METHYLATION STUDIES IN HIGHER PLANTS AND ANIMALS

By

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VITA

The author was born March 13, 1927 in Youngstown, Ohio, and received his secondary education at Woodrow Wilson High School in Youngstown. He served as a laboratory technician in the United States Navy Medical Corps for two years, and entered Youngstown College in January 1947. He transferred to Ohio University in September 1948, and was graduated in June of 1950 with a Bachelor of Science Degree. He enrolled in the Graduate School of Michigan State University in the Fall of 1950 as a Teaching Assistant in Chemistry, remaining at that position until recalled to naval service in June of 1951. After completing a year and a half of duty as a biochemistry instructor, he resumed his studies at Michigan State University in the Fall of 1952 as a Special Graduate Research Assistant under an Atomic Energy Commission Grant. He received the Master of Science Degree in June of 1953, presenting as his thesis "The Role of the Alpha Carbon of Glycine in Methylation Studies in Tobacco Plants." He is married to the former Maritta Floyd of Beaufort, S. C., and has a daughter, Sebette Ann. Upon graduation the author will be associated with Eli Lilly and Company as a research biochemist.

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AN ABSTRACT

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ABSTRACT

These investigations were conducted to study three aspects of methylation reactions in higher plants and animals: 1) A study of possible N-methyl group precursors for nicotine in tobacco plants; 2) A comparative study of O-methyl group precursors for tobacco lignin; 3) A study of the extent of incorporation of formaldehyde into the C-methyl group of thymine and the uracil carbons of the purines of rat deoxyribonucleic acid.

D,L-serine-3-C^{14} and glycine-1-C^{14} were administered to tobacco plants, and the nicotine was isolated from the plants and checked for radioactivity. The nicotine from the serine-fed plants possessed radioactivity, whereas the nicotine from the glycine-fed plants possessed no radioactivity. Most of the radioactivity of the nicotine isolated from serine-fed plants was located in the methyl group. Therefore the beta carbon of serine appears to be a precursor for the methyl group of nicotine, whereas the carboxyl carbon of glycine does not seem to have a role in nicotine synthesis under the conditions used. When compared with other nicotine methyl group precursors, the beta carbon of serine seems to be incorporated to a lesser extent than formaldehyde and the alpha carbon of glycine, and to about the same extent as the methyl group of methionine, choline, and betaine, and the alpha carbon of glycolate. It was proposed that formaldehyde, the alpha carbon of glycine, and the beta carbon of serine enter the nicotine methyl group by way of an "active formaldehyde" intermediate.

Lignin was isolated from tobacco plants fed formaldehyde-C^{14}, D,L-serine-3-C^{14}, glycine-2-C^{14}, glycine-1-C^{14}, glycolate-2-C^{14}, formate-C^{14}, betaine-methyl-C^{14}, D,L-methionine-methyl-C^{14}, and choline-methyl-C^{14}, and all of the respective lignin samples were found to possess radioactivity. Demethylation of lignin from plants fed formaldehyde, methionine, serine, glycolate, formate, and glycine-2-C^{14} demonstrated that most of the radioactivity was located in the methoxyl group. Low recoveries of radioactivity upon demethylation of lignin from plants fed choline and betaine are unexplained at present, whereas the carboxyl carbon of glycine seems to enter the lignin molecule randomly. The beta carbon of serine seems to be incorporated into the O-methyl group of lignin to the greatest extent, and formaldehyde appears to be incorporated to the next best extent. A proposed "active formaldehyde" intermediate is attractive for the formation of O-methyl groups. However, the beta carbon of serine may possibly enter the O-methyl group by a second pathway involving the hydroxyl oxygen attached to the beta carbon.

Formaldehyde-C^{14} injected intraperitoneally into rats gave rise to radioactivity in the pyrimidines and purines of deoxyribonucleic acid isolated from the rats. Thymine, adenine, and guanine possessed much greater activity than did cytosine. The radioactivity of thymine was assumed to be located in the methyl group because of the low activity of cytosine and the results of other "1-carbon" compound studies. When compared with studies involving formate, the methyl group of methionine,

and the beta carbon of serine, formaldehyde appears to be intermediate between formate and either the methionine methyl group or the serine beta carbon as a precursor for the thymine methyl group. Formaldehyde may enter the thymine methyl group by way of an "active formaldehyde" intermediate, which may be converted to an "active formate" compound to give rise to the uracil carbons of the purines.

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INTRODUCTION

The investigations undertaken here were concerned with some of the possible metabolic methylation reactions in higher plants and animals. The first study was to examine compounds which might supply information about precursors for the N-methyl group of nicotine in tobacco plant metabolism. The second investigation was a comparative study of the possible O-methyl group precursors for the lignin molecule in the tobacco plant. The third study dealt with the possible incorporation of formaldehyde into the C-methyl group of thymine and the ureide carbons of the purines.

