STUDIES ON THE BIOSYNTHESIS OF NICOTINE AND LIGNIN

By

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VITA

The author was born February 17, 1927 in Kalamazoo, Michigan. He received his secondary education at the W. K. Kellogg High School near Augusta, Michigan. After graduation he served in the United States Navy for sixteen months. In September 1946 he entered Kalamazoo College and was graduated in June 1950 with a Bachelor of Arts Degree. During the summers between the school years at Kalamazoo College, he worked in the plant breeding research program at the A. M. Todd Company in Kalamazoo. In the fall of 1950 he enrolled in the Graduate School of Michigan State College as a Teaching Assistant in Chemistry and remained at that position until July 1951 when he was made a Special Graduate Research Assistant under an Atomic Energy Commission grant. He held this position during the remainder of his graduate program at Michigan State College.

ABSTRACT

Using methionine, doubly labeled in the methyl group with C^{14} and deuterium, it has been shown that the methyl group of methionine can give rise to the methyl group of nicotine through transmethylation. Also in similar experiments the methoxyl groups of the lignin of both tobacco and barley have been shown to arise from the methyl group of methionine through transmethylation. The latter transmethylation involves the transfer of methyl groups from sulfur to oxygen, a reaction which is not known to occur in animal metabolism. In addition it has been indicated that the direct transfer of the methyl group of methionine to form the methoxyl group of lignin represents a synthesis of part of the lignin molecule. From the findings of these and other studies it is concluded that transmethylation reactions are of general importance in the metabolism of higher plants.

Calcium glycolate-2-C^{14} has been shown to give rise to radioactive nicotine when administered to tobacco plants. Most if not all of the radioactivity of the nicotine was localized in the methyl group. A comparison of the rate of incorporation of the alpha carbon of glycolate into the methyl group of nicotine with rates obtained in previous methylation studies, where methionine, formate, bicarbonate, choline and glycine were administered, has been made. From the comparison the following conclusions were drawn: 1) the alpha carbon of glycolate is not converted to either formate or bicarbonate before its incorporation

into the methyl group of nicotine, 2) glycine is not converted to glycolate before the incorporation of the alpha carbon of glycine into the nicotine methyl group and 3) glycolate may first be converted to glyoxylate or the alpha carbon may first be converted to formaldehyde before entering the nicotine methyl group.

By administering ornithine-2-C^{14} hydrochloride to tobacco plants it has been shown that part of the nicotine molecule may arise from ornithine. Half of the radiocarbon of the isolated nicotine was shown to be present in the 2 position of the pyrrolidine ring and the other half somewhere in the 3, 4 and 5 positions of the pyrrolidine ring. It was postulated that the second half of the radiocarbon was located in the 5 position of the pyrrolidine ring. Therefore, the conclusion was drawn that ornithine is converted to a symmetrical intermediate before its incorporation into the pyrrolidine ring of nicotine. Less than two per cent of the radioactivity of the nicotine was found in the methyl group and none was found in the pyridine ring.

When lysine-2-C^{14} hydrochloride was administered to tobacco plants under the same conditions as were employed in the ornithine study, the nicotine isolated was considerably less radioactive. It was postulated that lysine is not incorporated directly into the nicotine molecule but is first converted to some precursor of proline or ornithine such as glutamic acid.

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In the investigation described here two distinct metabolic processes in higher plants have been studied. First, it was desired to establish whether or not transmethylation, the transfer of an intact methyl group from one compound to another, is a general metabolic reaction in higher plants. Secondly, various compounds have been tested as possible precursors of the nicotine molecule in an attempt to establish a pathway for the biosynthesis of nicotine.

Transmethylation as an important metabolic process in the higher animal has been well established by the use of isotopically labeled compounds (1) and with isolated enzyme systems (2). Although the importance of the transmethylation process has been recognized for some time in animal metabolism, no direct attempt to demonstrate this metabolic process in higher plants has been published up to the present time. Indirect evidence, however, has been presented by several investigators. Brown and Byerrum (3) have shown that the methyl group of methionine is more rapidly incorporated into the nicotine methyl group than is the carbon of formate and have suggested transmethylation as a process to account for these results. Byerrum and Wing (4) using choline, another important methyl group donor in animal metabolism, have shown that this compound is equally as effective in giving rise to the methyl group of nicotine as methionine. Studies reported by Marion and co-workers (5) show that the methionine methyl carbon enters more rapidly into the

methyl groups of the barley alkaloids, N-methyl tyramine, hordenine and gramine, and of the choline of barley than does the carbon of formate. However, choline labeled in the methyl group with carbon-14 did not give rise to radioactive hordenine indicating that choline does not serve as a methyl donor in hordenine formation. Dubeck and Kirkwood (6) have found that the methionine methyl group serves as a precursor of the O- and N-methyl groups of the castor bean alkaloid, ricinine, but that neither the methyl groups of choline nor the carbon of formate give rise to the methyl groups of this alkaloid. The failure of choline to donate methyl groups in the plants studied was attributed to the lack of an enzymic system, which would oxidize choline to betaine. The betaine appears to be the actual methyl donor at least in animal metabolism (7). Another study by Flokstra (8) has shown that the methyl group of methionine is incorporated more rapidly into the methoxyl carbon in the lignin of barley plants than is the carbon of formate. Barrenscheen and von Vályi-Nagy (9) have found that methionine and glycocyamine administered to ground wheat germ increase six- to eight-fold the synthesis of creatine. Finally, Ahmad and Karim (10) have demonstrated that methionine and creatinine stimulate the biosynthesis of choline in the seedling of the chick-pea by about 30 per cent. It has been postulated that the formation of nornicotine in the leaves of Nicotiana glutinosa at the expense of nicotine involves a transmethylation process (11). Most of the investigators of plant material mentioned above also have suggested that transmethylation is a metabolic reaction in higher plants

but final proof has been lacking previous to this study. It therefore seemed of interest to administer methionine doubly labeled in the methyl group with carbon-14 and deuterium to intact plants and determine whether or not the methyl group was transferred as an entity to form the methyl group of nicotine and the methoxyl group of lignin.

In addition to the transmethylation studies there has been considerable interest in the origin of methyl groups from sources other than the known methyl donor compounds. The formate studies have already been mentioned above. Another compound which has been studied in both animals (12) and plants (13) in this connection is glycine. Hamill demonstrated that the alpha carbon of glycine is incorporated into the methyl group of nicotine at about the same rate as the methyl group of methionine. Since the glycine alpha carbon was incorporated into nicotine more rapidly than formate, one proposal advanced to explain the methylating action of the alpha carbon of glycine was that the glycine may be hydrolytically deaminated to give glycolic acid, the alpha carbon of which could then be reduced to give methyl groups without going to formate. In studying the methylation of guanidineacetic acid Barrenscheen and Gigante found that glycolic acid had neither an activating nor inhibiting effect when glycolic acid and guanidineacetic acid were incubated with an enzyme preparation from etiolated wheat seedlings (14). Similarly, Steensholt has found that glycolic acid did not methylate guanidineacetic acid to creatine when they were incubated together with suspensions of various animal tissues or when perfused through isolated rabbit heart (15).

In view of these negative findings in isolated systems from both plants and animals on the ability of glycolic acid to serve as a methylating agent it seemed of interest to investigate the rate of incorporation of the alpha carbon of glycolic acid into the nicotine methyl group in intact plants. In addition it would be possible to compare the rates of incorporation of the alpha carbons of glycine and glycolic acid into the methyl group of nicotine. The methylating ability of glycolic acid has been studied in the present work using calcium glycolate labeled with carbon-14 in the alpha position.

An even more extensive literature than that in the methylation field has accumulated in the search for precursors of the pyridine and pyrrolidine rings of nicotine. Before the general availability of carbon-14 the studies on the biosynthesis of alkaloids involved mainly the feeding of supposed precursors to plants and then measuring the increase in alkaloid content in the treated plants over control plants. The results of these studies were often open to question either because the alkaloid increase was small or in more recent years because aerial portions of plants were employed in the original studies and Dawson (16) has shown that in the case of the tobacco alkaloid, nicotine, synthesis occurs in the roots. Klein and Linser (17) were able to show an increase in nicotine content of tobacco plants when solutions of proline and ornithine were injected into the stems of the plants. Dawson (18) has reported that l-proline, nicotinic acid, l-pyrrolidonecarboxylic acid and d-glutamic acid increased the nicotine content of the leaves of

tobacco shoots, which were cultured in aqueous solutions of these acids. However, Dawson (19) has since employed initially nicotine-free tobacco leaves and has failed to obtain a synthesis of nicotine from proline and nicotinic acid. More recently Petrosini (20) has reported that proline, glycine, 3-indole acetic acid, tryptophan, alanine, aspartic acid, glutemic acid and nicotinic acid caused the appearance of nicotine in germinating seeds of tobacco cultured in the dark.

Other studies have taken the form of speculative proposals for alkaloid biogenesis using analogies from organic reactions which were known to take place in the laboratory. Robinson's (21) scheme for alkaloid biogenesis is a good example of this approach. In his scheme for the biosynthesis of nicotine Robinson proposed that 2-hydroxy-N-methyl pyrrolidine condensed with acetone dicarboxylic acid, which supposedly would arise from citric acid. The condensation product would be decarboxylated and then reacted with two moles of formaldehyde and one mole of ammonia to form 4 keto-3-(2'-N-methyl pyrrolidine) piperidine. The latter then would be converted to nicotine by the loss of the keto group and 2 moles of hydrogen.

Later Trier (22) proposed a pathway for the origin of both the pyridine and pyrrolidine rings of nicotine from a single amino acid, proline. More recently Mortimer (23) has suggested tryptophan as a likely precursor of nicotine and has presented a mechanism for the incorporation of at least part of the tryptophan molecule into nicotine. James (24) and Dawson (19) have prepared reviews which present a more extensive survey of the studies on alkaloid biogenesis than can be given here.

Finally the recent studies by Dawson and co-workers (25) and Bowden (26) in which compounds labeled with carbon-14 were tested as possible precursors of nicotine should be mentioned. When the synthesis of nicotine in root cultures was studied with nicotinic acid-carboxyl-C^{14} as a precursor, Dawson et al. were not able to isolate radioactive nicotine indicating that the carboxyl group of nicotinic acid is unavailable for nicotine synthesis in tobacco root cultures. Similarly when DL-tryptophan labeled in the beta position of the side chain with carbon-14 was administered to tobacco plants by Bowden no radioactivity was found in the isolated nicotine. Neither of these studies, however, rules out the possibility that the pyridine ring of nicotine may arise from nicotinic acid or tryptophan.

