studies of Dewey (21), it has been assumed that the bicarbonate is essentially the product of carbons 3, 4, and 5.

Hence, after the bicarbonate was converted to barium carbonate, (Line 2, Table III) the original count of the barium carbonate was multiplied by three in computing its specific activity, to permit a comparison of the barium carbonate and nicotine. The specific activity of the barium carbonate is seen to be about half that of the nicotine, and in the same order of activity as was found for the nicotinic acid

(Line 3). This closely resembles the pattern of incorporation of ornithine-2-C^{14} into the pyrrolidine ring, reported by Dewey (21).

When the nicotinic acid was decarboxylated, the carboxyl carbon (originally carbon 2 of the pyrrolidine ring), was isolated as barium carbonate and counted. The specific activity of this barium carbonate (Line 4, Table III) was about 90 per cent of that of the nicotinic acid. The remaining activity of the nicotinic acid was associated with the pyridine ring, which was counted as pyridine picrate (Line 5, Table III).

In previous studies with ornithine-2-C^{14} by Dewey and Leete, Dewey (21) found no activity in the pyridine (isolated as the picrate), following decarboxylation of nicotinic acid. Leete (20) decarboxylated nicotinic acid by refluxing it in quinoline with a copper chromite catalyst. The activity of the carboxyl carbon was essentially the same as that of the nicotinic acid; hence, no activity was assumed to be present in the pyridine, which, however, was not isolated.

The biogenesis of the pyridine ring of nicotine has never been fully elucidated. Recently certain evidence (28) has indicated that this ring may arise from "active acetate" or, perhaps, from some intermediary product of acetate metabolism. Studies of nicotine biogenesis, involving ribose-1-C^{14} (29) and pyruvate-3-C^{14} (30), suggest that the pyridine ring precursor is related to the intermediates of glycolysis or of the tricarboxylic cycle. Since glutamic acid is a very active metabolite in living systems, it may quite conceivably be linked with this precursor of the pyridine ring.

In the previous studies of nicotine biogenesis by Dewey and Leete, working with ornithine-2-C^{14}, the nicotine was demethylated to isolate the N-methyl grouping of the pyrrolidine ring. Dewey (21) found less than two per cent of the total radioactivity of the nicotine with this carbon atom; Leete (20) reported no significant activity at this position. Hence, no attempt was made in the present study to demethylate nicotine.

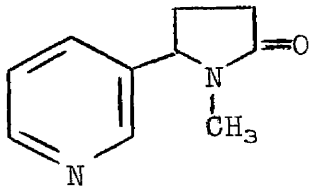
From these studies of nicotine biogenesis, it has been postulated (20,21) that the pyrrolidine ring is formed from some type of symmetrical intermediate. The studies with ornithine-2-C^{14}, mentioned above, have shown that half of the radioactivity of the nicotine molecule is located at carbon 2 of the pyrrolidine ring, with essentially all of the remaining radioactivity associated with carbons 3, 4, and 5 of this ring. If the pyrrolidine ring is formed from some type of symmetrical intermediate, without a previous cleavage of the carbon chain of the glutamic acid or ornithine precursor, the remaining radioactivity would probably be located at carbon 5 of this ring, rather than with carbons 3 or 4.

Hence, a further series of degradations were undertaken to investigate this possibility. The primary purpose of the intended study was to isolate carbon 5 of the pyrrolidine ring, using more of the radioactive nicotine, labelled by glutamic acid-2-C^{14}. In the event that carbon 5 would be found radioactive, it was hoped that a comparison of its radioactivity might be made with the residual carbon skeleton of the nicotine molecule.

Studies of Nicotine Degradation

Huber, Etard, Pinner, and others, discovered several methods for degrading the pyrrolidine ring of nicotine, when the structure of this compound was under study. The oxidation with permanganate, described by Laiblin (27) has already been discussed. However, the nature of the $C_5H_{10}N$ residue, attached to the pyridine ring of nicotine, was actually established by a study of the action of bromine on nicotine. This study, pursued at times by several investigators (31,32) was more thoroughly investigated by Pinner (33). Pinner succeeded in isolating several brominated products, which were in turn subjected to further degradative treatments. Although many of these products were obtained only in small quantities, this was sufficient for Pinner's purpose of elucidating the structure of nicotine.

When nicotine is treated with bromine in acetic acid, an oily perbromide is formed. Reduction of the perbromide with zinc and hydrochloric acid forms compound I, which Pinner named "cotinine."

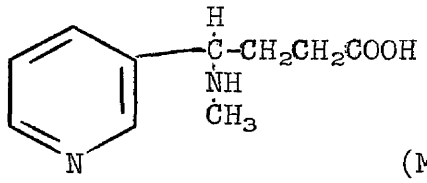


Compound I

Cotinine is a stable compound which, according to Pinner (33), is not affected by heating with barium hydroxide or by the action of hydrochloric acid at 180°C.

Prior to the present study, an attempt was made at this laboratory (34) to isolate carbon 5 of the pyrrolidine ring as the carboxyl carbon

of benzoic acid. Cotinine was treated with phenyl magnesium bromide and subsequently oxidized with potassium permanganate. These reactions, successful in degradative studies of hyoscyamine (18), were unsuccessful when applied to cotinine degradation. In other studies, through which the lactam structure of cotinine was confirmed, Frankenburg and Vaitenkunas (35) degraded the pyridine ring of cotinine to obtain N-methyl-5-pyrrolidone-2-carboxylic acid. These reactions, however, are not relevant to the problem under study. Recently McKennis, Turnbull, Wingfield, and Dewey (37) have reported the hydrolysis of cotinine. The product of hydrolysis has been identified as 4-(3'-pyridyl)-4-methylaminobutyric acid which is represented thus:



Compound II (MAPBAcid)

4-(3'-pyridyl)-4-methylaminobutyric acid

Hence, the 5 carbon of the pyrrolidine ring of nicotine may be converted to the carboxyl carbon of this substituted butyric acid. Since decarboxylation of this compound should yield the desired carbon atom as carbon dioxide, it was decided to prepare this material to permit further decarboxylation studies.

Isolation of Cotinine

The procedure employed for the isolation of cotinine was essentially that of Pinner (33). Occasionally small modifications were introduced as seemed of advantage in the present studies.

Thirty-two ml. of bromine in 80 ml. of 80 per cent acetic acid was added slowly with cooling to 14 ml. of 95 per cent nicotine dissolved in 65 ml. of 80 per cent acetic acid. Almost immediately the perbromide separated as a dark reddish oil. After a few minutes of heating, this mixture was added slowly to 375 ml. of hot water to effect solution of the perbromide. Upon cooling, the perbromide crystallized as orange-yellow crystals. The perbromide was separated by filtration (Whatman No. 2 paper on Buchner funnel), transferred to a beaker and covered with 50 ml. of 6N hydrochloric acid. The transfer was completed by rinsing the remaining perbromide crystals into the beaker from the paper and filtering with about an equal volume of water. Zinc dust was added in small amounts to the mixture to reduce the perbromide. The customary practice has been to place the perbromide-acid mixture in an ice bath, which was permitted to return to room temperature as the reaction proceeded. The beaker was covered to exclude light because, on the basis of several experiments, it was believed that side reactions were thereby reduced, yielding a less colored product. Then the zinc dust was added periodically until the perbromide had dissolved and a colorless or pale yellow solution resulted. The excess zinc was removed by filtration and the solution was neutralized with sodium hydroxide. The resulting zinc hydroxide was removed by filtration, washed with water several times[7] and the washings were added to the filtrate. This solution was transferred

[7]Usually the operation was continued until no positive test was obtained with silicotungstic acid.

to a separatory funnel, made strongly alkaline with sodium hydroxide, and extracted repeatedly with chloroform.[8] Upon removing the chloroform, (which was usually recovered by distillation), crude cotinine remained as a brownish oil. The crude material was transferred to a 50-ml. Claisen flask (a few ml. of water were added to facilitate this transfer), which was connected to a distillation flask (39), which served as a receiver. The distillation apparatus was evacuated, heat was supplied with a small heating mantle, and the cotinine distilled. The cotinine usually separated as a colorless or pale yellow opalescent oil. The yield of cotinine by this procedure ranges from about 45 to 65 per cent.

Isolation and Identification of 4-(3'-pyridyl)-4-methylaminobutyric Acid

The method (38) commonly employed in the isolation and purification of the 4-(3'-pyridyl)-4-methylaminobutyric acid is outlined below.