Transmethylation was first shown to be a reaction in animal metabolism by du Vigneaud in 1940 (1). Since that time a number of compounds have been examined in animals and have been shown to give rise to methyl groups either by direct transmethylation or by reduction of a "one-carbon unit" to a methyl group. The origin of methyl groups in higher plants was not studied until later, although Barrenscheen and von Vályi Nagy in 1943 (2) found that the creatine content of wheat germ was increased upon the administration of methionine and glycocyamine. In 1952 Brown and Byerrum (3) showed that formate and the methyl group of methionine could serve as N-methyl group precursors for the alkaloid, nicotine, in the tobacco plant, methionine being incorporated to a greater extent than formate into the methyl group. At about the same time, Marion, Kirkwood, and co-workers (4) found that

methionine could enter the methyl groups of the barley alkaloids, hordenine, N-methyl tyramine, and gramine to a greater extent than formate. They however noted that the methyl group of choline did not give rise to the methyl group of hordenine. Byerrum and Wing (5) in studies using tobacco plants, observed that the methyl group of choline could act as a precursor for the methyl group of nicotine and was incorporated at about the same rate as methionine. That methionine could serve as a precursor for the O-methyl and N-methyl groups of the castor bean alkaloid, ricinine, was noted by Dubeck and Kirkwood (6), but it was also found that choline and formate failed to give rise to these methyl groups. The reason postulated for the failure of choline to serve as a methyl group precursor in the barley plant was the possibility of the absence of an enzyme system to convert choline to betaine since betaine has been shown to be a methyl group precursor in animals (7) and recently in plants (8).

Direct transmethylation has been shown to occur in higher plants by Byerrum, Flekstra, Dewey and Ball (9,10), who by the use of methionine doubly labeled with carbon-14 and deuterium observed the same deuterium to carbon-14 ratio in the methyl group of nicotine and the methoxyl group of lignin from both tobacco and barley as was in the methyl group of methionine. It was also shown that methionine entered the methoxyl group of barley lignin to a greater extent than formate (10). The alpha carbon of glycine, when studied in the animal, showed little ability to serve as a methyl group precursor. However when studied in the tobacco

plant (11), it was shown to be as good if not better than methionine and choline as a methyl group precursor of nicotine. The alpha carbon of glycolate (12) also has been shown to be a methyl group precursor. Serine, glycine, and glycolate appear to be interrelated in plant metabolism (13), and other studies (14,15) have shown the interconversion of glycine and serine, with the alpha carbon of glycine becoming the beta carbon of serine. It then was of interest to ascertain whether serine was a methyl group precursor in plants. Serine has been shown to act as a methyl group precursor in animals. In the present study serine labeled with carbon-14 in the beta position therefore was administered to tobacco plants, the nicotine isolated, and the radioactivity observed compared with the radioactivity of nicotine after administration of glycine-2-C^{14} (11) to see if a possible metabolic pathway might be suggested.

The unusual incorporation of the alpha carbon of glycine into methyl groups in plant metabolism also led to the possibility that the carboxyl carbon of glycine might have a role in nicotine synthesis. Previous studies (15) in the animal have indicated that the carboxyl carbon is oxidized to carbon dioxide and the carbon dioxide is not reduced to formate or any other one-carbon unit. However, since in plant metabolism the carboxyl carbon may react differently than in the animal, it was decided to feed glycine labeled with carbon-14 in the carboxyl carbon to tobacco plants, isolate the nicotine, and determine its radioactivity.

The O-methyl group is unknown in animal metabolism but does occur in the higher plant. As mentioned previously Byerrum, Flokstra, Dewey,

and Ball (10) found that methionine and formate could give rise to the methoxyl group of barley lignin, whereas Dubeck and Kirkwood (6) similarly showed that methionine gave rise to the O-methyl group of ricinine. The latter investigation however failed to show that formate and choline gave rise to the ricinine O-methyl group. Further studies on lignin isolated from the barley plant (10) and the tobacco plant have demonstrated the occurrence of transmethylation between methionine and the methoxyl group. Previous to these studies little was known about the origin of the lignin methoxyl group, although Klason (16) suggested that formaldehyde might serve as a methoxyl group precursor. It therefore seemed of interest to investigate a number of possible precursors to determine whether they could give rise to methoxyl groups and to compare the extent of incorporation of these compounds into the methoxyl group. The present study dealt with the isolation of lignin from the tobacco plants to which had been administered DL-serine-3-C^{14}, formaldehyde-C^{14}, DL-methionine-methyl-C^{14}, glycine-2-C^{14}, glycine-1-C^{14}, choline-methyl-C^{14}, formate-C^{14}, glycine betaine-methyl-C^{14}, and glycolate-2-C^{14}.