In view of this lack of definite knowledge concerning the biosynthesis of nicotine and considering the fact that ornithine often has been suggested as a precursor of nicotine it was decided to administer both ornithine and lysine labeled with carbon-14 to intact tobacco plants and determine whether or not they are incorporated into the nicotine molecule.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

Synthesis of Labeled Precursors

DL-methionine isotopically labeled in the methyl group was synthesized by reducing DL-homocystine with sodium in liquid ammonia and treating the reduction product with labeled methyl iodide essentially according to the method of du Vigneaud, Dyer and Harmon (27) with the exception that the modified apparatus designed by Brown (28) was used in the synthesis, and the purification procedure outlined by Melville and co-workers (29) was employed. In the case of the DL-methionine labeled with deuterium in the methyl group deuterated methyl iodide was used while for the preparation of DL-methionine labeled with carbon-14 in the methyl group C^{14}-methyl iodide was employed. Both labeled samples of methyl iodide were purchased from Tracerlab, Inc., Boston. The doubly labeled methionine was then obtained by mixing the C^{14} labeled methionine with the deuterated methionine in the ratio of 10 to 90 per cent (by weight) respectively.

Calcium glycolate-2-C^{14} was synthesized from bromoacetic acid, which was obtained from Nuclear Instrument and Chemical Corporation, Chicago, according to the procedure outlined by Hughes, Ostwald and Tolbert (30) for the preparation of calcium glycolate from chloroacetic acid. About 30 mg. of bromoacetic acid-2-C^{14}, 470 mg. of inactive bromoacetic acid, 2 g. of calcium carbonate and 10 ml. of water were

placed in a round bottom flask and heated on the steam bath for 2 1/2 days. At the end of this time the reaction mixture was filtered while hot using no wash water and the filtrate placed in the refrigerator. The calcium carbonate was then washed thoroughly and the wash saved for a second crop. At the end of 24 hours the first crop of crystals was filtered off, washed with absolute alcohol and dried in a vacuum desiccator. The calcium glycolate was shown to contain only one radioactive compound by paper chromatography and radioautographs. The paper chromatographs were developed with phenol saturated with an aqueous solution containing 6.3 per cent sodium citrate and 3.7 per cent sodium dihydrogen phosphate in a chamber saturated with acetic acid vapor (31).

Ornithine-2-C^{14} hydrochloride and lysine-2-C^{14} hydrochloride were purchased from Tracerlab, Inc., Boston and were used as such in the experiments described later.

Determination of the Isotopes

All radioactivity measurements obtained in these studies were made in a windowless flow counter (Tracerlab, Inc.) using a Nuclear Instrument and Chemical Corporation scaler. The gas used in the flow counter was a mixture of helium (99.05 per cent) and isobutane (0.95 per cent) and was purchased from The Matheson Co., Inc., East Rutherford, New Jersey. The over-all efficiency of the counter was about 19 per cent as determined using a National Bureau of Standards sodium bicarbonate sample. All samples were plated in "infinitely thick" layers on aluminum dishes with

an area of 2.83 square cm. for counting. The counts were corrected for self-absorption (see Appendix I) except where noted to the contrary and then converted to counts per minute (c.p.m.) per mM.

For the determination of the radioactivity in the experiments where methionine doubly labeled with deuterium and carbon-14 was fed the methionine, the nicotine dipicrate and the quaternary iodide obtained from the lignin methoxyl group as described later were burned at 700°C. in a micro-combustion tube. The combustion tube was packed with the "combination filling" of copper oxide, lead peroxide[1] and platinized asbestos described by Niederl and Niederl (32) with the inclusion of silver wire immediately before the packing as well as at the end. When burning the iodide, it was found necessary to replace the silver wire after 3 or 4 combustions because after that time, impurities came through with the water which were difficult to remove in the purification procedure. The CO_2 of combustion was precipitated as barium carbonate from a barium hydroxide solution in a special trap in the combustion train. The barium carbonate was collected, washed with hot water and dried at 110°C. for one hour. To plate the barium carbonate an alcohol slurry of the compound was made on an aluminum dish and the alcohol was evaporated over a heat lamp. Since only a comparison of radioactivities was desired in the transmethylation studies, 40 mg. of barium carbonate were counted until 10,000 counts were obtained in each case and the count was not corrected for self-absorption.

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[1] The lead peroxide was omitted in later combustions with equally good results.

A trap immersed in a solid carbon dioxide-methyl cellosolve bath was placed in the combustion train before the barium hydroxide solution to freeze out the water of combustion. The water was purified and its deuterium content determined by the gradient tube method described by Linderstrom-Lang et al. (33). The water from the combustion was transferred from the trap to a pyrex tube (interior diameter about 5 mm. and length 12 cm.) sealed at one end and containing about 3 mg. of sodium peroxide and 3 mg. of potassium permanganate carefully introduced previously with a long thin paper funnel so that none of the reagents came in contact with the tube walls. Then the tube was quickly bent in the middle at a 45° angle and sealed. The water was distilled from the oxidizing mixture into the receiving arm and then poured back again. This distillation procedure was repeated several times to insure proper purification. At the end of the last distillation the receiving arm was cut off and the water analyzed immediately as described below.

An H_2O-D_2O mixture was standardized by determining the density of the solution by means of a pycnometer and using a micro-balance for weighings and then reading the per cent D_2O in the solution from the straight line plot of the densities of pure H_2O and pure D_2O against per cent of D_2O. The atom per cent excess D in the water of the standard was calculated from the per cent of D_2O in the standard. Various secondary standards of from 0.0746 to 1.045 atom per cent excess D were prepared by diluting the primary standard with appropriate amounts of redistilled H_2O.

The gradient tube in which the deuterium analyses were made was half-filled with a mixture of redistilled bromobenzene and odorless kerosene having a density slightly larger than the heaviest secondary standard. The remainder of the tube was carefully filled with another mixture of these two liquids having a density slightly less than the lightest secondary standard. Both the kerosene and bromobenzene were distilled before use in an all glass apparatus and then dried over calcium chloride. The kerosene fraction boiling from 210 to 240°C. and the bromobenzene fraction boiling at 152 to 154°C. were used. The two solutions in the tube were mixed slightly at their interphase with a long glass rod terminating in a loop, and the tube was allowed to stand several days in a constant temperature bath to establish a linear density gradient. To determine the atom per cent excess D in an unknown sample various secondary standard solutions covering the interval of density expected in the unknown were introduced into one side of the gradient tube with a micro-pipette and the unknown was introduced into the other side. At the end of a given time, usually 0.5 hour, the distance the unknown had fallen and the distances the standards had fallen were read with the aid of a cathetometer. The atom per cent excess D of the unknown was then obtained by simple interpolation assuming a linear relationship between the density of the water droplets and the distance they had fallen in the medium. Analysis of methionine, synthesized from methyl iodide of known deuterium content, by the gradient tube method just outlined indicated that the method was accurate within a range of about $\pm$ 0.03

atom per cent deuterium with the average of several analyses falling close to the known deuterium content.

Preparation of the Plants

The treatment of the plants employed in the several studies described in this investigation varied somewhat with the separate study. For the studies involving nicotine and the lignin of tobacco plants, Nicotiana rustica, var. humilis, a high nicotine strain, was used. A variety of Hordeum vulgare known as Bay barley was used in the transmethylation study involving barley lignin. Barley and tobacco seeds were planted in flats containing vermiculite,[2] which provided a base for the growing plants but gave no nutrients. The plants were grown until they had attained a height of 13-18 cm. (about one month for the barley and from two to three months for tobacco). The plants were watered twice a week with a nutrient solution composed of 1 g. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4, 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$ and four liters of tap water.

To prepare the plants for the hydroponic administration of the radioactive materials the plants were removed from the flats and the roots were carefully freed of vermiculite by soaking and washing in tap water. The roots of the plants were then immersed in a 0.1 per cent solution of detergent germicide[3] for 0.5 hour, with occasional agitation,

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[2] Vermiculite is a commercially available heat expanded mica.

[3] Wyandotte detergent germicide No. 1528 was obtained from the Wyandotte Chemicals Corporation, Wyandotte, Michigan.

to reduce the bacterial population. After rinsing under tap water, the plants were placed in 125 ml. erlenmeyer flasks containing 50 ml. (25 ml. for the barley plants) of an inorganic nutrient medium prepared by diluting, with two parts of water, one part of a stock solution which had the following composition: water 1 l.; calcium nitrate, 1 g.; potassium chloride, 250 mg.; potassium dihydrogen phosphate, 250 mg.; magnesium sulfate, 250 mg.; ammonium sulfate, 250 mg.; ferric chloride, 2 mg. The weights are of the anhydrous salts. Six drops of one per cent germicide solution were added to each flask. Twice a day for two minutes a stream of oxygen was passed through the nutrient solution to provide aeration for the root systems and to prevent wilting of the plants. In the studies with calcium glycolate, ornithine and lysine the treatments with germicide and oxygen were omitted. Instead of the six drops of germicide solution, 0.5 ml. of an aureomycin solution (50 mg. per 50 ml. of solution) was added to each flask as a means of reducing the number of root microorganisms.

In the first experiments with ornithine and lysine the radioactivity recovered in the isolated nicotine was too low, when the treatment of the plants outlined above was followed, to permit degradative studies on the nicotine. It had been noted in the earlier studies that the tobacco plants developed many new roots when they had been in the nutrient medium for a week or more. Since nicotine synthesis has been shown to occur in the roots (16), it was decided to force the tobacco plants to develop new root systems after their transfer from the vermiculite in

an attempt to obtain an increased synthesis of nicotine and therefore an augmentation in the amount of carbon-14 in the isolated nicotine. To induce the development of new root systems the roots of the tobacco plants were cut off after the plants had been removed from the vermiculite. The plants were then placed in the erlenmeyer flasks containing the hydroponic medium described earlier. At the end of two weeks the plants had produced completely new root systems and at this time the radioactive materials were added as will be described later. No improvement in this procedure occurred when the cut plants were treated with a commercial plant hormone powder[4] before being placed in the inorganic nutrient solution; so the hormone treatment was not utilized.

During the administration of the radioactive compounds the plants were grown in a hood. Two 36-inch, 30-watt fluorescent tubes and a 100-watt incandescent bulb were placed about 14 inches above the tops of the plants as a source of illumination. A light intensity at the level of the upper leaves of about 200 foot-candles was thus obtained. The lights were left on 12 hours out of 24 during the entire administration period. Additional nutrient solution was added to the flasks to keep the volume constant.