The cotinine, prepared as described above, was transferred to a 100-ml. round-bottomed flask. Ten ml. of water and 10 gms. of $Ba(OH)_2.8H_2O$ were added to the flask and the mixture refluxed for about twelve hours. The flask was then permitted to cool, and the flask contents were concentrated to dryness under reduced pressure at 40 to 50°C. The solid obtained was extracted several times with acetone to remove non-hydrolyzed cotinine. A small quantity of water was added to the flask and the resulting solution was saturated with carbon dioxide to separate the barium as barium carbonate. This precipitate was removed by filtration with a sintered glass filter, the precipitate was washed with water,

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[8] Ibid.

and the washings and filtrate were combined. This solution was placed in an ice bath and the carbon dioxide saturation was repeated once or twice, until no further barium carbonate precipitation was observed. After removal of the remaining barium carbonate, the filtrate was concentrated to dryness under reduced pressure at 25-30° C., using a flash evaporator. The product, usually a yellow oil, was dissolved in a minimal amount of absolute alcohol at 60°C., and an equal volume of acetone was added. It was found convenient to filter the alcoholic solution through a sintered glass filter into a 50-ml. suction flask. Excess solvent was removed by drawing a stream of air through the flask and gently warming the solution. Acetone was added after the temperature had been brought to 60° and the solution reduced to a minimal volume (usually a trace of crystalline product appeared at this stage). The product crystallized from the solution as white needles; after several hours of refrigeration, it was separated by filtration, washed with a little acetone and ethyl ether, and dried in a vacuum desiccator.

When crude cotinine was hydrolyzed, the final product isolated was frequently gummy and impure; hence, it was found to be more practical to distil the cotinine prior to the hydrolysis.

No consistent melting-point values could be obtained, but this difficulty may have been related to the hygroscopic nature of the MAPBAcid and its tendency to crystallize as a hydrate. Melting-point figures ordinarily ranged from 120 to 125°, although a value of 132-133° has been reported (37). Anal. Per cent calculated for $C_{10}H_{14}N_2O_2.1/2$

H_2O: O, 59.08; H, 7.44; N, 13.78; per cent found: C, 59.21, 59.30; H, 7.51, 7.44; N, 13.82, 13.77.

Evidence supporting formula II was obtained by a neutralization equivalent. Since MAPBAcid may be considered a substituted N-methyl amino acid, the determination was carried out in 85 per cent ethanol. After a quantity of the crystalline material had been dried for 10 hours in a vacuum desiccator over Drierite, a 0.1051 g. sample was transferred to a small beaker and dissolved in 50 ml. of 85 per cent ethanol. The solution was stirred by a magnetic stirring-bar and bubbles of nitrogen were introduced by a capillary to reduce interference of carbon dioxide. The electrodes of a Beckman glass-electrode pH meter, which had been calibrated against a standard buffer solution, were placed in contact with the solution and standardized sodium hydroxide was added dropwise. A pH reading was taken for each 0.05 ml. addition of the standard base. A plot was made of pH vs. volume of sodium hydroxide added. From the point of inflection of the curve, the volume of standard sodium hydroxide was determined, which permitted calculation of the neutralization equivalent. Theoretical N.E.: 194; Experimentally determined N.E.: 195. Although the elemental analysis would suggest a theoretical molecular weight of 203 (considering a half molecule of water of hydration), the drying conditions employed prior to the elemental analyses may have been less rigorous than those described for the neutralization equivalent determination, which could account for this discrepancy. McKennis et al. (37) have obtained the MAPBAcid as a monohydrate after air drying.

Upon drying in a vacuum desiccator (1 mm.) over potassium hydroxide, the water of hydration was readily removed. Hence, it seems feasible that the water of hydration was lost by drying the MAPBAcid as described above.

McKennis and co-workers (40) have reported that compound II develops a red color when exposed to vapors of cyanogen bromide. A red color developed when some of the crystalline material, prepared as described above, was exposed to cyanogen bromide vapors.

Some Attempted Degradations of 4-(3'-pyridyl)-4-methylaminobutyric Acid

After a survey was made of some possible methods of decarboxylating the MAPBAcid, several preliminary experiments were carried out in an effort to select the most practical method. An effort to convert the carboxyl grouping to an amide by means of thionyl chloride and concentrated ammonium hydroxide yielded a brownish solid; however, when some of the characteristic amide tests were applied to this material, the results were negative. An attempt to form the silver salt by adding silver nitrate or silver acetate to an aqueous solution of the MAPBAcid was not successful. (In a later study, the silver salt was prepared by the method described by Anker (41). However, as will be discussed, considering the difficulties encountered with the silver salt of a derivative of MAPBAcid, the author believes the silver salt of MAPBAcid to be of doubtful value as a degradative intermediate.)

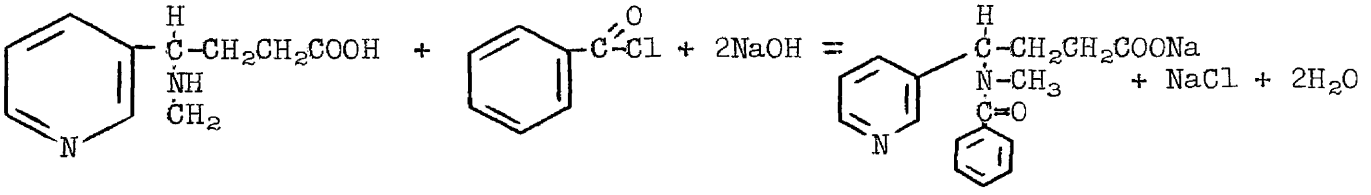
The preparation of the barium salt was then attempted, since aliphatic acids are frequently decarboxylated by heating barium or

calcium salts. Seventy mg. of the MAPBAcid was heated with a slight excess of $Ba(OH)_2.8H_2O$, under an atmosphere of nitrogen. The hydrate decomposed at 78°, dissolving the MAPBAcid. The temperature was held at about 80° until the water had evaporated, then raised slowly to about 250°. After acidifying the residue and sweeping nitrogen through the flask into barium hydroxide, only a trace of barium carbonate was observed. A blank sample of the barium hydroxide hydrate was treated in the same manner; about an equal amount of carbonate was formed. Abderhalden and Kantzsch (42) have reported that glutamic acid is not decarboxylated by heating its calcium salt; rather, a lactam is formed at 180-185°. Possibly cyclization also occurs upon heating the barium salt of MAPBAcid. McKennis et al. (40) have observed the cyclization of the MAPBAcid side chain in acidic or neutral aqueous solutions, whereby cotinine is reformed. Since most of the conventional methods of decarboxylation involve acidic or neutral conditions, it seemed very desirable to consider methods of blocking the methylamino grouping of the MAPBAcid before attempting a decarboxylation. Some of the common reagents employed to this end--acetyl chloride, benzoyl chloride, and 3,5-dinitrobenzoyl chloride--were tested briefly, but no derivatives were isolated. However, since the compound MAPBAcid possesses a carboxyl and a pyridyl grouping, its derivative would remain in ionized form under acidic, basic, or neutral conditions; hence an acetylated or benzoylated derivative is not readily separated by an adjustment of pH. Nevertheless, since the Schotten-Baumann reaction has been a successful means of benzoylating primary and secondary amino groups, it was

considered quite probable that a derivative could be formed with MAPBAcid by this method. Furthermore, during the preliminary tests with benzoyl chloride, a striking difference in the colors of the solutions was noted when MAPBAcid was compared with a cotinine or water blank. Hence it was decided to study this reaction further and attempt to isolate the benzoylated derivative.

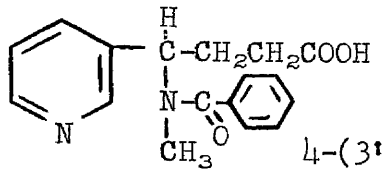
Isolation and Identification of 4-(3'-pyridyl)-4-N-methylbenzoylamino-butyric Acid

The Schotten-Baumann reaction, commonly carried out in 10 per cent sodium hydroxide, frequently involves the reaction of benzoyl chloride with a primary or secondary amino grouping. If the MAPBAcid would undergo this reaction, it might be depicted as shown:



An extensive series of experiments were carried out in an effort to isolate the benzoylated derivative. Since benzoic acid is a product of the hydrolysis of excess benzoyl chloride, the benzoic acid was usually removed by filtration, after adjusting the pH to about 3.5. In one experiment, after removal of benzoic acid, a blue precipitate was formed when a solution of copper acetate was added. Assuming the material to be a copper salt, it was slurried in water, treated with hydrogen sulfide, and thereby decomposed. Upon removal of the water, a white solid remained which appeared to be organic in nature, melted at about 170°, and gave an acidic reaction when it was dissolved in

water and tested with litmus. This product was later identified as 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid which has been abbreviated to N-benzoyl MAPBAcid and is indicated as structure III.