The biosynthesis of the methyl group of thymine has attracted interest in recent years and has led to investigations of possible precursors in animals. Formate (18) has been shown to give rise to the methyl group of thymine and to the carbons 2 and 8 of the purines. Methionine (18) has been demonstrated to serve as a thymine methyl group precursor, and a source of the ureide carbons of the purines. Serine, as studied by Elwyn and Sprinson (19), has also been shown to

be a precursor for the methyl group of thymine, and for the ureide carbons of the purines. It has been indicated that the carbons and nitrogen of glycine enter the purine molecule (19), and the alpha carbon into the thymine methyl group. Formaldehyde also has been suggested to have a role in the thymine methyl group syntheses, but no data to confirm this suggestion has appeared. Recent work in the rat by Herrmann, Fairley, and Byerrum (49) in which methionine and formate were studied, indicated that formate was used to only twice the extent of methionine as a source of the thymine methyl group, but to about ten times the extent of methionine as a source of the ureide carbons of the purines. They also noted that the methyl group of methionine appeared to enter the methyl group of thymine to a greater extent in relation to the purine ureide carbons than did the formate carbon. These studies along with the serine work suggest the possibility of the role of "active 1-carbon units" at the oxidation states of formate and formaldehyde. As a result of this postulation, formaldehyde labeled with carbon-14 has been administered to rats and the pyrimidines and purines, isolated from the deoxyribonucleic acid of the rat, were examined for radioactivity. The extent of incorporation of formaldehyde as compared with formate and the methyl group of methionine was ascertained.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

Preparation of Plants

The tobacco plants used in the nicotine and lignin studies were a high nicotine strain, Nicotiana rustica L. var. humilis. The seeds were planted in flats containing vermiculite as a non-nutrient supporting material for the plants which were transplanted after a period of two to three weeks. The plants were grown in the greenhouse until a height of about 6 inches was attained, usually after about a 90 day growing period. They were fed twice a week with a nutrient solution which was composed of 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$, 1 g. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4, in 4 liters of tap water. Budding and flowering were noted in some plants during the feeding period, but for the most part were absent.

To prepare the plants for hydroponic administration of the radioactive compound to be studied, the plants were removed from the flats and freed of vermiculite as completely as possible. They were then rinsed carefully with tap water to remove most of the remaining extraneous material and to avoid damage to the roots. The roots were soaked in 0.1 percent solution of a detergent germicide (Detergent germicide No. 1528 manufactured by Wyandotte Chemical Co., Wyandotte, Michigan) for an hour, with occasional agitation, to reduce the number of bacteria present. The roots were then rinsed with distilled water and placed in 125 ml. Erlenmeyer flasks containing 50 ml. of an inorganic nutrient solution. The nutrient solution was a 1:3 dilution of the stock nutrient solution shown in Table I. All the weights are of the anhydrous salts and only

C. P. grade chemicals were used. To prevent the destruction of the administered organic compounds by microorganisms, 0.5 ml. of 1:1000 solution of aureomycin was also added to each flask.

TABLE I

COMPOSITION OF THE STOCK NUTRIENT SOLUTION

Water	1000 ml.	Magnesium sulfate $MgSO_4$	250 mg.
Calcium nitrate $Ca(NO_3)_2$	1 g.	Ammonium sulfate $(NH_4)_2SO_4$	250 mg.
Potassium Chloride KCl	250 mg.	Potassium dihydrogen phosphate	250 mg.
Ferric chloride $FeCl_3$	2 mg.		

The administration of the various experimental methyl group precursors will be described later. All the experiments were carried out in a special fume hood to avoid any health hazard from radioactive material. Artificial lighting was used in all the experimental work for 12 hours each day. The source of light consisted of a 100 watt incandescent bulb and two 36 inch, 30 watt fluorescent tubes, placed about 1½ inches above the top of the leaves and had a light intensity of 200-250 foot candles at the top of the plants. A stream of oxygen was passed through the nutrient solution of each plant for two minutes twice a day to provide aeration for the root systems. Additional nutrient solution was added as needed to keep the volume constant.

Isolation and Purification of Nicotine

Following the period of administration of the possible methyl group precursors, the plants were removed from the flasks, the roots were

rinsed with distilled water, and the excess liquid blotted from the roots with a cheese cloth. The plants were then cut up into small pieces and immediately dried under infrared lamps. The temperature of the plants was left at 80°C. for an hour near the end of the drying period. The dried plant material was finely ground in a mortar, mixed with 20 percent of its weight of calcium hydroxide, and placed into a Kjeldahl flask. The material was steam distilled until the distillate no longer gave a precipitate 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 *in vacuo* to a small volume and the nicotine was purified by two successive azeotropic distillations into 2 ml. of 1 N HCl from alkaline medium, as described by Smith (20). the distillate was concentrated to dryness under reduced pressure and nicotine hydrochloride crystallized out. The salt was dissolved in methanol plus a small amount of water, and a saturated methanolic solution of picric acid was added in excess. After a half hour of standing the nicotine dipicrate crystals which had formed, were filtered off, washed with methanol and recrystallized from hot water. The melting point was 223-224°C (224°C literature (21)).

Methylation Study with Glycine -1-C^{14}

Several studies (3,5,11,12) in this laboratory have shown that tobacco plants can absorb various organic compounds through the roots. An earlier study using glycine -2-C^{14} (11) indicated that about 2 mg. of glycine could be absorbed by the roots in a period of about four days.

The hydroponic administration of the glycine -1-C^{14}, under study in the present experiments, seemed feasible.