Isolation and Purification of Nicotine

After the administration period the tobacco plants were removed from the nutrient medium and the roots were rinsed with distilled water, the

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[4] The hormone powder employed was Rootone, which is manufactured by the American Chemical Paint Co., Ambler, Pa.

excess being blotted off with cheesecloth. The plants then were cut into small pieces and immediately dried as rapidly as possible under infrared heat lamps. The temperature of the drying plants reached about 80°C. in 15 minutes. The dried material was ground in a mortar, mixed with 20 per cent of its weight of calcium hydroxide, and steam distilled in a Kjeldahl flask. The distillation was continued until no precipitate was obtained when the distillate was tested with silicotungstic acid indicating that no more nicotine was coming over. The distillate which was collected in 5 ml. of 6 N hydrochloric acid was concentrated to a small volume under reduced pressure, and purification of the alkaloid was accomplished by two successive azeotropic distillations with water from alkaline medium as described by Smith (34). The acid distillate was concentrated to dryness under reduced pressure and the nicotine hydrochloride residue resulting was dissolved in methanol plus a little water. Then a saturated methanolic solution of picric acid was added in excess. After standing a short time the precipitate of nicotine dipicrate was filtered off, washed with methanol and recrystallized from hot water. (m.p. 224-226°C.; recorded 224°C. (35).)

Transmethylation Experiments with Doubly Labeled Methionine

A. Nicotine Study

Administration of methionine. -- Previously it has been shown that tobacco plants absorb methionine from a nutrient solution through the root systems (3). Sixty tobacco plants were prepared for the hydroponic

administration of the labeled methionine as described earlier. They were allowed to grow in the nutrient solution for nine days to establish the root systems. On the tenth day three plants were discarded because of wilting, and then one ml. of a solution containing two mg. of doubly labeled methionine per ml. was added to the nutrient solutions in the flasks containing the remaining plants. During the rest of the growing period the plants grew as was evidenced by increase in stem length, production of leaves and growth of roots. Four and nine days later 2 mg. more of labeled methionine were added to the flasks making a total of 6 mg. of labeled methionine fed to each plant. This procedure was necessary to obtain a sufficient incorporation of deuterium to be detected accurately in the isolated nicotine. The labeled methionine had an atom per cent excess D of 24.5 and the total count received by each plant was 8×10^{8} counts per minute. Thirteen days after the administration of the first methionine the plants were removed from the flasks and treated as described earlier for the isolation of the nicotine.

Exchange study. -- Although the possibility of an uncatalyzed exchange of methyl groups between methionine and nicotine during the feeding period and isolation of the nicotine from the plants seemed remote, an experiment was set up to check this possibility. Seventy mg. of nicotine and 50 mg. of C^{14}-methylmethionine were dissolved in 250 ml. of a 0.05 M phosphate buffer, pH 6.6. After this mixture had stood for eight days at room temperature, the nicotine was separated by an azeotropic distillation with water and isolated from the distillate as the dipicrate. The dipicrate was counted in an aluminum dish in the

flow counter and no significant count over background was obtained. This experiment indicates that under conditions comparable to the conditions of the feeding experiments there was no detectable, uncatalyzed interchange of the methyl groups between nicotine and methionine.

Results. -- The atom per cent excess deuterium and the radioactivity of the fed methionine and the isolated nicotine are presented in columns (A) and (C) of Table I. In column (B) is shown the per cent excess D in the methyl groups of the labeled methionine and dipicrate. The data in column (B) are values calculated on the assumption that all of the excess deuterium is located in the methyl groups of the respective compounds. In the case of the methionine the compound was synthesized from deuterated methyl iodide and homocystine, and the methyl group is the only position where the excess deuterium may be located. In the case of the nicotine dipicrate it has been shown that within experimental error all of the carbon-14 in nicotine isolated from plants fed C^{14}-methyl methionine was located in the methyl group of the nicotine (3). The above assumption, therefore, seems justified in this study. By similar argument the radioactivity of the methyl groups of the two compounds is assumed to be the same as the radioactivity of the respective compounds. The radioactivity of the compounds and methyl groups are given in columns (C) and (D) respectively.

The ratios of the deuterium to the radioactivity in the methyl group of the nicotine dipicrate and the methionine are shown in the column subtitled (B/D) in Table I. In the last column it is shown that the ratio of deuterium to C^{14} in the methyl group of the nicotine

TABLE I

DEUTERIUM AND C^{14} IN THE FED METHIONINE AND IN THE NICOTINE DIPICRATE

Compound	Per Cent Excess D		C^{14} Content in cpm/mM		Ratio of D in Methyl Group to C^{14} in Methyl Group	Per Cent Nicotine Dipicrate Ratio of Methionine Ratio
	Compound	Methyl Group	Compound	Methyl Group		
	(A)	(B)	(C)	(D)	(B/D)	
DL-doubly-labeled methionine	24.5	89.8	2.07×10^{6}	2.07×10^{6}	4.34×10^{-4}	--
Nicotine dipicrate	0.243*	1.62	3.96×10^{3}	3.96×10^{3}	4.08×10^{-4}	94

* The author is indebted to Dr. David Rittenberg and Laura Ponticorvo for this deuterium analysis.

dipicrate is 94 per cent of the ratio of deuterium to C^{14} in the methyl group of the methionine. This percentage represents the per cent of the deuterium, originally present in the methionine methyl group, which was transferred with the methyl carbon. If transmethylation occurs then, of course, 100 per cent of the deuterium would remain in the methyl group. However, if the methyl group of methionine is oxidized to the state of formaldehyde and later reduced only 67 per cent of the original deuterium can remain with the methyl carbon. Since 94 per cent of the deuterium remained with the methyl carbon during its transfer from methionine in the present study, it may be concluded that a large proportion of the methyl groups, which are transferred from methionine, are transferred through a transmethylation process.

B. Lignin Study

Administration of methionine. -- Flokstra (8) has demonstrated that barley plants absorb methionine from a nutrient solution through the root systems and so the hydroponic technique of administering methionine was used in the present study. Sixty barley plants were prepared for the feeding experiment as was outlined earlier. One ml. of a solution containing two mg. of doubly labeled methionine per ml. was added to each flask. At the end of five days two more mg. of labeled methionine were added to each flask making a total of four mg. of methionine received by each plant. The labeled methionine had an atom per cent excess D of 24.5 and the total count received by each plant was 5.6×10^3 c.p.m. Twelve days after the administration of the first

methionine the plants were removed from the nutrient solution, the roots were discarded and the remaining part of the plants was dried. The dried material was then subjected to the treatment described below for the isolation of the lignin.

In the case of the lignin studies on tobacco plants the plant material, which had been steam distilled to recover the nicotine as outlined earlier, was dried again and subjected to the lignin isolation procedure.

Isolation of lignin. -- Flokstra (8) has described a method for the isolation of lignin from freshly dried plant material, and his procedure was used in the present study. The procedure consists of a series of solvent extractions, which were performed on the plant tissue in a Waring Blendor, followed by treatment with sulfuric acid. The series of solvent extractions as suggested by MacDougall and DeLong (36) included two 15-minute extractions with ether-saturated water to remove some nitrogen containing materials, one 20-minute extraction with five per cent acetic acid in water for the removal of carbohydrates, and two 15-minute extractions with ethanol-benzene (1 + 2) to remove lipids. These solvent extractions were followed by treatment of the remaining white, fibrous material with 70 per cent sulfuric acid for 18 hours at 5°C. The sulfuric acid was then diluted to 3 per cent and the mixture boiled gently for two hours, keeping the volume constant by adding water. After cooling, the lignin, which settled out, was filtered, washed with distilled water and dried in a vacuum desiccator for future use.

Demethylation of lignin. -- For a comparison of the C^{14} and D in the methyl group of the fed methionine to the C^{14} and D in the methoxyl group of the isolated lignin, which has an unknown chemical structure, it was necessary to obtain a compound with a well characterized structure by degrading the lignin. Since only the methyl groups in the lignin were of interest in the present study, the lignin was demethylated utilizing Flokstra's modification (8) of the Phillips' method (37). The demethylation apparatus employed here was the modified form of the Pregl apparatus (38) described by Brown (28). About 60 mg. of the lignin to be demethylated was weighed on cigarette paper, which previously had been shown to yield no methyl iodide in the demethylation procedure, and was introduced into the reaction flask. Then two ml. of phenol, which acted as a solvent for the lignin, and four ml. of 47.3 per cent periodic acid were placed in the reaction vessel. A gas-washing bubbler containing 1.5 ml. of the 5 per cent $CdSO_4$-$Na_2S_2O_3$ solution, recommended by Pregl to remove any hydriodic acid and iodine carried over, was attached to the reaction vessel. A tube from the bubbler led to the receiving vessel, which contained a five per cent solution of triethylamine in ethanol, cooled in a methyl cellosolve-solid carbon dioxide bath to about -75°C.

During the demethylation procedure the reaction flask was heated in a copper oxide bath. A stream of nitrogen was slowly run in through the side arm of the reaction flask. The temperature of the bath was kept at about 150°C. for 45 minutes, then raised to 200° and held for 30 minutes. The bath was allowed to cool for 15 minutes during which

time a more rapid stream of nitrogen was passed through the apparatus. Next the tip of the delivery tube was rinsed with ethanol into the receiving vessel, which was then stoppered and allowed to stand overnight at room temperature. The next day the ethanolic solution was taken nearly to dryness by heating and the last traces of ethanol and triethylamine were removed in a vacuum desiccator leaving the white, solid methyltriethylammonium iodide. The isotope content of the iodide was measured by the method already described in the section on the determination of isotopes.

Results. -- The data from two trials in this particular study are presented in Table II. Since all of the deuterium in the compounds analyzed is present in the methyl group of the respective compounds, the values in column (B) are calculated from the values in column (A) by employing the appropriate conversion factor. The radioactivity of the methyl groups is the same as the radioactivity of the respective compounds so the values in column (D) are obtained directly from the values in column (C). It will be noted that the D to C^{14} ratio obtained in the methyltriethylammonium iodide from the barley lignin methoxyl groups was about 94 per cent of the D to C^{14} ratio in the methyl group of the fed methionine. This percentage again represents the per cent of the deuterium, originally present in the methionine methyl group, which was transferred with the methyl carbon. The D to C^{14} ratio in the methyltriethylammonium iodide obtained from lignin isolated from tobacco plants was about 95 per cent of the D to C^{14} ratio in the fed methionine as ascertained from two independent deuterium analyses.

TABLE II

DEUTERIUM AND C^{14} IN THE FED METHIONINE AND IN THE METHYLTRIETHYLAMMONIUM IODIDE OBTAINED FROM THE ISOLATED LIGNIN

Compound	Atom % Excess D		C^{14} Content in cpm/mM		Ratio of D in Methyl Group to C^{14} in Methyl Group	Per Cent Quaternary Iodide Ratio of Methionine Ratio
	Compound	Methyl Group	Compound	Methyl Group		
	(A)	(B)	(C)	(D)	(B/D)	
DL doubly labeled methionine	24.5	89.8	2.07×10^{6}	2.07×10^{6}	4.34×10^{-4}	--
Methyl triethyl ammonium iodide						
Trial 1 (Barley)	.212	1.27	3.12×10^{3}	3.12×10^{3}	4.07×10^{-4}	94
Trial 2 (Tobacco) (a)	.334*	2.00	4.81×10^{3}	4.81×10^{3}	4.16×10^{-4}	96
(b)	.329	1.97	4.81×10^{3}	4.81×10^{3}	4.10×10^{-4}	94

* I am indebted to Dr. David Rittenberg and Louis Ponticorvo for this deuterium analysis.