4-(3'-pyridyl)-4-N-methylbenzoylamino-butyric acid

(III) (N-benzoyl MAPBAcid)

However the yield of the above experiment was very low. In subsequent experiments, the proportions of reagents used and strength of the sodium hydroxide were varied in attempts to improve yields. Later it was discovered that, probably due to the presence of the benzoyl grouping, the white product can be crystallized from water. The method which was eventually adopted to obtain the N-benzoylated compound is outlined below; the procedure of Steiger (43) was followed in the initial steps of the benzoylation.

Six hundred mg. (about 3 m moles) of MAPBAcid was dissolved in 3 ml. of 1N sodium hydroxide in an 8-inch test tube. A magnetic stirring-bar was placed in the tube, which was placed in an ice bath. Four ml. of 1N sodium hydroxide and 0.4 ml. of benzoyl chloride were introduced dropwise into the solution over a period of 15 to 20 minutes, during which time the solution was stirred vigorously. The solution at this stage developed a bright red color. The stirring-bar was then removed, the test tube was stoppered, and placed on a shaker. The tube was shaken in an ice bath which was permitted to return to room temperature.

After the benzoyl chloride has dissolved and its odor disappeared (usually 2 to 3 hours, occasionally longer), the sample was removed from the shaker. The red color had faded at this stage to a yellow-brown. The solution was transferred to a small beaker, placed in an ice bath, and acidified with dilute hydrochloric acid to a pH of 3.5 (a Beckman pH meter was used to measure the pH in this operation). The precipitated benzoic acid was removed by filtration through a sintered glass filter, washed with a little cold water, and the washings added to the filtrate. The filtrate was then made alkaline with dilute ammonium hydroxide and concentrated to dryness under reduced pressure, at 40-50° C. Extraction of the residue with cold n-butanol separated the benzoylated product from the bulk of inorganic salts present. The salts were removed by filtration and the butanol extract was concentrated to dryness with a flash evaporator at 50-60°C. A few ml. of water were added to this residue to facilitate removal of the remaining butanol as an azeotrope. The yellowish residue was dissolved in methanol and filtered with a sintered glass filter into a 50-ml. suction flask containing 5 to 10 ml. of water. A stream of air was drawn through the filter into the flask and the flask was warmed to about 50 to 60° C. to facilitate removal of the methanol. When the flask was permitted to cool to room temperature, the solution clouded; with refrigeration, a white crystalline product separated. Since a trace of benzoic acid may be present in the product, it was purified by recrystallization from water.

The N-benzoyl MAPBAcid crystallized as white needles in about 20-30 per cent yield; the melting point was 170°. Anal. Per cent calculated

for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.40; per cent found: C, 68.42, 68.45; H, 6.09, 6.14; N, 9.38, 9.31. Unlike the MAPBAcid, the benzoylated compound was not hygroscopic, and did not appear to decompose when a sample was heated on a steam bath at a pH of 2. No change in color was observed when this compound was exposed to cyanogen bromide vapor. From an inspection of the structures of various compounds which give the positive red color with cyanogen bromide (44) including MAPBAcid, it appears that a primary amino or methylamino grouping is commonly present. The negative test with the N-benzoyl MAPBAcid may be explained by the fact that the N-methyl grouping of MAPBAcid has been altered through benzoylation. Further evidence that the crystalline product is represented by structure III was obtained by determination of a neutralization equivalent. After a quantity of the product was dried in a vacuum desiccator, 0.1211 g. was dissolved in 50 ml. of 85 per cent ethanol. The remaining steps of the determination were carried out in essentially the same manner as has been described for the determination of the neutralization equivalent of MAPB Acid. The neutralization equivalent was calculated on the basis of the data obtained. Theoretical N.E, 298; Experimentally determined N.E., 300. The plot of pH vs. ml. sodium hydroxide indicates that the N-benzoylMAPBAcid behaves as a typical weak acid, with a pK value of approximately 6.6. The compound appears to be quite soluble in methanol, ethanol, and chloroform; difficultly soluble in water; almost insoluble in ether and carbon tetrachloride.

Some Attempted Reactions with 4-(3'-pyridyl)-4-N-methylbenzoylamino-butyric Acid

Decarboxylation of the N-benzoylated MAPBAcid was attempted initially by means of the Hunsdiecker reaction. This method involves treatment of the silver salt of a carboxylic acid with bromine in an organic reagent, such as carbon tetrachloride. The general reaction is shown:

$$R\text{-}CH_2\text{-}COOAg + Br_2 \xrightarrow{CCl_4} R\text{-}CH_2\text{-}Br + CO_2 + AgBr$$

Since the decarboxylation apparatus and reagents must be moisture-free, bromine was shaken with concentrated sulfuric acid and carbon tetrachloride was dried over phosphorus pentachloride before the experiments were begun. All glassware was oven-dried at 110° C.

The silver salt of the N-benzoyl MAPBAcid was prepared from silver nitrate and ammonium hydroxide, as described by Anker (41). When the silver salt was dried in a vacuum oven at 110°, as has been recommended with certain preparations by the Hunsdiecker reaction (41), the salt decomposed after a few hours. Several attempts were made to effect the drying by lyophilizing the salt, or by combining a lyophilizing with a vacuum-oven drying at 70-80° for 10 to 12 hours.

In carrying out this reaction, the evolved carbon dioxide was swept into a saturated solution of barium hydroxide by a stream of nitrogen, which had been passed through calcium chloride. Usually 150 to 300 mg. quantities of the silver salt were slurried in 10 ml. of the carbon tetrachloride with a calculated equivalent quantity of bromine. The apparatus used for these attempted decarboxylations will be described later, when the Hofmann reaction is discussed.

When the salt was dried by lyophilizing (up to 15 hours), no significant decarboxylation could be effected through the Hunsdiecker reaction. With heating, the silver salt frequently tended to decompose. Although this method of decarboxylation may be quite effective for this compound under the correct experimental conditions, it was abandoned, in view of the unpromising results, as another method was concurrently being investigated.

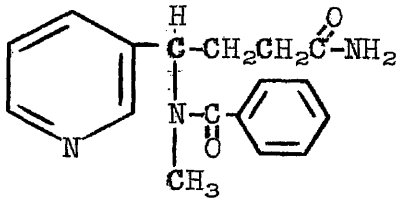
The Hofmann method of degrading an aliphatic amide to an amine may be represented by the following equation:

$$R\text{-}CH_2CO\text{-}NH_2 + Br_2 + 4NaOH = R\text{-}CH_2\text{-}NH_2 + Na_2CO_3 + 2NaBr + 2H_2O$$

The first attempts to convert N-benzoyl MAPBAcid to an amide were by conversion of the carboxylic acid to an acid chloride by means of thionyl chloride. The amide was obtained from the acid chloride by treatment of the acid chloride with concentrated ammonium hydroxide or with benzene saturated with ammonia. The general procedure was to dissolve the N-benzoyl MAPBAcid in a few ml. of dry chloroform, add an equivalent of thionyl chloride, and reflux the mixture for periods varying from 30 minutes to two hours. The chloroform was removed under reduced pressure before the ammonium hydroxide (or ammonia in benzene) was added. Although positive hydroxamate tests were obtained with hydroxylamine.HCl and ferric chloride, to indicate some amide synthesis, the yields were of very doubtful significance. No crystalline amide could be isolated, nor did preliminary attempts to decarboxylate the amide preparations yield more than traces of carbon dioxide. Hence, another method of synthesizing the amide was investigated.

Isolation and Identification of 4-(3'-pyridyl)-4-N-methylbenzoylamino-butyric Acid

Methyl esters of carboxylic acids are frequently synthesized in quantitative yields by the action of diazomethane on carboxylic acids. In the present studies, ethereal solutions of diazomethane (45) were employed to synthesize the methyl ester of the N-Benzoyl MAPBAcid. No attempts were made to purify or crystallize the ester. Following the method of Yang and Rising (46) for the conversion of alpha-amino acid esters to amides, N-Benzoyl MAPBAmide (Compound IV) was prepared by the action of ammonia on the methyl ester of N-Benzoyl MAPBAcid.



(IV) 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide

(N-Benzoyl MAPBAmide)

After several experiments, involving various proportions of reagents and the N-Benzoyl MAPBAcid, and comparing ether and chloroform as esterification media, the following general procedure was adopted to synthesize Compound IV:

One-hundred-fifty mg. of dry N-Benzoyl MAPBAcid (0.5 mmole) was transferred to a 125-ml. suction flask, placed in a hood. About 30 to 40 ml. of a solution of diazomethane in ether was then added to the flask. The diazomethane was prepared by adding 400 mg. of nitrosomethyl-urea[8] to 20 ml. of 40 per cent potassium hydroxide, covered with about

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[8]The author is indebted to Mr. Arleigh Dodson for a preparation of this compound.