Administration of Glycine -1-C^{14}

Two experimental feeding trials were run with glycine -1-C^{14},* using 30 tobacco plants in each experiment. The plants were prepared as described previously and 1.33×10^{-5} moles of glycine with a radioactivity of 1×10^{6} counts per minute was added to the nutrient solution in each flask. The molar quantity and radioactivity was calculated to be equal to compounds previously administered (3,5,11,12). After the seven day administration period, the nicotine was isolated as the dipicrate as described in the previous section. The nicotine dipicrate was ground finely with a mortar and pestle, and 60 mg. of it was plated on tared aluminum discs for counting.

Results

No radioactivity was found in the nicotine dipicrate from either trial after feeding glycine -1-C^{14}, indicating that the carboxyl carbon of glycine does not enter into nicotine synthesis under the experimental conditions used.

Methylation Study with Serine -3-C^{14}

Uptake of DL - Serine

As with other possible methyl group precursors, it was decided to feed serine to the tobacco plants from a nutrient solution through the

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* Obtained from Tracerlab, Inc., Boston, Mass.

roots. This procedure would then make it possible to compare the extent of incorporation into nicotine and lignin of the beta - carbon of serine with other methyl group precursors. However, before the administration of the radioactive serine, it was necessary to ascertain the absorption rate of serine, and to determine whether serine was toxic in low concentrations, or whether microorganisms on the roots destroy or change the amino acid before absorption.

Therefore 2 mg. of DL - serine was added to 10 flasks containing nutrient solution. Tobacco plants were placed in 6 flasks, and 6 root fragments 1 cm. long placed in each of 2 flasks. The last 2 flasks were used as uninoculated controls. After two days, the plants and roots were removed from the various flasks, and the solutions analyzed for remaining serine by the ninhydrin method of Harding and MacLean (22). This method was considered adequate since it was shown that plants growing in nutrient solution without added serine excreted nothing to produce color with ninhydrin under the conditions used. The analyses of the solutions containing the plants showed that after two days all of the serine had disappeared. The solutions containing the roots, when compared with the uninoculated controls, showed no decrease in the concentration of serine. These results indicate that serine may be readily absorbed through the roots of tobacco plants from a nutrient solution and that no destruction of the serine by microorganisms occurred under the conditions of feeding.

The non-toxicity of DL - serine in the concentration used was evidenced by the normal growth of the plant stem, production of leaves,

and growth of roots. The above findings agree with the results obtained by Ghosh and Burris (23) which indicate that DL - serine can be utilized by tobacco plants for growth and nitrogen metabolism when administered in a nutrient solution in the presence of ammonium ions.

Administration of DL - Serine -3-C^{14}

In order to duplicate the conditions of previous methylation studies so that valid comparisons of the extent of incorporation into methyl groups could be made between serine and the other compounds, the DL - serine* was administered in the same molar quantity and radioactivity as had been done previously. The DL - serine was fed to 2 groups of 30 tobacco plants as described in the preceeding section of Preparation of Plants. Each plant received 1.6×10^{-5} moles of DL - serine with a radioactivity of 1×10^{6} counts per minute as measured using a thin-end window Geiger-Müller tube.

After the seven day administration period, the nicotine was isolated as the dipicrate as described earlier. The dipicrate was finely ground in a mortar, plated on aluminum counting discs, and counted for radioactivity.

Demethylation of Nicotine

As will be indicated later the nicotine from serine - 3-C^{14} fed plants was radioactive, and therefore it was desirable to determine whether or not the radioactivity was localized in the methyl carbon. The demethylation of the nicotine was done by Brown's (24) modification

* Obtained from the California Foundation for Biochemical Research, Los Angeles, California.

of Pregl's method (25), and the methyl group isolated as methyltriethylammonium iodide, a white solid suitable for counting.

Since the nicotine dipicrate was quite insoluble and unsuitable for demethylation, 200 mg. of nicotine dipicrate was dissolved in sodium hydroxide and the nicotine was azeotropically distilled into 6 N hydrochloric acid. The distillate was placed into the reaction flask of the demethylation apparatus and concentrated to dryness under reduced pressure.

The reaction flask was then attached to the demethylation train and the following reagents added to the flask on the basis of 50 mg. of nicotine: 45 mg. of ammonium iodide, 2 drops of 5 percent gold chloride solution, and 3 ml. of 47.3 percent hydriodic acid. The gas-washing bubbler contained 1.5 ml. of 5 percent sodium thiosulfate-cadmium sulfate solution to remove hydriodic acid and iodine. The delivery tube dipped below the surface of a 5 percent ethanolic solution of triethylamine in the receiving vessel, which was cooled to about -70°C in a methyl cellosolve-carbon dioxide bath. A constant stream of nitrogen, introduced into the side arm of the reaction flask, was passed slowly through the entire apparatus during the demethylation process.