As was indicated earlier if all of the methyl groups of methionine had been oxidized to the state of formaldehyde, the least possible oxidation, and then reduced to give the methoxyl groups of lignin, the ratio of D to C^{14} in the methyl of the isolated quaternary salt would be 67 per cent of the D to C^{14} ratio of the fed methionine. It will be noted that the ratio of D to C^{14} in the methyl group of the quaternary iodide in all cases is well over 67 per cent of the D to C^{14} ratio in the fed methionine. Since this is the case it may be concluded that some direct transfer of the methyl group, i.e. transmethylation, occurred from the sulfur of methionine to the oxygen of lignin in barley and tobacco plant metabolism. In this connection it should be pointed out that Flokstra (8) has shown that the radioactivity recovered from the lignin by demethylation was present in the methoxyl groups of the lignin and not in N-methyl or S-methyl groups, which might be present in impurities in the lignin.

Methylation Experiments With Calcium Glycolate

Several studies in this laboratory (3,4,13) have shown that tobacco plants can absorb various organic compounds through their root systems from a nutrient solution. The hydroponic procedure of administering labeled compounds therefore was adopted in the present study since it was desired to duplicate the previous studies as closely as possible in order to make valid comparisons between the glycolate experiment and the former experiments. The calcium salt of glycolic acid was used in the present study not only because it occurred as the final product of the glycolate

synthesis mentioned earlier but also because it is a more convenient compound to handle than glycolic acid itself. Before the metabolic studies with glycolate could be undertaken it was necessary to ascertain whether or not the glycolate would be absorbed by the plants and also whether or not microorganisms would destroy the glycolate in the nutrient solution.

Uptake of calcium glycolate. -- To determine the rate of glycolate uptake by the root systems a sensitive method for the analysis of glycolate solutions was needed. The method of analysis chosen was that of Calkins (39), a colorimetric method based on the formation of a violet-red color when glycolic acid and 2,7 dihydroxynaphthalene are heated together in concentrated sulfuric acid. The solution to be analyzed was made 2 N with respect to sulfuric acid and 0.2 ml. was introduced into a test tube. The tube was cooled in an ice bath and then 2 ml. of a 0.01 per cent solution of 2,7 dihydroxynaphthalene were added from a microburet. The contents of the tube were made homogeneous by shaking and then heated in a boiling water bath for 20 minutes. After cooling again in an ice bath, the solution was diluted with four ml. of 2 N sulfuric acid, shaken vigorously after the heat of reaction had subsided and the color read at 530 mμ with a Beckman spectrophotometer. Various standard solutions containing from 10 to 70 micrograms of calcium glycolate per ml. were used to prepare a standard curve.

To test the uptake of glycolate four plants were prepared as described previously, using 40 ml. of nutrient solution, 2.5 ml. of calcium glycolate standard (1 mg./ml.) and 0.5 ml. of aureomycin solution

(1 mg./ml.). The aureomycin effectively inhibited the growth of bacteria (4,13). Four other flasks prepared in the same manner were inoculated by the addition of root fragments, and four flasks containing only the three solutions were used as controls. At the end of 48 hours the plants were removed, the roots were rinsed and all solutions were filtered through Whatman number 42 filter paper. The filtrates were analyzed for glycolate as outlined earlier. It was found that at the end of 48 hours over 95 per cent of the glycolate had disappeared from the nutrient solution in which plants had been placed. There was no loss due to the growth of microorganisms since over 90 per cent of the glycolate was recovered in both the controls and the inoculated solutions. In addition samples of nutrient solution, which had served as media for the administration of radioactive glycolate, were evaporated to dryness after removal of the plants and the residue was tested for radioactivity. There was no significant count over background in the residue. From these results it was concluded that glycolate was readily absorbed through the roots and therefore that the hydroponic technique was entirely satisfactory for its administration.

Administration of the radioactive glycolate. -- The tobacco plants were prepared for the hydroponic administration of the glycolate as was outlined earlier. In the first trial each of 30 tobacco plants was given 1.27 mg. of calcium glycolate (corresponding to the 2 mg. of methionine administered by Brown (3)) having a radioactivity of 1.87×10^6 c.p.m.[5]

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[5] This was determined by counting an "infinitely thin" layer (0.009 mg./square cm.) in the flow counter.

Since an activity of 1×10^6 c.p.m. was administered in the previous methylation studies, the Trial I data presented in Table III have been corrected to correspond to a dosage of 1×10^6 c.p.m. In Trial II 25 plants each received 1.27 mg. of calcium glycolate having a count of 1.06×10^6 c.p.m. Trial III was conducted in the same manner as Trial II. At the end of one week the plants were removed from the flasks and the nicotine was isolated as the dipicrate, according to the procedure discussed earlier, and found to be radioactive.

Demethylation of the nicotine. -- As in the past methylation studies it was necessary in the present case to determine whether or not the radioactivity of the nicotine was localized in the methyl group. The demethylation procedure used to establish this point was the Brown modification (28) of the Pregl (38) methyl- and ethyl-imino determination. Using this procedure the methyl group is isolated as methyltriethylammonium iodide, a solid compound suitable for counting.

Two hundred mg. of nicotine dipicrate were decomposed with sodium hydroxide and the nicotine was recovered by azeotropic distillation with water through a Widmer column. The acidified distillate was concentrated under reduced pressure, the concentration being completed in the reaction flask of the demethylation apparatus. To the nicotine hydrochloride in the reaction flask were added 45 mg. of ammonium iodide, two drops of five per cent gold chloride solution and three ml. of hydriodic acid (specific gravity 1.5). The gas washing vessel in the apparatus contained 1.5 ml. of the five per cent $CdSO_4$-$Na_2S_2O_3$ solution described by Pregl.

The receiver contained a five per cent solution of triethylamine in ethanol and was cooled in a solid carbon dioxide-methyl cellosolve bath.

When the apparatus was completely assembled a slow stream of nitrogen was passed through, and the reaction flask was heated in a copper oxide bath to 200°C. in 20 to 25 minutes. The temperature was then raised slowly to 350-60°C. and held there for 45 minutes. The heat was removed and as the apparatus cooled it was flushed with a continuous stream of nitrogen. After the apparatus had cooled to room temperature the delivery tube was rinsed with ethanol into the receiving vessel, which was then stoppered, shaken, and allowed to stand overnight at room temperature. The next day most of the ethanolic solution was evaporated by heating and the last traces of ethanol and triethylamine were removed in a vacuum desiccator. A white, crystalline residue of methyltriethylammonium iodide was recovered.

The iodide was dissolved in a small amount of ethanol and transferred to a tared aluminum counting dish. The ethanol was evaporated and the dish weighed to acquire the weight of the plated compound. The counting of the sample followed the procedure already outlined before.

Results. -- The counts per minute per mM for both the nicotine dipicrate and the methyltriethylammonium iodide are presented in Table III for three experimental runs.

From this data it can be seen that most, if not all, of the radioactivity of the nicotine is localized in the methyl group of that compound. The relatively high values of Trial I may be due to seasonal influences since the plants were grown at different times of the year.

TABLE III

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER THE ADMINISTRATION OF CALCIUM GLYCOLATE-2-C^{14}

Trial No.	No. of Plants	Maximum Specific Activity (c.p.m./mM)		Per Cent Recovery in Methyl Group
		Nicotine Dipicrate	Methyltriethyl-ammonium iodide	
I	30	7.65×10^3	7.81×10^3	106
II	25	2.20×10^3	2.08×10^3	95
III	25	3.74×10^3	3.53×10^3	94

Biosynthetic Studies with Ornithine-2-C^{14}

Several investigators have felt that ornithine or compounds biologically related to ornithine may play an important role in the biosynthesis of nicotine as was pointed out earlier. However, up to the present time no one has been able to demonstrate conclusively that ornithine can serve as a precursor of nicotine in the tobacco plant. It therefore seemed of interest to establish whether or not ornithine could be incorporated into the nicotine molecule in tobacco plants by administering ornithine labeled with carbon-14.

Uptake of ornithine hydrochloride. -- As in the other feeding experiments it was necessary to find a convenient method of administering the labeled ornithine to the plants. Since the hydroponic administration of compounds had been successful in the past with methyl group precursors, it was attempted in this study also.

In testing the absorption of ornithine by tobacco roots the ninhydrin method of Bergdoll and Doty (40), originally used for the analysis of lysine solutions, was employed in the present study for the analysis of ornithine in the nutrient medium. A standard curve was prepared by mixing together three ml. of various standard ornithine hydrochloride solutions (10 to 70 γ per ml.), one ml. of ninhydrin solution[6] and five ml. of glycerol in large test tubes graduated at 35 ml. The standard solutions contained 15 ml. of phosphate buffer,[7] pH 7.4, per 100 ml. of solution. After mixing thoroughly the tubes were placed in a boiling water bath for 30 minutes. At the end of this time the tubes were removed, diluted to 35 ml. with 95 per cent ethanol and the per cent transmission read at 540 mμ with a Beckman spectrophotometer. A straight line was obtained when the logarithm of the per cent transmission was plotted against the concentration.

Six 125 ml. erlenmeyer flasks were set up with 40 ml. of the nutrient solution, 0.5 ml. of aureomycin solution (1 mg./ml.) and three ml. of standard ornithine hydrochloride solution (1.2 mg./ml.). Plants, whose root systems were freed of vermiculite and rinsed, were placed in two of the flasks. Two other flasks were inoculated with root fragments and the remaining two flasks were used as controls in the experiment. The flasks were placed in the hood for 48 hours and the lights were kept

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[6] This solution was prepared fresh each day by diluting 125 mg. of ninhydrin (triketohydrindene) and 6.57 g. of sodium chloride to 25 ml. with distilled water.

[7] The phosphate buffer was prepared by adjusting 250 ml. of 0.4 M KH_2PO_4 to pH 7.4 with 80 ml. of 1 N NaOH and diluting to 500 ml. with distilled water.

on 12 hours each day. At the end of this period the plants were removed from the flasks and the roots were washed with a fine stream of water into the flasks from which they had been removed. The nutrient solutions were filtered through Whatman number 42 filter paper. Seven and one-half ml. of phosphate buffer, pH 7.4, were added to the filtrates and the solutions were diluted to 50 ml. with distilled water in volumetric flasks. The analysis for ornithine in these solutions was conducted as described above for the preparation of the standard curve. In both the controls and the solutions inoculated with roots almost 100 per cent of the ornithine was recovered while in the solutions which had been exposed to the plants only five per cent of the ornithine remained. In a 48 hour period, then, practically all of the ornithine was assumed to be absorbed by the tobacco plants, and there was no evidence of ornithine destruction by microorganisms. As an added check the nutrient solution in one of the erlenmeyer flasks from a C^{14}-labeled ornithine feeding experiment was evaporated to dryness and the residue was tested for radioactivity. There was only a slight count above background indicating essentially complete absorption of the amino acid from the solution.