25 ml. of ether, in an Erlenmeyer flask. This mixture was placed in an ice bath until the solid nitrosomethylurea had reacted. The yellow solution of diazomethane in ether was partially decanted into a beaker containing a few pellets of potassium hydroxide. Ether was added to the Erlenmeyer flask and decanted into the beaker until the ether layer in the flask was colorless; essentially all of the diazomethane had then been transferred to the beaker. After a drying period of 15 to 20 minutes, the ethereal solution of diazomethane was added to the sample of N-Benzoyl MAPBAcid, as has been mentioned.

A glass-covered magnetic stirring-bar (3/8" x 3/8") was then placed in the solution to slurry the N-Benzoyl MAPBAcid. Since the ester is soluble in ether, whereas the acid is not, the course of esterification could be conveniently followed by visual observation. Bubbles of nitrogen were evolved as esterification proceeded. Occasionally it was necessary to prepare and add more of the diazomethane solution; however, the N-Benzoyl MAPBAcid was usually dissolved within six to ten hours. At the end of this period, the ether and diazomethane were removed by drawing a stream of air through the flask by means of a water aspirator. The residue in the flask, which was usually a pale yellowish glassy material, was dissolved in about 25 ml. of methanol, which had been saturated with ammonia at 0°C. The stoppered flask was permitted to stand at room temperature, with occasional shaking, for about 100 hours. The methanol was then removed under reduced pressure, at 30-40°C., and the residue was dissolved in a few ml. of chloroform. Charcoal was usually added to the solution at this stage. Although it was difficult

to remove all of the color, a partial decolorizing appeared to give rise to a purer product without seriously affecting yields. With no addition of charcoal, a brownish impurity was frequently precipitated with the desired product.

The charcoal was removed by filtration and the volume of chloroform was reduced by heating the solution. Ether was added to the hot solution until a white cloud had formed. With refrigeration, the N-Benzoyl MAPBAmide crystallized as white plates, which melted at 141-142° C. The average yield of the amide was about 85 per cent.

Evidence supporting structure IV was obtained by elemental analysis. Anal. Per cent calculated for $C_{17}H_{19}N_3O_2$: C, 68.65; H, 6.45; N, 14.13; per cent found: C, 68.72, 68.80; H, 6.47, 6.53; N, 14.23, 14.09. Further confirmation of amide synthesis was obtained by a ferric hydroxamate test. The characteristic wine-red color developed after a few minutes.

Decarboxylation

The Hofmann degradation of the N-Benzoyl MAPBAmide was carried out in a three-necked, 125-ml. pear-shaped flask. A 1-ml. pipette had been sealed to a 24/40 joint and cut to such length that, when fitted into the flask, it extended to about 4 cm. from the base of the flask. The joint was also connected by ground glass fittings to a small tube of ascarite, which was, in turn, joined to a tank of nitrogen by tygon tubing. A glass piece, containing a stopcock, was fitted by a ground glass joint to a second neck of the flask; the piece was also connected

by tygon tubing to a small funnel. Glass fittings led from the third neck of the flask to a saturated solution of barium hydroxide. Hence, a slow stream of carbon dioxide-free nitrogen could be swept through the flask into the barium hydroxide to trap evolved carbon dioxide.

A carbonate-free solution of sodium hydroxide (approximately 0.25 N) was prepared by adding 20 ml. of 0.5 N barium chloride to 500 ml. of 0.5 N sodium hydroxide and diluting the resulting solution to one liter. After the barium carbonate had settled, the supernatant was decanted into a liter bottle, which was stoppered with a siphon assembly connected to a tube of carbon dioxide absorbent.

The general procedure employed in the decarboxylation (47) is outlined below.

One hundred fifty mg. (0.5 m mole) of N-Benzoyl MAPBAmide was transferred to the pear-shaped flask. The flask, connected to the decarboxylation assembly, was clamped into position in a small beaker of ice-water, which had been placed on a magnetic stirring base. A glass-covered magnetic stirring-bar was placed into the flask; another magnetic stirring-bar and a thermometer were placed into the beaker. The system was then flushed with nitrogen (which is not passed through barium hydroxide at this stage). A sodium hypobromite solution was prepared by dissolving 0.025-0.030 ml. of bromine (approximately 0.6 m mole) in 12 ml. of the carbonate-free sodium hydroxide, in an ice bath. The clear yellow solution of sodium hypobromite was introduced into the flask by pipette, as rapidly as possible, to minimize the transfer of atmospheric carbon

dioxide into the system. The stirring was then begun, to dissolve the solid N-Benzoyl MAPBAmide in the sodium hypobromite. After another brief period of flushing, the nitrogen was directed through the solution of barium hydroxide. When no barium carbonate formation was evident in the barium hydroxide, the beaker of ice-water was warmed with a microburner. When the temperature rose to 50-60° C., the clear yellow solution began to cloud due to the formation of barium carbonate (from the reaction of the residual barium ions in the hypobromite with the carbon dioxide of decarboxylation). The temperature was maintained at 70-80° C. for about twenty minutes to complete the reaction. The flask was then permitted to cool to room temperature and 3 ml. of 1N hydrochloric acid was introduced through the funnel to make the solution slightly acidic. A little water was used to rinse the acid into the flask; this column of water also served to prevent escape of carbon dioxide when the stopcock of the connecting glass piece was opened.

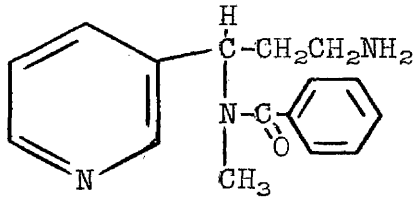
The system was flushed with nitrogen for about two hours. At the end of this period, the barium carbonate was collected on a 2.1 cm. filter paper (Whatman 540), using a stainless steel filtering apparatus. The barium carbonate was washed with a little water and dried to a vacuum desiccator (or in an oven at 110°, if desirable). Yields of barium carbonate ranged from 40 to 60 per cent.

Isolation and Identification of the Amine Derivative

The acidified medium, remaining in the flask after the decarboxylation, may contain some unreacted N-Benzoyl MAPBAmide with the presumed

product of the reaction, 3-(3'-pyridyl)-3-N-methylbenzoylaminopropylamine designated by the abbreviation N-benzoyl MAPPAmine. Some of the N-Benzoyl MAPBAcid may also be present, due to hydrolysis of the amide. It seemed desirable to isolate the amine from the reaction mixture, since identification of the amine would serve as evidence that the desired carbon atom had been separated from the amide. Furthermore, in later degradations of radioactive materials, the radioactivity of the N-benzoyl MAPPAmine plus that of the isolated carbon atom should total the radioactivity of the N-Benzoyl MAPBAmide.

Separation of the amine, (indicated by structure V) from the other constituents of the reaction mixture was accomplished by preparing a thiourea derivative of the amine.



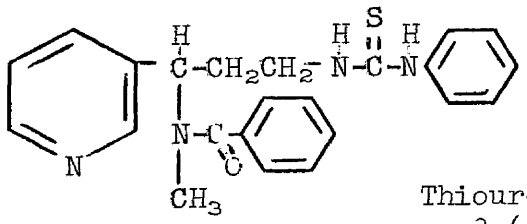
(V)

3-(3'-pyridyl)-3-N-methylbenzoylaminopropylamine
(N-benzoyl MAPPAmine)

The general procedure that has been followed is described below:
The acidified solution remaining in the pear-shaped flask was made basic to litmus with 1N sodium hydroxide, the solution was transferred to a separatory funnel, and extracted with chloroform until the extracts gave no positive tests with the silicotungstic acid solution. (In the experiments performed, this chloroform extract has given basic tests with litmus). The chloroform was removed under reduced pressure, and the residue was dissolved in 2 or 3 ml. of methanol. Then 0.06 ml. of

phenyl isothiocyanate was added and the mixture refluxed gently for about 10 minutes. Water was added dropwise to the hot solution until a white cloud formed. Upon cooling, shiny white plates of the amine derivative appeared. These were recrystallized from water and methanol, washed with water, and dried in a desiccator. M.p., 197-198°.