The reaction flask was embedded in a copper oxide bath and was heated to 200°C in 20-25 minutes. Then the temperature was raised slowly to 350-60°C and held there for 45 minutes. After the heat was removed, the flask was allowed to cool and was flushed for 15 minutes with a faster stream of nitrogen. The receiver was then disconnected and the delivery tube was rinsed with absolute ethanol. The rinsings were placed into the receiving flask. This flask was stoppered, the contents mixed,

and allowed to stand overnight at room temperature. The following day, most of the ethanol was evaporated over an infrared lamp with a slow stream of air directed across the liquid surface. The last of the ethanol and triethylamine were removed in a vacuum desiccator. The methyltriethylammonium iodide remaining was a white crystalline compound.

The quaternary iodide was dissolved in a small amount of ethanol and plated with a micropipette on a tared aluminum disc. The ethanol was evaporated over an infrared lamp, and the disc reweighed to obtain the weight of the compound to be counted.

Results

The radioactivity of the nicotine isolated from serine -3-C^{14} fed tobacco plants is presented in Table II. The results are expressed in counts per minute per millimole at "infinite thinness". The column labeled "methyltriethylammonium iodide" shows that most of the radioactivity of the nicotine after the administration of radioactive serine is located in the methyl carbon. The difference in radioactivity of the nicotine in the two experiments was probably due to seasonal variations in growth and metabolism since the plants were raised at different times of the year. Some incorporation of serine into the rings of nicotine may be indicated by the fact that not all the radioactivity was recovered upon demethylation.

TABLE II

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER THE ADMINISTRATION OF SERINE -3-C^{14}

Experiment	Number of Plants	Maximum Specific Activity (Counts per minute per millimole) Nicotine Dipicrate	Methyltriethyl-ammonium iodide
1	30	4.1×10^3	3.5×10^3
2	30	2.2×10^3	2.1×10^3

A sample of the calculations is shown in the appendix.

Lignin Studies

Source of Plant Material

The tobacco plants used in this comparative study were of the strain Nicotiana rustica L. var. humilis. The nicotine of the plants previously had been removed by steam distillation in the presence of calcium hydroxide, and the resulting residue dried. Several groups of plants had been fed different carbon-14 labeled compounds. The labeled compounds, fed in equimolar quantities, and in equal radioactivity, were as follows: DL-methionine-methyl-C^{14} (3), sodium formate-C^{14} (3), choline-methyl-C^{14} (5), glycine betaine-methyl-C^{14} (8), DL-serine -3-C^{14}, glycine-2-C^{14} (11), glycine-1-C^{14}, calcium glycolate-2-C^{14} (12), and formaldehyde-C^{14} (36).* The moles of each compound fed per tobacco plant was 1.4×10^{-6} and the radioactivity was 1.0×10^8 counts per minute.

* I am indebted to S. A. Brown, R. L. Ringler, C. S. Sato, R. E. Wing, and L. J. Dewey for providing radioactive tobacco materials.

Lignin Isolation

Ten to fifteen grams of the dried tobacco plant residue was treated by the method suggested by MacDougall and Deleng (26) to isolate a low nitrogen containing lignin. This method consisted of two 15 minute extractions with ether saturated water to remove nitrogen containing materials, a 20 minute extraction with 5 percent acetic acid to remove some carbohydrates, and finally two 15 minute extractions with an ethanol-benzene (1:2) mixture to remove the fatty materials. A Waring Blender was used to disperse the tobacco plant residue in the solvents, and cheese cloth used as a filter to remove the liquid portion.

The residue obtained from the series of solvent extractions, light brown in color, was broken up into fine pieces, placed in a large beaker and covered with a measured amount (usually 50 ml.) of 70 percent (v/v) sulfuric acid. This suspension was allowed to stand 18 hours at 5°C to hydrolyze and prevent carbonization of the carbohydrates. Then the suspension was diluted to three percent, and boiled gently using glass beads as anti-bump materials for two hours to complete the hydrolysis. The volume was kept constant by the addition of distilled water.

The resulting suspension of lignin in acid was cooled and then filtered on a sintered glass funnel under reduced pressure. Difficulty was encountered in the filtering process since the funnel rapidly became clogged. This trouble was avoided by allowing the lignin to settle, and decanting the supernate before filtration. The dark brown lignin was washed thoroughly with distilled water, partially dried on the filter with suction, and then transferred to a vacuum desiccator to complete the drying process.

Demethylation of lignin

Since lignin isolated from plants fed all of the compounds listed above was radioactive, as will be indicated later, it was of interest to ascertain whether any radioactivity was located in the methoxyl group. For such studies it was necessary to cleave the methyl group and obtain it in a suitable compound for counting. Flokstra's modification (27) of Phillips' method (28) was used to split the methyl group from the oxygen to yield methyl iodide. The methyl iodide was swept into an ethanolic solution of triethylamine to form methyltriethylammonium iodide quantitatively.