Administration of the radioactive ornithine. -- Initially a procedure similar to that employed in the administration of the doubly labeled methionine mentioned earlier was followed in the feeding of the labeled ornithine. The plants were prepared as outlined before. They were allowed to grow in the nutrient solution four days and then each plant was given 0.25 mg. of DL-ornithine-2-C^{14} hydrochloride having an activity

of 4×10^6 c.p.m. After eleven days the plants were removed, and the nicotine was isolated as the dipicrate. The nicotine dipicrate possessed an activity of 8.0×10^3 c.p.m/mM. However, only about 200 to 300 mg. of nicotine dipicrate are usually obtained from the number of plants employed in these studies, and it was found that at least two g. of the picrate were required for the degradation studies. It was necessary therefore either to use many more plants or to increase the radioactivity of the nicotine isolated. The former is not too feasible because of the expense of the labeled compound so the latter alternative was explored.

In order to increase the amount of labeled ornithine incorporated into the nicotine molecule it was necessary to increase the amount of nicotine synthesized by the plants during the feeding period. This was accomplished by removing the old roots from the plants before placing them in the nutrient solution and thereby forcing the plants to produce entirely new root systems prior to the administration of the radioactive ornithine.

In experiment number two the roots of 46 plants were removed, and the plants were placed in erlenmeyer flasks containing 50 ml. of nutrient solution and 0.5 ml. of the aureomycin solution. As was mentioned earlier some plants were treated with a plant hormone, but since no better results were obtained than with the untreated plants, the hormone treatment was discontinued. At the end of two weeks the plants had developed rather extensive root systems so 37 of the best appearing plants were chosen for the administration of the labeled ornithine.

Each plant received 0.25 mg. of ornithine-2-C^{14} hydrochloride which had an activity of 4×10^6 c.p.m. At the end of five days each plant was supplied with another 0.25 mg. of labeled ornithine. Nine days after this last administration the plants were removed from the flasks and the nicotine was isolated as has been described previously. The nicotine dipicrate was counted and possessed an activity of 1.21×10^5 c.p.m./mM. This count represents over a ten-fold increase in the radioactivity of the nicotine when compared with the count obtained in the first experiment. It was therefore possible to dilute the radioactivity of the nicotine dipicrate ten times by mixing one part of C^{14}-nicotine dipicrate with 9 parts of non-radioactive nicotine dipicrate to obtain sufficient material for the degradations. The data presented in Table IV are the counts derived from the diluted sample. The third experiment was conducted in the same manner as the second experiment except that 48 plants were given the labeled ornithine. The radioactivity of the dipicrate isolated was also diluted ten times.

Degradation of the radioactive nicotine. -- 1) Oxidation with potassium permanganate. Of the several methods mentioned in the literature for the oxidation of nicotine to nicotinic acid the potassium permanganate method described by Laiblin (41) appeared to be more applicable in the present study than the others. In this method CO_2 from the oxidation remains in the oxidation mixture as potassium bicarbonate and can be recovered later for counting by acidifying the medium and collecting the CO_2 as barium carbonate by bubbling through barium hydroxide.

To carry out the oxidation the nicotine (0.47 g.) from 1.8 g. of the diluted, radioactive nicotine dipicrate was recovered by the azeotropic distillation with water previously described except that no hydrochloric acid was added to the receiving vessel. The 250 ml. of distillate obtained were treated with 100 ml. of $KMnO_4$ solution (3 g./100 ml. distilled water), which were added in 5 ml. amounts at intervals of 3-5 minutes, at room temperature. After each addition the mixture was thoroughly mixed until the permanganate color had disappeared. Toward the end of the addition the permanganate color persisted for longer periods, and the mixture was heated on the steam bath for 3 to 4 hours to complete the oxidation. At the end of this time the solution was removed from the steam bath and allowed to cool. The solution was then filtered and the MnO_2 was washed thoroughly with hot water. The washings and filtrate were combined and concentrated to dryness under reduced pressure.

The residue remaining after the concentration consisted mainly of the potassium salt of nicotinic acid and potassium bicarbonate. Although the bicarbonate was not identified as such it seems probable that under the conditions of the oxidation the CO_2 is present mainly as potassium bicarbonate and not potassium carbonate. For purposes of identification, counting and further degradation it was desired to isolate the nicotinic acid as the free acid. Initially the residue was dissolved in 40 ml. of water and treated with Dowex-50 ion exchange resin (a resin containing sulfonic acid groups) in the acid form to remove the potassium ions and free the nicotinic acid. However, the

ion exchange resin adsorbed most of the nicotinic acid as well as the potassium ions so this procedure was abandoned.

Another procedure, which was used to isolate the nicotinic acid in experiment number two reported in Table IV, was based on the relative insolubility of the copper salt of nicotinic acid. The residue of potassium nicotinate and potassium bicarbonate was again dissolved in 40 ml. of water, treated with acid to remove the bicarbonate and then neutralized with ammonium hydroxide. A saturated solution of copper sulfate was then added slowly and a blue precipitate was obtained. This precipitate of copper nicotinate was collected and washed thoroughly. To free the nicotinic acid the copper salt was suspended in water and treated with hydrogen sulfide. The copper sulfide precipitate was filtered off and the filtrate was decolorized with charcoal and then evaporated to a small volume on the steam bath. Upon cooling the nicotinic acid precipitated. However, only 18 mg. of the nicotinic acid were recovered so further purification was not possible. The apparent low recovery of counts in this sample of nicotinic acid may be due not only to errors in counting this small a sample but also to some impurities in the nicotinic acid itself.

Since the yield of nicotinic acid was very low when the copper sulfate procedure was followed, a further search was made for a better precipitating agent. Finally the precipitation of the silver salt was adopted as the best procedure. The residue of potassium bicarbonate and potassium nicotinate dissolved in 40 ml. of water was first acidified with dilute nitric acid to liberate the CO_2. Next the solution was

neutralized with dilute ammonium hydroxide and the silver salt of nicotinic acid precipitated by adding 0.1 N $AgNO_3$ until no more precipitate formed. The silver salt was collected, washed with distilled water and suspended in 50 ml. of water. The suspension was treated with hydrogen sulfide and the precipitate of silver sulfide removed by filtration. The filtrate was decolorized with charcoal and then evaporated to a small volume on the steam bath. Upon cooling the nicotinic acid precipitated as fine, white needles. It was collected, recrystallized from hot water and dried in a vacuum desiccator. Anal. Calcd. for $C_6H_5NO_2$: C, 58.53%; H, 4.09%; N, 11.38%; found: C, 58.72%; H, 3.98%; N, 11.26%.[a]

The nicotinic acid was plated and counted as has been described earlier. Since there was not enough nicotinic acid recovered to prepare a self-absorption curve, the count from the nicotinic acid was corrected for self-absorption using the self-absorption curve prepared for the nicotine dipicrate. This procedure may introduce some error but the magnitude of the error is probably low (42).

The CO_2 liberated by the acidification of the potassium nicotinate and potassium bicarbonate solutions was always swept into a saturated solution of barium hydroxide by a stream of nitrogen. The barium carbonate precipitate was collected, washed with hot distilled water and dried at 110°C. for one hour. In determining the radioactivity of the barium carbonate the procedure outlined previously was used.

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[a] The microanalyses reported in this thesis were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.

2) Decarboxylation of the radioactive nicotinic acid. Since the nicotinic acid obtained from the oxidation of the radioactive nicotine possessed considerable radioactivity, it was necessary to determine the location of this source of radioactivity in the nicotinic acid molecule if possible. To ascertain whether or not any of the carbon-14 was located in the carboxyl group the nicotinic acid was decarboxylated. In the early work on the determination of the structure of nicotine it was found that the dry distillation of the calcium salt of nicotinic acid mixed with calcium hydroxide yielded pyridine and calcium carbonate (43). In the present study the nicotinic acid (usually about 50 mg.) was mixed with an excess of calcium oxide and placed in the specially constructed flask used in the demethylation apparatus. A delivery tube led from the reaction flask into a conical centrifuge tube containing 5 ml. of a saturated solution of picric acid in methanol. To sweep the pyridine into the receiver a slow stream of nitrogen was passed through the distillation apparatus. The flask was heated slowly at first with a micro-burner and then more rapidly as the distillation proceeded. Toward the end of the distillation the flask was heated vigorously with a bunsen burner. After about twenty minutes the heating was stopped and the flask was allowed to cool. The delivery tube was removed and rinsed several times with methanol into the receiving tube. The pyridine precipitated as the picrate almost immediately. After standing about thirty minutes the pyridine picrate was collected on a sintered glass funnel, washed several times with methanol and dried in a vacuum desiccator. For further purification it was recrystallized from hot water.

Anal. Calcd. for $C_{11}H_8N_4O_7$; C, 42.86%; H, 2.60%; N, 18.18%; found: C, 42.94%; H, 2.51%; N, 18.03%. The pyridine picrate was counted and found to contain no radioactivity, and therefore further degradation of the pyridine ring was unnecessary in the present study.

The radioactivity of the nicotinic acid then was located solely in the carboxyl group. As an added check the carboxyl carbon was recovered from the calcium carbonate residue in the reaction flask. The reaction flask was flushed out with nitrogen and then a delivery tube leading into a saturated aqueous solution of barium hydroxide was connected to the flask. Dilute nitric acid was added in the side arm of the flask at intervals and was forced into the flask by reconnecting the tube to the nitrogen tank and passing nitrogen gas through the apparatus. The liberated CO_2 was swept into the barium hydroxide solution by the nitrogen gas. The resulting barium carbonate precipitate was recovered and counted as has been described previously.

3) Demethylation of the radioactive nicotine. Although as will be shown later essentially all of the radioactivity from the nicotine was recovered in the nicotinic acid and barium carbonate from the nicotine-permanganate oxidation, the possibility that some radioactivity might reside in the N-methyl group was investigated nevertheless. In addition it was of interest to compare the rate of incorporation of the alpha carbon of ornithine into the methyl group of nicotine with the rate of incorporation of previously studied compounds. To ascertain whether or not the methyl group contained any carbon-14 the nicotine from a 200 mg. sample

of the diluted, radioactive nicotine dipicrate was demethylated in the same manner as was outlined in the calcium glycolate study.