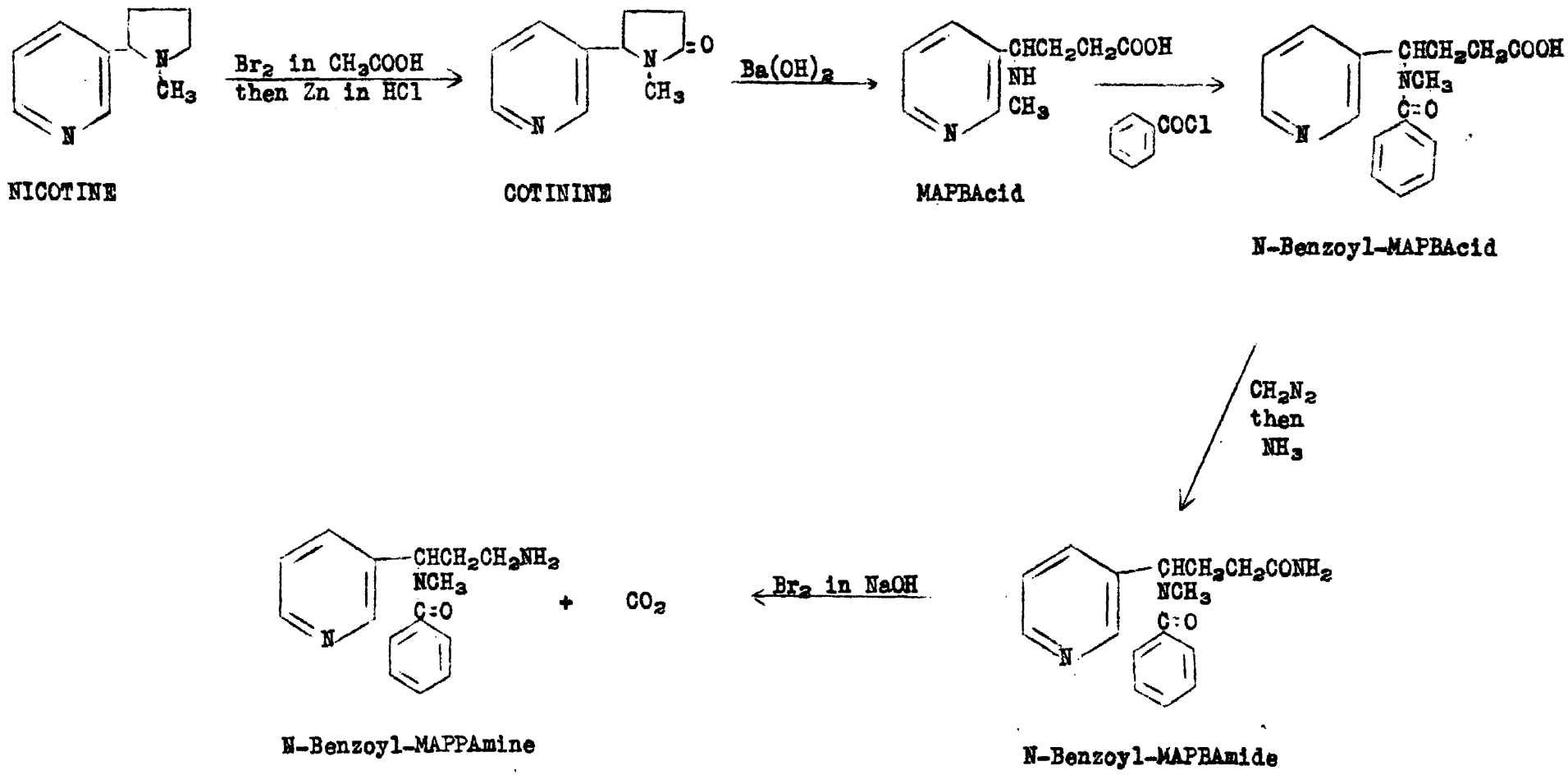
The constitution of the derivative, structure VI, was supported by elemental analysis. Anal. Per cent calculated for $C_{23}H_{24}N_4OS$: C, 68.29; H, 5.98; N, 13.85; per cent found: C, 68.42; H, 6.08; N, 13.97. The derivative was also tested qualitatively for the presence of sulfur. A sodium fusion was carried out on a few mg. of the material, dissolved in methanol. When the fusion mixture was filtered and the filtrate tested with 0.1 per cent sodium nitroprusside and a solution of lead acetate, the positive results gave evidence of the presence of sulfur in the derivative.



Thiourea derivative of
3-(3'-pyridyl)-3-N-methylbenzoylpropylamine

(VI)

It should be noted that, although the N-benzoyl MAPPAmine has not been identified as such and crystallized, this compound could serve as a useful intermediate if further degradative studies should be desirable. The presence of the amino grouping should facilitate attempts to shorten the sidechain by one carbon atom. Hence it is conceivable that each