Although the lignin demethylation procedure was similar in many respects to the nicotine demethylation procedure there were certain important differences. For this reason the procedure for the demethylation of lignin will be presented in detail for the sake of clarity. The demethylation was carried out in a modified form (24) of the apparatus described by Pregl (25). Approximately 60 mg. of lignin was accurately weighed on cigarette paper, which was previously shown to yield no methyl groups in the demethylation procedure, and placed in the reaction flask. Two ml. of phenol to act as the solvent and 4 ml. of 47.3 percent hydriodic acid were also added to the reaction flask. A gas-washing bubbler attached to the flask contained 1.5 ml. of 5 percent cadmium sulfate-sodium thiosulfate solution recommended by Pregl to remove iodine and hydriodic acid. The delivery tube was below the surface of the 5 percent ethanolic solution of triethylamine in the receiving vessel, which was cooled in a methyl cellosolve-carbon dioxide bath to about -70°C.

The reaction flask was embedded in a copper oxide bath during the process of demethylation. A constant stream of nitrogen was run slowly through the side arm of the reaction flask and then through the entire apparatus. The reaction mixture was brought slowly to 150°C, and held there for 45 minutes, then slowly raised to 200°C and held for 30 minutes. At the completion of the heating, the bath was allowed to cool for 15 minutes, and at the same time a faster stream of nitrogen was used to sweep the reaction train. After cooling, the receiving vessel was disconnected and the delivery tube washed with absolute ethanol into the receiving vessel which was then stoppered, mixed and allowed to stand overnight at room temperature. The contents of the receiving flask were transferred to a small beaker and evaporated almost to dryness over an infrared lamp with a slow stream of air blowing across the surface of the liquid. The last of the ethanol and triethylamine were removed in a vacuum desiccator, yielding a white solid, methyltriethylammonium iodide. The compound was weighed and counted for radioactivity.

Determination of radioactivity

The lignin samples were ground very finely in a mortar and 40 mg. amounts were weighed on tared aluminum discs. The sample surface was made as smooth as possible with the end of a spatula. The 40 mg. weight was used throughout the counting of lignin to facilitate the comparison of radioactivity between different samples.

The methyltriethylammonium iodide was dissolved in a small amount of absolute ethanol, and plated on tared aluminum discs with a micropipette in such a manner to obtain a smooth surface. The discs were

then reweighed to obtain the sample weight and kept in a desiccator until counted.

All the counts were made on a Model 163 Scaling Unit manufactured by Nuclear Instrument and Chemical Corporation. The discs containing the samples were placed on the top shelf of the end window counting assembly. To correct for self absorption, the counts were converted to "infinite thickness" by reference to curve based on the activity of a standard C^{14} sample.

Results

Lignin is not a chemical entity so that specific activity on a molar or millimolar basis could not be used. The radioactivity of the lignin shown in Table III was therefore expressed as counts per minute based on a definite weight of lignin counted. The weight as shown in the "Lignin" column was 60 mg. After demethylation, the methyltriethylammonium iodide recovered from 60 mg. of lignin was weighed and a weighed portion counted. The column labeled "Methyltriethylammonium iodide" gives the counts per minute of total quaternary iodide from the demethylation of 60 mg. of lignin. A sample of the calculations for obtaining counts per minute at "infinite thinness" is shown in the appendix.

The results indicate that all of the compounds examined enter the lignin molecule, but they are incorporated in varying degrees. The beta carbon of serine appears to be incorporated to a greater extent than any of the other compounds administered to the plants. The carbon of formaldehyde was second in extent of incorporation when compared to the other substance fed. The other compounds studied give results that are

similar to those obtained in the nicotine studies (3,5,11,12) with the exception of formate which seems to be incorporated into lignin faster than into nicotine when compared to the other precursors.

The values obtained for choline and betaine should be multiplied by three for a better comparison since only one methyl group in each compound was labeled with carbon-14. This involves the assumptions that 1) only one methyl group is removed from each compound and 2) the labeled methyl group is not preferentially removed or retained so that each compound has a one to three chance of losing the labeled methyl group.

Examination of the values in the methyltriethylammonium iodide column indicate that the majority of the carbon-14 in the labeled compounds entered the methoxyl group of lignin. The beta carbon of serine would therefore appear to be the best O-methyl group precursor studied thus far, with formaldehyde being second best. Methionine (3), the alpha carbon of glycine (11), and the alpha carbon of glycolate (12) give results similar to the N-methyl group of nicotine precursor studies. As stated above in the lignin results, formate appears to be incorporated to a greater extent into the O-methyl group than into the N-methyl group when compared to the other precursors. It is not known why such low recoveries of counts in the methoxyl group was encountered in the choline and betaine studies, but these are being investigated at present. The carboxyl carbon of glycine probably goes to carbon dioxide and random distribution is obtained since the percent radioactivity of the lignin recovered on demethylation was similar to the recovery obtained after feeding bicarbonate (29).

The difference in counts observed from a particular compound in two different experiments may be due to seasonal variations in growth and metabolism.