Results. -- The data obtained from two experiments in which an attempt to locate the radioactivity in nicotine after feeding ornithine-2-C^{14} to tobacco plants was made are presented in Table IV. The oxidation of nicotine with potassium permanganate in neutral solution yields potassium bicarbonate, nicotinic acid and methyl amine. The potassium bicarbonate presumably arises from the 3,4 and 5 carbons of the pyrrolidine ring although it is not definitely known whether or not any of the methyl carbon is oxidized to carbonate. In this connection it should be noted that Laiblin (41) was able to isolate methyl amine from the oxidation mixture, and there is always a strong odor of methyl amine during the oxidation procedure. In the present study it has been assumed that essentially only the 3, 4 and 5 carbons of the pyrrolidine ring of nicotine are recovered in the potassium bicarbonate. Therefore, since three moles of potassium bicarbonate are obtained in the oxidation, the original count of the barium carbonate recovered from the potassium bicarbonate is multiplied by three to make it comparable to the count of the nicotine. The counts of the barium carbonate calculated in this manner are given in column (B) for the two experiments. It will be noted that around 50 per cent of the total count of the nicotine dipicrate is recovered in the barium carbonate in each case. Therefore, about half of the radioactivity of the original nicotine resides somewhere in the 3, 4 and 5 positions of the pyrrolidine ring.

TABLE IV

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER THE ADMINISTRATION OF ORNITHINE-2-C^{14} HYDROCHLORIDE

Experiment Number	Maximum Specific Activity (Counts per Minute per Millimole)					
				Decarboxylation Products of Nicotinic Acid		
	Nicotine Dipicrate (A)	$BaCO_3$ from Nicotine-MnO_4^- Oxidation* (B)	Nicotinic Acid (C)	Pyridine Picrate (D)	$BaCO_3$ (E)	Methyltriethyl-ammonium iodide (F)
2	1.27×10^4	6.60×10^3	4.61×10^3	0	4.32×10^3	--
3	1.78×10^4	8.31×10^3	9.64×10^3	0	1.21×10^4	3.32×10^2

* These values are calculated on the assumption that three moles of $BaCO_3$ are obtained from the oxidation of one mole of nicotine (see text).

In column (C) the radioactivity of the nicotinic acid obtained from the permanganate oxidation of the nicotine is presented. In experiment number two 36 per cent of the radioactivity of the original nicotine is recovered in the nicotinic acid while in experiment number three there is a recovery of 54 per cent of the total count. The recovery of the radioactivity in the nicotinic acid of experiment number two apparently is low since the total recovery of counts in the barium carbonate and nicotinic acid in experiment number two is about 88 per cent of the count present in the original nicotine while in experiment number three 100 per cent of the radioactivity of the nicotine is recovered in the barium carbonate and nicotinic acid. Therefore, it may be concluded that within experimental error half of the radioactivity of the nicotine recovered from plants fed ornithine-2-C^{14} is located in the nicotinic acid obtained from that nicotine and half is located somewhere in the 3, 4 and 5 positions of the pyrrolidine ring. It will be necessary to perform further degradations on the radioactive nicotine to ascertain where the carbon-14 is located in the 3, 4 and 5 positions of the pyrrolidine ring. A procedure is being developed at the present time in this laboratory whereby the 3, 4 and 5 carbons of the pyrrolidine ring may be recovered individually for counting. As will be discussed in more detail later it seems very likely that the location of the radioactivity will eventually prove to be in the 5 position.

Since the nicotinic acid was found to be radioactive, it was necessary to degrade it in an attempt to locate the position of the radioactive carbon. As has already been mentioned the first degradation

attempted on the nicotinic acid was a decarboxylation to yield pyridine and carbon dioxide. The results of this degradation are presented in columns (D) and (E). It will be noted that no radioactivity was recovered in the pyridine picrate indicating that all of the carbon-14 present in the nicotinic acid was located in the carboxyl group. The data presented in column (E) confirm this finding since essentially all of the radioactivity of the nicotinic acid is recovered in the barium carbonate derived from the carboxyl group. From these results it is now known that the 2 position in the pyrrolidine ring contains approximately half of the radioactivity present in the original nicotine while the other half is located outside of the 2 position but still in the pyrrolidine ring.

In column (F) the counts of the methyltriethylammonium iodide obtained from the demethylation of the radioactive nicotine are given. It can be seen that less than two per cent of the total radioactivity of the nicotine is located in the methyl group. The rate of incorporation of the alpha carbon of ornithine into the methyl group of nicotine is markedly lower than the rate of incorporation of the methyl groups of methionine (3) and choline (4), the alpha carbon of glycine (13) and glycolate and the carbon of formate (3). In this connection it should be pointed out that the plants in the present study received at least eight times the amount of radioactivity administered to the plants in past methylation studies and were grown for a longer period of time in contact with the carbon-14.

Biosynthetic Studies With Lysine-2-C^{14}

It has been shown that lysine can be converted to pipecolic acid (piperidine-2-carboxylic acid) in both plants (44) and animals (45). It seemed conceivable that pipecolic acid might give rise to the pyridine ring of the nicotine molecule if the pipecolic acid were first dehydrogenated and decarboxylated. Thus lysine through conversion to pipecolic acid could be a precursor for the pyridine ring portion of the nicotine molecule. To determine whether or not lysine could serve as a precursor of the nicotine molecule, lysine-2-C^{14} was administered to intact tobacco plants using the same procedures as were followed in the ornithine experiments.

It was necessary first to show that lysine could be absorbed from the nutrient medium by the tobacco plants. Using the same analytical procedure employed for the ornithine analyses, it was found that at the end of 48 hours about 100 per cent of the lysine was recovered both in the flasks inoculated with the roots and in the control flasks while only 10 per cent of the lysine was recovered in the flasks containing the plants. It was concluded that within the feeding period practically all of the lysine would be absorbed by the tobacco plants. In addition the contents of one of the flasks used in administering the radioactive lysine was evaporated to dryness after the growing period, and when the residue was tested for radioactivity there was only a slight count above background indicating practically complete disappearance of the amino acid from the nutrient medium. There was no evidence of the growth of microorganisms in the nutrient solutions.

When 0.25 mg. of DL-lysine-2-C^{14} hydrochloride containing 4 x 10^5 c.p.m. was administered to 20 tobacco plants under the same conditions as were described in experiment number one with the ornithine-2-C^{14} hydrochloride, the nicotine isolated from the plants possessed an insignificant count over background. Under these conditions no detectable amount of lysine is incorporated into the nicotine molecule.

When the lysine labeled with carbon-14 was administered employing the technique used for experiments number two and number three in the ornithine studies, the nicotine which was isolated from the plants as the dipicrate possessed a count of 2.00 x 10^3 c.p.m./mM. The nicotine isolated from plants, which were fed ornithine-2-C^{14} under comparable conditions, possessed a count of 1.21 x 10^5 c.p.m./mM. The rate of incorporation of ornithine into the nicotine molecule then is about 60 times the rate of incorporation of lysine when the two were administered under essentially identical conditions. Because of this low rate of lysine incorporation it was concluded that lysine does not give rise to nicotine through a direct pathway but only indirectly. Since the radioactivity of the nicotine dipicrate isolated from the plants fed lysine was so low, it was not possible to obtain sufficient material by mixing unlabeled nicotine dipicrate with the labeled sample for degradation studies so the degradation procedure used in the ornithine experiments was not undertaken in the present study.

One way in which the alpha carbon of lysine could enter the nicotine molecule is through the conversion of lysine to glutamate or some other compound closely related to ornithine. Borsook and co-workers (46)

have demonstrated that guinea pig liver homogenates are able to convert lysine to glutaric acid. Glutaric acid through oxidation to α-ketoglutaric acid followed by a transamination reaction could in turn be converted to glutamic acid. More recently Miller and Bale (47) have shown that DL-lysine-6-C^{14} is converted to glutamic acid, arginine and ornithine in intact dogs, and they suggest that glutarate or α-ketoglutarate is the likely common intermediate for the formation of these three amino acids from lysine. Glutamic acid has been shown to act as a precursor of proline and arginine in rats (48) and of proline in E. coli (49). In addition Fincham (50) has demonstrated that enzyme preparations from Neurospora crassa mycelia can catalyze the synthesis of ornithine from glutamic -γ- semialdehyde and also from glutamate. Although there is no evidence at present that lysine can be converted to glutamic acid and then to proline or ornithine in higher plants it seems quite possible that this conversion could occur.

Therefore one mechanism, which would be consistent with the above findings and which would explain the incorporation of the alpha carbon of lysine into the nicotine molecule, could be based on the supposition that lysine is first converted to ornithine or some closely related compound before being incorporated into the nicotine molecule in the intact tobacco plant.

In view of the results of the ornithine experiment and the similarity between lysine and ornithine, it is interesting to speculate on the origin of the alkaloid anabasine (α-(β-pyridyl)-piperidine)

in tobacco plants. Il'in (51) has suggested that anabasine is formed from nicotine by the expansion of the 5-membered pyrrolidine ring at the expense of the methyl group. However, it seems more likely that the piperidine ring of anabasine is formed from lysine in a manner analogous to the formation of the pyrrolidine ring of nicotine.

DISCUSSION

DISCUSSION

Transmethylation studies. -- The results of the experiments in which methionine doubly labeled in the methyl group with carbon-14 and deuterium was administered to barley and tobacco plants provide evidence that the intermolecular transfer of methyl groups is a reaction in the metabolism of higher plants. The widespread occurrence of methylated compounds in the plant kingdom indicates the importance of transmethylation reactions in the metabolic processes of higher plants. It should be pointed out that transmethylation at least where methionine and choline are involved is probably not the only reaction which can give rise to methyl groups in higher plants since there is evidence that the alpha carbon of glycine may be incorporated into the methyl group of nicotine at a faster rate than the methyl carbons of either methionine or choline (13).