NICOTINE

COTININE

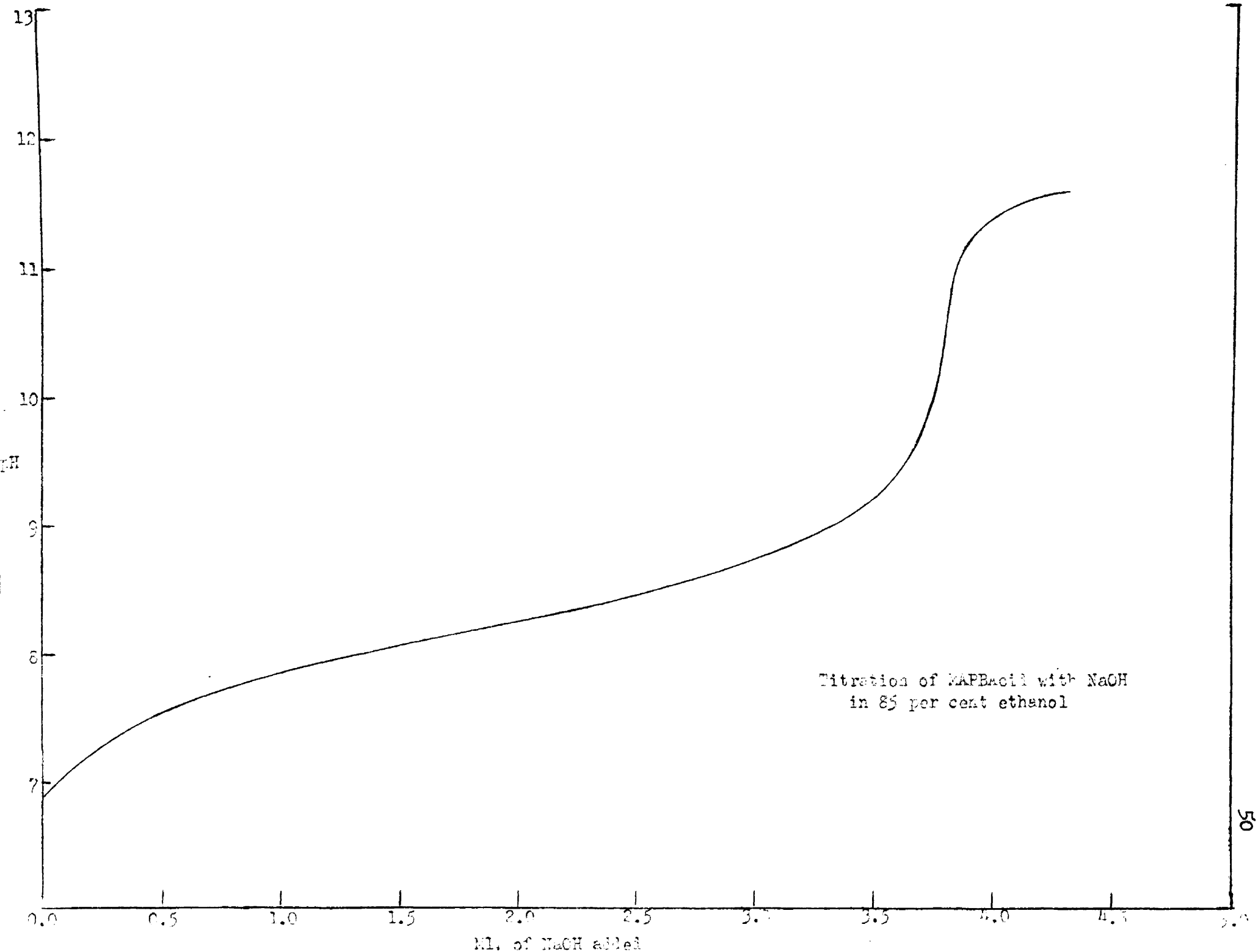
MAPBAcid

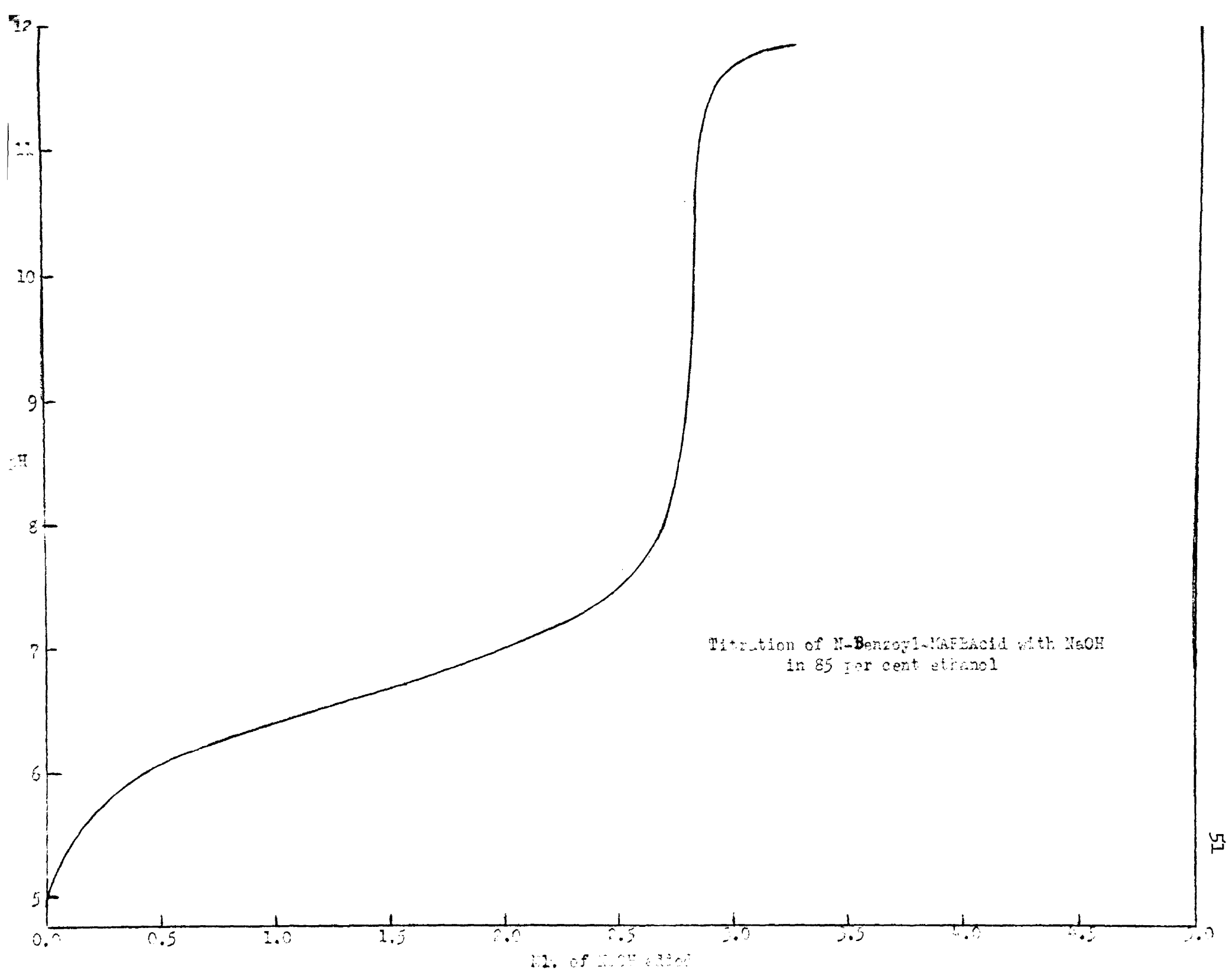
N-Benzoyl-MAPBAcid

N-Benzoyl-MAPPAmine

N-Benzoyl-MAPBAmide

DEGRADATION OF NICOTINE WITH ISOLATION OF CARBON FROM POSITION 5 OF PYRROLIDINE RING


13
12
11
10
9
8
7
pH
0.0
0.5
1.0
1.5
2.0
2.5
3.5
4.0
4.5
Ml. of NaOH added
Titration of NAPBAcid with NaOH
in 85 per cent ethanol



Titration of N-Benzoyl-MAFBAcid with NaOH
in 85 per cent ethanol

carbon atom in the pyrrolidine ring of the nicotine molecule may be recovered separately.

Degradation of Nicotine Labelled by Glutamic Acid-2-C^{14}

The radioactive nicotine, isolated from the tobacco plants which had received glutamic acid-2-C^{14}, yielded a total of 3.8 g. of nicotine dipicrate. For the purpose of degradation, this sample was mixed with 10.2 g. of non-radioactive nicotine dipicrate and the material was ground in a mortar. The nicotine dipicrate (equivalent to 3.66 g. of nicotine) was mixed thoroughly in a 250-ml. round-bottomed flask; three samples were then removed, plated, and counted. Since the sample counts agreed within experimental error, sodium hydroxide was added to the flask and the nicotine was recovered by an azeotropic distillation with water through a Widmer column. After about 250 ml. of distillate had been collected in a beaker containing 15 ml. of 6 N hydrochloric acid, and the distillate showed no further traces of nicotine, the solution was concentrated to dryness on a flash evaporator. The resulting nicotine hydrochloride was dissolved in 10 ml. of water and 38 ml. of glacial acetic acid was added. The perbromide was formed by the addition of 8 ml. of bromine in 20 ml. of 80 per cent acetic acid to the nicotine solution. Seventy-five ml. of hot water dissolved the oily perbromide; upon cooling, the characteristic orange-red crystals were formed.

The perbromide was covered with 45 ml. of 2N hydrochloric acid and reduced with zinc dust. To preserve the radioactive material as much as possible, the zinc was not removed as the hydroxide; rather, the sample

was made strongly alkaline, so that the hydroxide, initially precipitated, was redissolved. The crude cotinine was separated by extraction with chloroform. Because of the small quantity of radioactive material, a special distillation apparatus was constructed by joining two 10-ml. distilling flasks (39). After the crude cotinine was transferred to this apparatus, and the system had been evacuated by a vacuum pump, the flask containing the crude material was lowered into a Wood's metal[9] bath, which was heated with a microburner until the cotinine was distilled. (Under these conditions, cotinine distilled at a bath temperature of 170-175° C.) The cotinine (2.0 g.) was transferred to a 100-ml. round-bottomed flask, to which was added 15 ml. of water and 10 g. of $Ba(OH)_2 \cdot 8H_2O$, and the mixture was refluxed for twelve hours. The MAPBAcid (0.465 g.; m.p., 120-121° C.), obtained in the manner previously described, was plated and counted.

The MAPBAcid (0.450 g.) was benzoylated, as has been described. The product was recrystallized from water and samples of this N-Benzoyl MAPBAcid (0.163 g.; m.p., 169-170° C.) were plated and counted.

The N-Benzoyl MAPBAcid was then esterified with diazomethane, and the ester was permitted to stand for 130 hours under 20 ml. of methanol saturated at 0° with ammonia. The N-Benzoyl MAPBAmide (0.142 g.; m.p., 141-142° C.) was crystallized from chloroform and ether. After several samples were plated and counted, the amide was decarboxylated and 0.056 g. of barium carbonate was isolated, in the manner previously described. Samples of the barium carbonate were plated and counted.

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[9]An alloy of bismuth, lead, cadmium, and tin.

The amine derivative was isolated and purified (0.078 g.; m.p., 197-198° C.). Samples of the derivative were plated and counted.

Results

The data obtained upon counting the various samples are presented in Table IV.

TABLE IV

ISOLATION OF CARBON 5 OF THE PYRROLIDINE RING OF NICOTINE AFTER THE ADMINISTRATION OF GLUTAMIC ACID-2-C^{14}

Compound	Maximum Specific Activity (cpm/mM x 10^{-3})
1) Nicotine dipicrate	6.7
2) MAPBAcid	6.4
3) N-Benzoyl MAPBAcid	6.3
4) N-Benzoyl MAPBAmide	6.4
5) Barium carbonate	3.1
6) N-Benzoyl MAPPAmine derivative	2.9

The specific activities of the intermediates in the degradation procedure, of lines 2, 3, and 4, are approximately equal to the specific activity of the nicotine dipicrate. Hence, essentially all of the original radioactivity was present in the amide (line 4). Upon degradation of the amide, one carbon atom was separated from the molecule, and was isolated as barium carbonate (line 5). This carbon atom--originally carbon 5 of the pyrrolidine ring of nicotine--contained approximately half of the radioactivity of the amide. The amine derivative (line 6), containing the residual carbon skeleton of the amide, possessed a

specific activity equal to that found with the barium carbonate. From the previous study of nicotine degradation by potassium permanganate, it is evident that the greater part of this radioactivity of the amine is associated with the carbon atom joining the pyridine ring and the sidechain (i.e., the 2 carbon of the pyrrolidine ring).

Degradation of Nicotine Labelled by Ornithine-2-C^{14}

The series of degradations to be discussed was initiated on 2.06 g. of dibromocotinine (3,5-dibromo-5-(3'-pyridyl)-N-methyl-2-pyrrolidone). The radioactive nicotine from which the dibromocotine[10] was synthesized was isolated from tobacco plants (var. N. rustica), to which ornithine-2-C^{14} had been administered hydroponically.