TABLE XII

COMPARISON OF VARIOUS COMPOUNDS AS METHOXYL GROUP PRECURSORS IN TOBACCO LIGNIN

Trial	Compound Fed	Lignin (cpm/60 mg)	Methyltriethyl-ammonium iodide (cpm)	Percent Recovery of Counts
1	Serine -3-C^{14}	8100	7240	89.5
2	Serine -3-C^{14}	5360	4660	87
3	Formaldehyde -C^{14}	4415	4350	98.5
4	Formaldehyde -C^{14}	4020	3930	97.8
5	Methionine-methyl -C^{14}	1820	1770	97.4
6	Methionine-methyl -C^{14}	1520	1492	98.2
7	Glycine -2-C^{14}	1820	1600	88
8	Glycine -2-C^{14}	1650	1527	92.5
9	Glycolate -2-C^{14}	1740	1263	72.5
10	Formate -C^{14}	700	709	101
11	Choline-methyl -C^{14}	573	114	20
12	Betaine-methyl -C^{14}	665	188	28.3
13	Glycine -1-C^{14}	716	23	3.2
14	Glycine -1-C^{14}	675	11	1.6

Incorporation of Formaldehyde into Thymine and the Purines

Administration of formaldehyde -C^{14}

Two male albino rats, weighing approximately 160 g. each, were used in each trial. Each rat was injected intraperitoneally with 1 ml. of a water solution containing 0.1mM of formaldehyde* having a radioactivity of 0.1 mc. After twenty-four hours the rats were killed by etherification, immediately decapitated, and the blood allowed to drain out of the bodies. The rats were then cut open and the viscera removed, washed, and immediately frozen on solid carbon dioxide. The stomachs and intestines were cut open and cleaned of food particles before freezing.

Isolation of the Mixed Polynucleotides

The frozen viscera from two rats were cut into small pieces and placed in a Waring Blender. Two hundred ml. of cold absolute ethanol was also added to the blender and the mixture homogenized for five minutes. The homogenate was then centrifuged in a refrigerated centrifuge at 1340 x g., and the supernatant liquid discarded. The tissue was extracted with 200 ml. of a 3:1 ethanol-ether mixture in a boiling water bath for five minutes to remove lipids. The extraction was repeated three times, the extract being discarded each time. The tissue was finally washed twice with ether and air dried.

The dry residual material was placed in a mortar and sufficient 10 percent sodium chloride solution added to make a paste. Then 0.5 g. of

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* Obtained from the Isotopes Specialties Co., Glendale, California.

120 mesh carborundum powder was added and the mixture ground for 15 minutes with a pestle. The mixture was then transferred to 2 centrifuge bottles with 300 ml. of 10 percent solution of sodium chloride. The suspension in the 2 bottles was heated on the steam bath for 20 minutes, and then stirred slowly with mechanical stirrers for 24 hours. The suspension was centrifuged and the supernatant liquid was decanted into a beaker. One hundred ml. of the 10 percent sodium chloride solution was added to each bottle, heated again for 20 minutes on the steam bath, and stirred for 12 hours. After centrifuging and decanting, the residue was washed with a small amount of the sodium chloride solution, and the washings and supernatant fluids combined in a large beaker. Two and a half volumes of ethanol was added to the contents of the beaker, the mixture stirred and allowed to stand overnight in the cold. A white fibrous material formed immediately upon the addition of the alcohol and had settled to the bottom of the beaker by the next day. The mixture was centrifuged, and then the residue washed 3 times with ethanol. Finally it was washed twice with ether. The mixed polynucleotides were allowed to dry in air and the white residue was weighed. (Yield of about 1 g. obtained.)

Isolation of Deoxyribonucleic Acid

The deoxyribonucleic acid was isolated by the method of Hammarsten (31). The mixed polynucleotides were placed in an Erlenmeyer flask and sufficient 0.1 N NaOH added to make a 1 percent solution. The mixture was heated in a boiling water bath for two hours, the polynucleotide

dissolving seen after being heated. The solution was adjusted to pH2 with 2 N hydrochloric acid and a gummy precipitate formed. The suspension was transferred to a centrifuge bottle, one-tenth volume of 0.1 M lanthanum nitrate added to precipitate the deoxyribonucleic acid, and the mixture centrifuged. The supernatant solution containing the mononribonucleotides was discarded, and the precipitate was transferred to a 15 ml. centrifuge tube with the aid of 2 ml. of 0.01 M lanthanum nitrate. The precipitate was washed twice with 0.01 M lanthanum nitrate. One ml. of 1 M potassium carbonate solution and 4.5 ml. of water were added to the precipitate and heated for five minutes to decompose the lanthanum precipitate. After centrifuging and cooling the supernatant fluid gelled, but liquefied upon warming. The precipitate was decomposed two more times with 0.5 ml. of 1 M potassium carbonate solution and 2.5 ml. of water. The supernatant solutions were combined in a beaker, adjusted to slightly acid pH with glacial acetic acid, and boiled to remove carbon dioxide. The deoxyribonucleic acid was precipitated by the addition of 4 volumes of ethanol and allowed to stand overnight in the cold. The mixture was then centrifuged and the precipitate washed 3 times with ethanol, twice with ether, and allowed to air dry. The deoxyribonucleic acid, a nearly white powder, was then weighed (usually a weight of about 180 mg. was obtained).