The question as to whether or not the origin of the methyl group of nicotine through transmethylation from methionine actually represents a net biosynthesis of nicotine still remains unproved. In this connection it is of interest to consider Dawson's work on the formation of nornicotine in Nicotiana glutinosa (11). It was shown that nicotine is synthesized in the roots of N. glutinosa and then translocated to the aerial portions of the plant where it is demethylated to form nornicotine. In an attempt to reverse the demethylation reaction and form nicotine from nornicotine and some methyl precursor in N. glutinosa, Dawson

reported in a later publication (52) that there was no detectable synthesis of nicotine when nornicotine alone or when nornicotine and either choline or methionine were administered to excised leaves, which were made essentially free of alkaloid by grafting glutinosa shoots on tomato root stocks. From these results the conclusion was drawn that the pathway of nicotine demethylation contains at least one irreversible step. It is doubtful that this situation obtains in N. rustica, the plant used in the present study, since there appears to be essentially no nornicotine in N. rustica (53). Il'in (51,54) has confirmed Dawson's findings concerning the formation of nornicotine in N. glutinosa, and although the acceptor of the methyl group from nicotine was not identified, it was indicated that nicotine may participate in the general metabolism of the plant. More recently Kuzin and Merenova (55) have shown that when excised tobacco leaves are exposed to CO_2 labeled with carbon-14 the nicotine isolated from these leaves is radioactive. Moreover all of the radiocarbon is located in the methyl group of the nicotine. The authors conclude that the leaves of Nicotiana thus carry on transmethylations with the participation of nicotine. In addition Culp (56) in this laboratory has found that intact N. rustica plants injected with a solution of sodium bicarbonate labeled with carbon-14 produce nicotine with a slightly larger amount of radiocarbon in the methyl group than in the rest of the nicotine molecule. The several studies mentioned above indicate that the methyl group of nicotine may be labile at least to a certain extent. In view of these findings it is difficult

at present to say whether the transmethylation reaction observed in the present study leads to a net synthesis of nicotine or whether it is merely involved in the general transmethylation reactions which are operative in the leaves of the plant. Since it has been shown that nicotine is synthesized mainly in the roots (16), one approach to this question would be to ascertain whether or not a net synthesis of nicotine could be obtained by incubating root enzyme preparations with methionine and nornicotine. Such a study is in progress at the present time in this laboratory.

The studies on the lignin of barley and tobacco plants provide the first conclusive evidence that methyl groups can be transferred intact from sulfur to oxygen in metabolic processes. The observations in the present study along with the finding of Kirkwood, et al. (57) that the methyl carbon of methionine may be a precursor of the methoxyl carbon of ricinine in castor beans and the methylenedioxy groups of protopine in Dicentra hybrids, would lend support to the hypothesis that the acceptance of methyls by oxygen, a reaction not observed in animal metabolism, is a general reaction in plant metabolism.

It is of interest to examine whether the transfer of methyl groups demonstrated in this study represented synthesis of part of the lignin molecule or merely some type of exchange reaction similar to that which may have been encountered in the nicotine study. Stone (58) has shown that in wheat plants fed $C^{14}O_2$ for a short time the total activity acquired by the syringaldehyde portion of lignin during that time remained constant throughout the growth of the plant, that is, the lignin

(as represented by syringaldehyde) was an end product and was not a participant in further metabolic reactions of the plant. Moreover, Stone demonstrated that part of the radioactivity in the lignin degradation products was located in the methoxyl groups. Stone's work would seem to show that the direct transfer of methyl groups demonstrated in the present study was a reaction in the synthesis of lignin.

Methylation study. -- The results of the study with calcium glycolate-2-C^{14} indicate that the alpha carbon of glycolic acid is capable of serving as a source of methyl groups in the metabolism of the tobacco plant. This is in contrast to the studies mentioned in the Introduction where it was pointed out that several isolated systems from both plants (14) and animals (15) were unable to use glycolic acid in methylating guanidineacetic acid. In the present study all of the radioactivity of the nicotine within experimental error was recovered in the methyl group indicating that under the conditions of the experiment none of the alpha carbon of glycolate enters the ring portions of the nicotine molecule.

The glycolate alpha carbon is more rapidly incorporated into the nicotine methyl group than either formate (3) or bicarbonate (56) but the difference between the rates of incorporation of the methyl group of methionine (3) and of the alpha carbon of glycolate is so small a definite conclusion cannot be drawn about which of the two enters more rapidly. On the other hand the rate of entry of the alpha carbon of glycine (13) is somewhat higher than the rate of entry of the glycolate

alpha carbon. It therefore seems probable that the glycine is not converted to glycolic acid before the alpha carbon is incorporated into the methyl group of nicotine. Hamill (13) has suggested two other possibilities for the mechanism of methylation by the alpha carbon of glycine which seem more probable in view of the present findings. These are: 1) glycine may be oxidatively deaminated to give glyoxylic acid the alpha carbon of which is then reduced without going to formate, or 2) the nitrogen of glycine may be incorporated directly into the pyrrolidine ring of nicotine and the alpha carbon remains to form the methyl group. The latter proposal is not too attractive in view of the experiments with ornithine-2-C^{14} to be discussed later, but the former proposal is a definite possibility.

It seems highly improbable that the alpha carbon of glycolate is converted to formate before its incorporation into the nicotine methyl group, but as was suggested above the glycolate may be converted first to glyoxylic acid, the carbonyl group of which could give rise to methyl groups through reduction. There is ample evidence that glycolic acid can be oxidized by higher plants to yield glyoxylic acid (59), and although this oxidation of glycolate to glyoxylate seems to be a roundabout pathway further studies are necessary to determine its validity. The rate of incorporation of the carbonyl carbon of glyoxylic acid into the nicotine methyl group is being studied in this laboratory at the present time, and the results should aid in clarifying this situation.

A further possibility concerning the methylating capacity of glycolic acid should be mentioned in view of the findings of Tolbert and

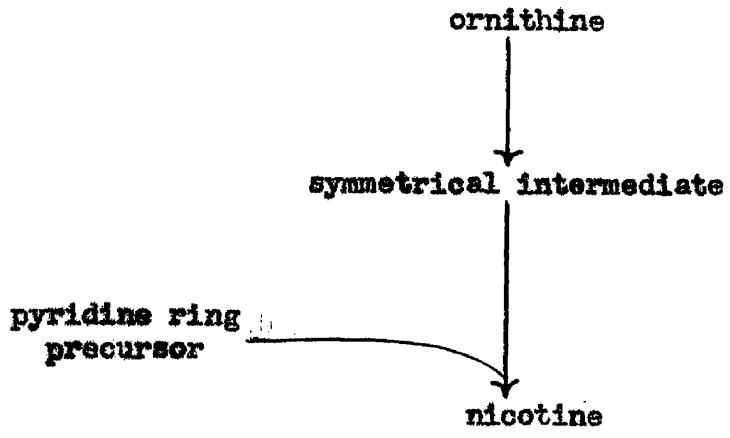
Cohan that the alpha carbon of glycolic acid can give rise to the beta carbon of serine in barley and wheat leaves (60). It is known that the beta carbon of serine can serve as a methyl group precursor for methionine, choline and creatinine in higher animals (61), and it is conceivable that at least part of the glycolic acid may enter the nicotine molecule by way of serine in higher plants. The administration of serine-3-C^{14} to tobacco plants is being conducted now in this laboratory and the results should give some indication as to the plausibility of such a pathway.

Finally, the possibility of the alpha carbon of glycolic acid being converted to formaldehyde before its conversion to methyl groups should be pointed out. Preliminary data in this laboratory indicates that formaldehyde enters the nicotine molecule more rapidly than any previously tested compound, but it still is not known whether all of the radiocarbon of the nicotine resides in the methyl group (62).

Glycolic acid is found in small amounts in higher plants (63), and as yet is the only two carbon organic acid, which has been found to accumulate carbon-14 during short time photosynthesis with $C^{14}O_2$ (64). The alpha carbon of glycolic acid may therefore be a rather important source of methyl groups in higher plants.

Studies with ornithine-2-C^{14}. -- As has already been pointed out half of the radiocarbon present in nicotine from plants fed ornithine-2-C^{14} is located in the 2 position of the pyrrolidine ring. The other half of the radiocarbon is located somewhere in the 3, 4 and 5 positions, and although it is not known definitely that the 5 position contains

the remaining half of the radioactivity this appears to be the logical position. This would call for a symmetrical intermediate in the formation of the pyrrolidine ring of nicotine from the ornithine molecule as is shown in Scheme I.



Scheme I

There are several possibilities for the formation of a symmetrical molecule from ornithine. The first possibility would be the formation of the symmetrical diamine, putrescine, directly by the decarboxylation of ornithine. A second pathway, which is more indirect, would involve the oxidative deamination of ornithine to give possibly glutamic semialdehyde, which on cyclization could yield Δ^1-pyrroline-5 carboxylic acid. The latter may be decarboxylated to give pyrroline, which would become symmetrical by shifting the double bond, or it may first be reduced to give proline, which would yield pyrrolidine, a symmetrical molecule, when decarboxylated. Finally, the possibility of ornithine being converted by decarboxylation followed by oxidative

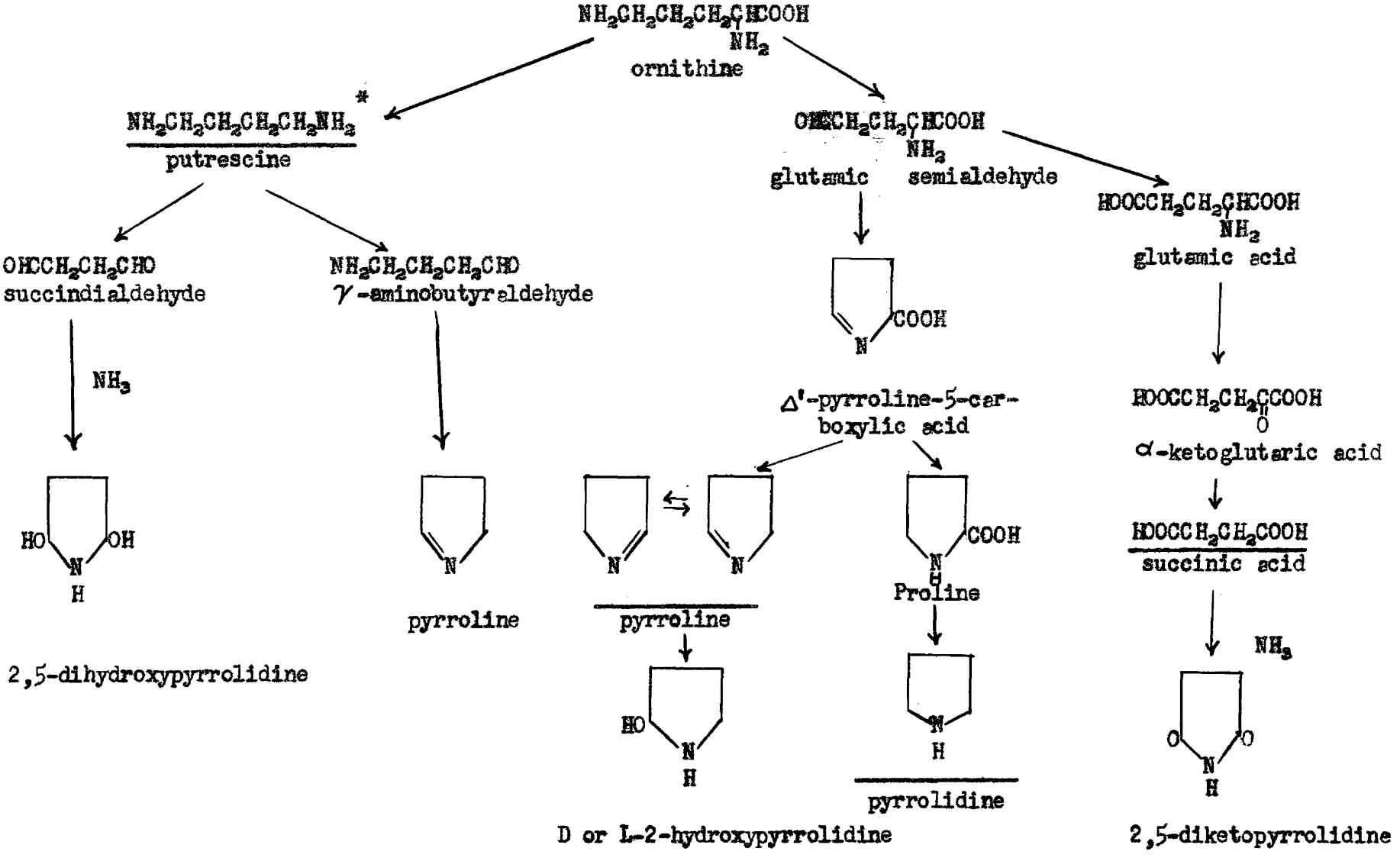
deamination to either succindialdehyde or succinic acid may be mentioned although it seems less likely than the two previously mentioned possibilities. The several possibilities are illustrated in Scheme II.