Samples of the original nicotine dipicrate were plated and counted. The dibromocotinine (33) (which may be obtained from the previously discussed perbromide by a sulfurous acid reduction) was covered with 10 ml. of 6N hydrochloric acid and reduced with zinc dust. The subsequent steps, resulting in the isolation of crude cotinine, were similar to those carried out with the radioactive glutamic and have been described previously. The crude cotinine was distilled with the apparatus discussed in the glutamic acid-2-C^{14} studies. The distillate (0.6 g.) was transferred to a 50-ml. round-bottomed flask to which 5 ml. of water and 5 g.

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[10]This sample was kindly furnished by Dr. L. J. Dewey.

of $Ba(OH)_2 \cdot 8H_2O$ were added. After this mixture was refluxed for 12 hours, the flask was permitted to cool and the MAPBAcid (0.350 g.; m.p., 121° C.) was isolated. Samples of the MAPBAcid were plated and counted.

The benzoylation was carried out on 0.330 g. of this preparation, dissolved in 1.7 ml. of 1N sodium hydroxide. Another 2 ml. of the 1N sodium hydroxide and 0.2 ml. of benzoyl chloride were added in the manner previously discussed; the recrystallized N-Benzoyl MAPBAcid (0.100 g.; m.p., 169-170° C.) was plated and counted. The 0.100 g. sample was esterified with diazomethane and the product was dissolved in 20 ml. of methanol, saturated with ammonia at 0°. After 100 hours, during which period the solution was shaken occasionally, the N-Benzoyl MAPBAmide (0.084 g.; m.p., 141-142° C.) was isolated; samples of this preparation were plated and counted.

Unfortunately the 84 mg. of the amide preparation seemed to be virtually the lower limit of quantity necessary for conducting a decarboxylation--at least, under the selected experimental conditions. However, it was decided to carry out the decarboxylation without diluting the amide preparation with non-radioactive carrier.

Using calculated quantities of reagents, the decarboxylation was effected and the carbon dioxide was separated as barium carbonate. However, only 15 mg. of barium carbonate were collected. This material was plated and counted. Because of the difficulties of counting small quantities, an 8.8 mg. sample of the barium carbonate was diluted with two parts of non-radioactive barium carbonate and the mixture was plated

and counted. The calculated specific activity of the original material, based on the count of the diluted sample, was not significantly different from the values obtained by counting the undiluted barium carbonate.

The amino derivative (0.027 g.; m.p., 194-195° C.) was isolated and recrystallized; samples were plated and counted.

Results

The data obtained upon counting the samples is shown in Table V.

TABLE V

ISOLATION OF CARBON 5 OF THE PYRROLIDINE RING OF NICOTINE AFTER THE ADMINISTRATION OF ORNITHINE-2-C^{14}

Compound	Maximum Specific Activity (cpm/mM x 10^{-3})
1) Nicotine dipicrate	8.1
2) MAPBAcid	8.2
3) N-Benzoyl MAPBAcid	8.3
4) N-Benzoyl MAPBAmide	8.1
5) Barium carbonate	3.0
6) N-Benzoyl MAPPAmine	2.9

A comparison of Tables IV and V reveals a striking similarity in the patterns of nicotine labelling by glutamic acid-2-C^{14} and ornithine-2-C^{14}. The intermediates of degradation of lines 2, 3, and 4, had the same radioactivity as the nicotine dipicrate of line 1. The barium carbonate of line 5--derived from carbon 5 of the pyrrolidine ring of nicotine--was radioactive, although this radioactivity was somewhat

less than half of the radioactivity found with the N-BenzoylMAPBAmide. However, the amine derivative (line 6) possessed approximately the same amount of radioactivity as the barium carbonate.

The reason for the rather low specific activities of the barium carbonate and the amine derivative has not been immediately apparent, although it is probably associated with the difficulty of handling and counting these small samples. It is possible that the radioactive barium carbonate was diluted by a small quantity of non-radioactive carbonate, originating during the isolation of the radioactive carbonate, from the contact of atmospheric carbon dioxide with the excess barium hydroxide being removed.

However, the studies of Dewey and Byerrum (19) and of Leete (20), with ornithine-2-C^{14}, (discussed in the Introductory portion of this thesis) have established that half of the radioactivity of nicotine labelled by ornithine-2-C^{14} is located at carbon 2 of the pyrrolidine ring. In another study with ornithine-2-C^{14}, Leete (48) oxidized the radioactive nicotine with nitric acid. A nitropyrazole, obtained as a by-product of nitric acid oxidation, possessed about half of the radioactivity of the nicotine. Since carbon 5 of the pyrrolidine ring is presumably expelled during formation of this nitropyrazole, this finding lends support to the data of Table V (i.e., that carbon 5 is radioactive, and possesses a specific activity equal to carbon 2 of the pyrrolidine ring).

Hence it is concluded, from these considerations and from the data presented in Tables IV and V, that the pyrrolidine ring of nicotine is

labelled in essentially the same manner by ornithine-2-C^{14} and glutamic acid-2-C^{14}.

DISCUSSION

DISCUSSION

From the preceding study, it is evident that the pyrrolidine ring of nicotine is labelled in essentially the same manner by glutamic acid-2-C^{14} and ornithine-2-C^{14}. The radioactivity of the pyrrolidine ring was found to be distributed equally between positions 2 and 5, when either glutamic acid-2-C^{14} or ornithine-2-C^{14} was employed as a precursor.

The studies on nicotine biogenesis of Dewey (21) and Leete (20) with ornithine-2-C^{14}, previously discussed, led to the postulation of a symmetrical intermediate in the pathway of biosynthesis of the pyrrolidine ring. This intermediate, which is evidently derived from the intact carbon chain of ornithine, has given rise to a certain amount of speculation, regarding its structure and manner of formation.

One consideration suggested that the intermediate might be putrescine, a four-carbon symmetrical diamine, which could arise directly from the decarboxylation of ornithine. Another possibility is the conversion of ornithine to glutamic-γ-semialdehyde by transamination or oxidative deamination. The semialdehyde could be oxidized to glutamic acid, which, by loss of the amino grouping, would give rise to alpha-keto glutaric acid. Decarboxylation of the latter compound, in the tricarboxylic acid cycle, would yield another symmetrical compound, succinic acid. The succinic acid could also conceivably result from the oxidation of succindialdehyde, which could arise from putrescine, through oxidative deamination.

Another pathway to a possible symmetrical intermediate involved cyclization of the semialdehyde to give Δ^1-pyrroline-5-carboxylic acid. Decarboxylation of this compound would produce pyrroline, which could become symmetrical by a shifting of the double bond. Also, the symmetrical compound pyrrolidine results from the decarboxylation of proline, which could arise from the reduction of Δ^1-pyrroline-5-carboxylic acid.

Although putrescine is generally associated with the metabolism of microorganisms, certain evidence suggests that it may also play a role in the metabolism of higher plants. Cromwell (49) reported the preparation of an enzyme system from A. belladonna, capable of oxidizing putrescine to ammonia and an aldehyde. Furthermore, he isolated small quantities of putrescine from the leaves and upper stems of A. belladonna and D. stramonium. When putrescine was injected into D. stramonium L., Cromwell (50) found an increased synthesis of hyoscyamine. On the other hand, Diaper, Kirkwood, and Marion (51) have reported that the hyoscyamine isolated from D. stramonium L., following administration of 1,4-C^{14} putrescine, was not radioactive. However, as has been mentioned earlier, Leete, Spenser, and Marion (18) demonstrated that ornithine-2-C^{14} is incorporated into the pyrrolidine ring of hyoscyamine. On the basis of these radioactive studies, the immediate precursor of the pyrrolidine ring of hyoscyamine would appear to be more closely related to ornithine than to putrescine.

Leete (52) has recently found putrescine to be a precursor of the pyrrolidine ring of nicotine; however, the efficiency of putrescine in this respect, as compared to ornithine, has not been reported.

From the studies of Cromwell (50) with hyoscyamine, it is conceivable that, in the biosynthesis of nicotine, putrescine may be oxidatively deaminated to γ-aminobutyraldehyde. This compound might then either cyclize to pyrroline or possibly condense with the precursor of the pyridine molecule before cyclizing. If, however, putrescine were converted to succindialdehyde through oxidative deamination at both ends of the molecule, condensation of the succindialdehyde with ammonia would yield the symmetrical compound 2,5-dihydroxy pyrrolidine. The pyrrolidine ring might then be formed by loss of water from this intermediate, followed by a reduction. Hence, it is conceivable that some synthesis of the pyrrolidine ring of nicotine may be effected by way of putrescine, although this pathway is probably of minor significance.

Succinic acid has also been mentioned as a possible symmetrical intermediate in the formation of the pyrrolidine ring of nicotine. Reduction of the succinic acid would yield succindialdehyde, which has been mentioned above. If a condensation of the succinic acid and ammonia were to occur, 2,5-diketopyrrolidone would be formed, which might possibly be considered a precursor of the pyrrolidine ring. Presumably glutamic acid could be incorporated into the pyrrolidine ring either by way of succinate or by way of ornithine and putrescine.

However, it has recently been reported (38) that carbon-14-labelled succinate was administered to tobacco plants. When the nicotine was isolated, no significant radioactivity was found to be associated with the pyrrolidine ring. Hence, it would appear that little, if any, pyrrolidine ring synthesis occurs by this pathway. The present study

would seem to confirm this finding. From Table I, it is seen that ornithine is incorporated into the pyrrolidine ring to a far greater extent than is glutamic acid. If succinate were an important intermediate in the biosynthesis of nicotine, the opposite result should have been found, since ornithine may be converted to succinic acid by way of glutamic acid.

Another possible pathway of pyrrolidine ring biosynthesis involves the intermediates glutamic-γ-semialdehyde and Δ^1-pyrroline-5-carboxylic acid, from which the symmetrical intermediates pyrroline or pyrrolidine might arise. It should be mentioned, in this connection, that pyrroline and pyrrolidine may also conceivably be formed from ornithine by way of Δ^5-pyrroline-5-carboxylic acid. Loss of the alpha-amino grouping of ornithine through oxidative deamination or transamination would yield alpha-keto delta-amino valeric acid, which, by cyclizing, would give the Δ^5-pyrroline-5-carboxylic acid. Glutamic acid could presumably form from the α-keto glutaric semialdehyde and α-keto glutaric acid. Some evidence for this sequence has been obtained from studies in vitro with animal tissue preparations (53).

However, the bulk of evidence shows that the former pathway is more important in animal systems and microorganisms--that is, by way of glutamic-semialdehyde and Δ^1-pyrroline-5-carboxylic acid. Stetten (54), for example, conducted comparable experiments in which two samples of ornithine, labelled in either the alpha or delta position with N^{15}, were fed to mice. Following the isolation of glutamic acid and proline from the animal proteins, it was apparent that the major loss of N^{15} had

been from the delta position of ornithine, which indicated that glutamic semialdehyde is the likely intermediate in this conversion of ornithine to glutamic acid or proline.

Other studies of E. coli, N. crassa, and T. utilis, have shown that the principal pathway in the conversion of glutamate to proline by these organisms is by glutamic-γ- semialdehyde and Δ^1-pyrroline-5-carboxylate (5). This finding has been established by isotopic competition experiments and by the administration of glutamic semialdehyde, prepared synthetically, to mutant strains of these microorganisms.

The present study suggests that one or more symmetrical intermediates are involved with the incorporation of glutamic acid or ornithine into the pyrrolidine ring of nicotine. This does not necessarily imply the function of a common intermediate for glutamic acid and ornithine, although, on the basis of the previous discussion, such a conclusion would seem reasonable. There is little information extant, regarding the glutamate-ornithine-proline relationship in higher plants, as was noted earlier. If, however, ornithine and glutamic acid give rise to glutamic semialdehyde in the tobacco plant, as would be plausible on the basis of animal and microorganism studies, two symmetrical intermediates could result--namely, pyrroline and pyrrolidine. At present, there is no conclusive evidence favoring one or more of these or other intermediates in the synthesis of the pyrrolidine ring of nicotine. The biosynthesis of the pyridine ring of nicotine has never been elucidated, although certain evidence (28,29,30) appears to link the immediate precursor of this ring to intermediates of glycolysis or the

tricarboxylic acid cycle. Furthermore, Dawson (55), in his studies of nicotine, has reported some incorporation of radioactive nicotinic acid into the pyridine ring.

Hence, in view of the uncertainty regarding the biosynthesis of this ring, the manner of condensation of the two rings of nicotine has never been explained. The precursor of the pyrrolidine ring may or may not cyclize before a condensation with the pyridine ring precursor. It is possible that the precursors of these rings may be linked before either ring has formed. However, such considerations are merely speculative at present and must necessarily await the evidence of further studies.

Present knowledge indicates that the carbon skeleton of the pyrrolidine ring probably originates with the carbon chain of glutamic acid. Calvin (56) has observed, that $C^{14}O_2$ is rapidly incorporated into glutamic acid, during the process of photosynthesis. Following the fixation of a molecule of $C^{14}O_2$ into the initial acceptor, ribulose diphosphate, the atom of carbon-14 may be traced through the intermediates of glycolysis into the tricarboxylic acid cycle, to alpha-keto glutaric acid. Glutamic acid is formed from alpha-ketoglutaric acid by reductive amination, in quantities that are contingent on the immediate requirements of the plant. This reaction is catalyzed reversibly by the enzyme, glutamic dehydrogenase (57), which is found in a wide variety of plant tissues (58).

Naylor and Tolbert (9) administered glutamic acid, uniformly labelled by carbon-14, to barley seedlings; then, after periods of

one-half to three hours, methanol-water extracts of the plant material were chromatographed. The greater part of the radioactivity of these extracts was found with glutamine, γ-amino butyric acid, and aspartic acid; no significant evidence of radioactive arginine, ornithine, or proline was reported.

However, current knowledge of living systems limits the source of the carbon chain of ornithine or proline to glutamic acid. In a study of E. coli, for example, Abelson (5) has found, by isotopic competition experiments, that glutamic acid is the precursor of ornithine and proline in this microorganism. It seems very likely that a similar relation obtains among these amino acids in higher plants, although little experimental evidence for this is available.

The experimental studies, mentioned above, suggest that the reservoirs of free ornithine or proline in the higher plants are probably small relative to the reservoir of glutamic acid. Commoner and Varda (59), and Roberts and Wood (60) have studied the free amino acids in tobacco leaves. Glutamic acid was found in significant quantities. Proline appeared to be present to a lesser extent, but no ornithine was reported, although traces of arginine were noted. If ornithine is synthesized from glutamic acid by way of glutamic-γ-semialdehyde, the reduction of the glutamic acid to the semialdehyde may be the rate-determining step. The experiments of Abelson (5) with E. coli would lend some support to this possibility in the bacterial system.

The mechanism of the reduction of glutamic acid to the semialdehyde is not known. However, Black and Wright (61) have reported

that aspartic acid forms B-semialdehyde by way of B-aspartyl phosphate in cell-free extracts of baker's yeast. By analogy, it is possible that γ-glutamyl phosphate is an intermediate in this conversion.

From the present study, it is evident that, when ornithine-2-C^{14} and glutamic acid-2-C^{14} are administered to tobacco plants in comparable experiments, the nicotine from the ornithine-fed plants is considerably more radioactive. These results may be explained if it is assumed that the conversion to the symmetrical precursor occurs more readily with ornithine than with glutamic acid, and that the reservoir of free ornithine in the plant is relatively small compared to glutamic acid, as seems very likely. The relation of proline to the immediate precursor of the pyrrolidine ring is uncertain. In his early work, Dawson (14) noted an increased synthesis of nicotine in tobacco shoots cultured in aqueous solutions of l-proline. However, a study of nicotine biogenesis with proline, labelled by carbon-14, is desirable. The capacity of proline to function as a precursor of the pyrrolidine ring might then be assessed in relation to the foregoing studies with ornithine and glutamic acid.

SUMMARY

SUMMARY

1. Tobacco plants that had been fed glutamic acid-2-C^{14} synthesized radioactive nicotine. The nicotine was considerably less radioactive than had been reported for a comparable study with ornithine-2-C^{14}.

2. A degradation of the radioactive nicotine demonstrated that about ten per cent of the carbon-14 resided with the pyridine ring. Half of the remaining radioactivity was located at position 2 of the pyrrolidine ring.

3. A procedure was developed for the degradation of the nicotine molecule which permits isolation of the carbon atom from position 5 of the pyrrolidine ring. The residual carbon skeleton of the molecule may be recovered intact as a derivative of a substituted aliphatic primary amine. The intermediate produced at each step of this degradation has been isolated and identified.

4. Nicotine labelled by glutamic acid-2-C^{14} was degraded by this procedure. Half of the carbon-14 was associated with position 5 of the pyrrolidine ring. The remaining radioactivity was found with the amine derivative. Another degradation demonstrated that ornithine-2-C^{14} effects the same pattern of C^{14}-incorporation into the pyrrolidine ring as was found in the glutamic acid study.

5. The similarity in this pattern of labelling by glutamic acid-2-C^{14} and ornithine-2-C^{14} supports the postulation of a symmetrical intermediate along the pathway of incorporation of the amino acid carbon chain into the pyrrolidine ring.

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APPENDIX

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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

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