Although putrescine is generally considered a product of the activity of microorganisms, there is some evidence that diamines may play some role in the metabolism of higher organisms. Zeller (65) and Best and McHenry (66) have reported the occurrence of a diamine-oxidase system in animal tissues. More recently Cromwell (67) has found that putrescine stimulates the synthesis of the alkaloid, hyoscyamine, when administered to both Atropa belladonna L. and Datura stramonium L. In a later publication (68) Cromwell reported the preparation of an enzyme system, which was capable of oxidizing putrescine with the formation of ammonia and an aldehyde, from the tissues of A. belladonna. Furthermore he was able to isolate small amounts of putrescine from the leaves and upper stems of A. belladonna and D. stramonium. On the basis of his results Cromwell postulated that putrescine could be converted to either succindialdehyde or γ-amino butyraldehyde, which could then be incorporated into the hyoscyamine molecule. If putrescine is considered as an intermediate in the formation of the pyrrolidine ring of the nicotine molecule, then it seems necessary to postulate an oxidative or possibly a hydrolytic deamination of the putrescine before cyclization can take place as Cromwell has done in the hyoscyamine study.

If one amino group of putrescine is lost by oxidative deamination, γ-amino butyraldehyde results. The latter could cyclize to give

SCHEME II

POSSIBLE INTERMEDIATES IN THE BIOSYNTHESIS OF NICOTINE FROM ORNITHINE



* The symmetrical intermediate occurring first in each series of reactions is underlined.

pyrroline or it may condense directly with a pyridine ring precursor and be cyclized later. On the other hand if oxidative deamination occurs at both ends of the putrescine molecule then succindialdehyde results. This dialdehyde might then condense with either ammonia or methyl amine to give a 2, 5 dihydroxy pyrrolidine derivative. The latter compound must undergo a dehydration and reduction before the nicotine molecule is finally formed. Putrescine is therefore a conceivable intermediate in the biosynthesis of nicotine since it is symmetrical and could undergo further reactions which would lead to the pyrrolidine ring.

Perhaps a more likely pathway for the incorporation of ornithine into the nicotine molecule is through the formation of proline or some compound closely related to proline. The mechanism for the conversion of ornithine to proline in animals has been rather well established. Schoenheimer and co-workers (69) have found that deuterated ornithine gives rise to labeled proline in mice. Schemes, which involve the conversion of ornithine to either α-keto- δ-amino valeric acid or glutamic semialdehyde followed by cyclization to give Δ^1 pyrroline-5-carboxylic acid and reduction of the latter to give proline, have been proposed by several investigators to explain the conversion of ornithine to proline in animals (70). More recently, Stetten (71) using ornithine labeled in either the alpha position or the delta position with N^{15} has shown that the delta amino group of ornithine is lost instead of the alpha amino group so that glutamic semialdehyde is the more likely intermediate in the conversion of ornithine to proline

in mice. It seems very likely that higher plants could also convert ornithine to proline although there is no conclusive evidence to support this supposition at the present time. If tobacco plants are able to perform this conversion, there are two symmetrical intermediates which could result, namely, pyrroline, which arises from the decarboxylation of Δ^1 pyrroline-5-carboxylic acid, or pyrrolidine from the decarboxylation of proline. A condensation between the pyridine ring precursor of nicotine and pyrroline or pyrrolidine or possibly one of their metabolites would then be necessary for the formation of the nicotine molecule.

The final possibility suggested for the formation of a symmetrical intermediate from ornithine is the conversion of ornithine to succindialdehyde or succinic acid. Both of these conversions involve the loss of all the nitrogen of ornithine and might provide a means for the entrance of the amino group along with the alpha carbon of glycine into the N-methyl group of nicotine as was suggested by Hamill (13). The succindialdehyde might be formed by the oxidative deamination of putrescine as has already been mentioned whereas the succinic acid might arise from α-ketoglutaric acid by oxidative decarboxylation. The α-ketoglutaric acid is derived from glutamic acid, which can arise from ornithine (69).

There is no basis for choosing among these various proposals at present so they can only be suggested as hypothetical schemes.

In view of the present findings Trier's (22) proposal that nicotine is derived from a single amino acid, proline, is only partially correct.

It is true that the pyrrolidine ring may arise from proline but the pyridine ring apparently does not. Robinson had suggested that ornithine was a likely precursor of the pyrrolidine ring in nicotine in 1917 (21). It is interesting that he suggested an N-methyl compound (N-methyl-2-hydroxypyrrolidine) as the compound, which would condense with the pyridine ring precursor (acetone dicarboxylic acid in his scheme). As yet it is not known exactly when the methylation reaction takes place, before the pyrrolidine and pyridine rings are joined or after they are joined. In any event it is possible as Robinson suggests that the pyrrolidine ring precursor may be methylated previous to the condensation with the pyridine ring precursor.

Mortimer (23) has suggested that part or all of the side chain of kynurenine, which can be derived from tryptophan in living organisms (72), may be incorporated into the pyrrolidine ring. It is clear that this is not too likely in view of the present findings. Indeed Bowden has already shown that the beta carbon in the side chain of tryptophan is not incorporated into the nicotine molecule (26). The present study also explains the finding of Dawson and co-workers that the carboxyl carbon of nicotinic acid is not available for nicotine synthesis (25). Nevertheless, the possibility that tryptophan or nicotinic acid may give rise to the pyridine ring of nicotine is not ruled out. It has recently been suggested (73) that some sort of a condensation between nicotinic acid and N-methylpyrrolidine, which has been found in the alkaloid fraction from tobacco plants (74), would seem to be a logical

step in the synthesis of nicotine. Trier (22) had proposed a similar condensation between nicotinic acid and proline many years before, but the proline molecule itself possesses asymmetry, and this condensation would be inconsistent with the findings of the present study. A condensation between nicotinic acid and some pyrrolidine ring precursor would be entirely consistent with the findings of the present study, but such a condensation would necessarily involve the loss of the carboxyl group of nicotinic acid and there seems to be no precedent for such a reaction.

One further point should be mentioned in connection with the union between the pyrrolidine and pyridine rings, and that is that only l-nicotine is formed in nature. Since the asymmetric carbon atom in nicotine is located in the pyrrolidine ring, either the ring precursor possesses an asymmetric carbon or the condensation of the pyridine and pyrrolidine rings results in an asymmetric synthesis. If an asymmetric pyrrolidine molecule is formed before the condensation it must be formed from a symmetrical compound to be consistent with the results of the present study. Such a compound could be 2-hydroxypyrrolidine which might arise either from the hydration of the double bond of pyrroline or the oxidation of pyrrolidine. If 2-hydroxypyrrolidine is formed then it is conceivable that the nicotine ring structure could be formed by splitting out a molecule of water between pyridine and 2-hydroxy-pyrrolidine.

At present it is impossible to propose a definite pathway for the biosynthesis of nicotine since the nature of the precursor of the

pyridine ring is unknown. In addition the nature of the immediate precursor of the pyrrolidine ring is not clear so that considerably more study is required before a definitive mechanism can be proposed.

Finally, the question arises as to whether ornithine is a natural metabolite in tobacco plants. James (75) has found that both ornithine and arginine when administered to detached leaves of belladonna plants give rise to an increased synthesis of the tropane alkaloids. Moreover, the increase in alkaloid when ornithine was fed was apparently in excess of the increase when arginine was fed. James was also able to show the presence of L-ornithine in the free state in young shoots of belladonna plants. Arginase has also been found in the extracts of roots and shoots of belladonna, tobacco and tomato (76). Since arginine is known to occur in the proteins of tobacco plants (77), it seems entirely possible that ornithine could be produced from arginine through the action of arginase and therefore could be available for the synthesis of nicotine.

SUMMARY

SUMMARY

1. A study in which methionine, doubly labeled in the methyl group with C^{14} and deuterium, was administered to intact tobacco plants has established that the methyl group of methionine can give rise to the methyl group of nicotine through transmethylation.

2. The methyl group of methionine was shown to be transferred as a unit to form the methoxyl groups of lignin in barley and tobacco, a reaction, which involves the direct transfer of methyl groups from sulfur to oxygen. It was indicated that the incorporation of the methyl group of methionine into lignin represented a synthesis of part of the lignin molecule.

3. After the administration of calcium glycolate-2-C^{14} to tobacco plants, radioactive nicotine has been isolated. Most if not all of the radioactivity has been shown to reside in the methyl group.

4. Tobacco plants fed ornithine-2-C^{14} hydrochloride produce nicotine, which has been shown to be radioactive. It has been found that half of the carbon-14 in the isolated nicotine is localized in the 2 position of the pyrrolidine ring of the nicotine. The other half of the radioactivity has been postulated to reside in the 5 position of the pyrrolidine ring, and therefore it was concluded that a symmetrical intermediate is formed from ornithine before its incorporation into the pyrrolidine ring of nicotine. None of the radioactivity has been found in the

pyridine ring of nicotine, and less than two percent of the radioactivity of the nicotine has been found in the methyl group.

5. Lysine-2-C^{14} hydrochloride when administered to tobacco plants under the same conditions as were employed in the ornithine study gives rise to nicotine possessing considerably less radioactivity. It has been postulated that lysine is not incorporated directly into the nicotine molecule but is first converted to some precursor of proline or ornithine such as glutamic acid.

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APPENDIX

APPENDIX

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 = 235.2 c.p.m., W = 67.3 mg., M = 620, T = 23.8 mg./cm^2, b = 0.27.

$$A_m = \frac{235.2 \times 620}{67.3 \times 0.27} = 8.03 \times 10^3 \text{ c.p.m./mM.}$$