Hydrolysis of Deoxyribonucleic Acid

The entire deoxyribonucleic acid sample was placed into a 10 ml. volumetric flask with a ground glass stopper. Two ml. of 7.5 N

perchloric acid was added to the flask and the mixture heated for one hour on the steam bath with occasional shaking. The mixture was cooled, transferred to a 15 ml. centrifuge tube with the aid of 1 ml. of water, and centrifuged. The residue was then washed with 0.5 ml. of water, the combined supernatant solutions placed in another centrifuge tube, and 4.7 ml. of 3.47 N KOH added to the solution to obtain a pH of 10-11. The potassium perchlorate precipitate, which resulted, was centrifuged off, and washed with 1 ml. of water. The supernatant solutions, containing the purines and pyrimidines, were combined and concentrated to 5 ml. by a stream of air directed across the liquid surface.

Separation of Nitrogen Bases

The separation and purification of the nitrogen bases were done by methods suggested by Cohn (30). Dowex 1 resin in a 2 by 30 cm. column was washed with 1 N hydrochloric acid and then with water. The hydrolysate was placed on the column and allowed to filter into the resin. The cytosine was eluted with a flow rate of 3 ml. per minute with 0.015 M ammonium formate buffer of pH 10.1. Thirty-five ml. fractions were collected. The cytosine elution was detected with a Beakmann Model DU Spectrophotometer by measuring the absorbancy at 274 mu. After the cytosine was off the column, 500 ml. of 0.015 M ammonium formate buffer of pH 9.1 was used to wash the column, checking absorbancies at 260 mu for any possible elution of thymine. No thymine elution was observed. To elute the thymine, 0.015 M ammonium formate buffer of pH 8.25 was used, and the elution was followed by measuring the absorbancy of the eluate

at 260 mu. After the elution of thymine, the adenine and guanine were eluted with 300 ml. of 1 N HCl.

Cytosine Purification

The cytosine eluate was concentrated to 5 ml. under reduced pressure and 3 drops of 12 N HCl added to the solution. The solution was placed on a Dowex 50 column (1 1/2 x 23 cm.) in the hydrogen ion form, and eluted with 1 N HCl. The elution was again followed by measuring the absorbancy of the eluate at 274 mu.

Adenine and Guanine Purification

The purine eluate was concentrated to dryness under reduced pressure several times to remove most of the hydrochloric acid, transferred to a beaker with a small amount of water, and evaporated to dryness with a stream of air. The residue was taken up in 4 ml. of 0.1 N HCl, and placed on a Dowex 50 column (1 x 12 cm.) in hydrogen ion form. The column was washed with 100 ml. of 0.1 N HCl, checking the absorbancy of samples at 260 mu to be sure that the purines were not eluted. The purines were then eluted with 3 N HCl, with the collection of 10 ml. samples at a flow rate of 2 ml. per minute. The purine separations were followed by measuring the absorbancies at 249 mu and 260 mu. Guanine gives a ratio of absorbancies of 249 mu to 260 mu of 1.3, and adenine gives a ratio of 0.82.

Radioactivity Measurement

Aliquots of the various fractions collected from the chromatographic columns were placed on platinum dishes and evaporated to dryness over an

infrared lamp. One ml. of the purified cytosine solution, 1 ml. of the thymine solution, and 0.5 ml. each of the purified guanine and adenine solutions were the aliquots used in each case. Since the amount of each nitrogen base plated was known from the optical density measurements and molar extinctions, the specific activity was expressed as counts per minute per micromole. The molar extinction of cytosine in acid solution was obtained from a prepared chart, and the molar extinctions of guanine and adenine in 3 N hydrochloric acid and of thymine in 0.015 N ammonium formate buffer, pH 8.25, were determined. A Tracerlab internal flow Geiger counter was used to measure the radioactivity.

Criteria of Purity

The chromatographic fractions of each nitrogen base were determined to be satisfactorily pure as judged by the ratio of radioactivity to absorbancy, and from the ratio of absorbancies at two selected wave lengths.

Results

The radioactivity of the deoxyribonucleic acid components isolated from rats fed formaldehyde-C^{14} is shown in Table IV. The specific activity was expressed as counts per minute per micromole, the calculation of which is shown in the appendix. These results indicate that formaldehyde is incorporated into thymine, adenine, and guanine to a much larger extent than into cytosine. If the dilution value is regarded as a measure of utilization, it is indicated that about 3 moles of formaldehyde enter the purines for each mole of formaldehyde which enters thymine.

The low radioactivity in the cytosine would seem to lend support to the assumption that most of the radioactivity of thymine is located in the methyl group. Therefore formaldehyde would be considered to have a major role in the methylation of pyrimidines. The radioactivity of the adenine and guanine is thought to be located in the 2 and 8 carbons as demonstrated by other workers using formate and serine, and would indicate that formaldehyde would be a relatively major source of the ureide carbons.

TABLE IV

INCORPORATION OF FORMALDEHYDE-C^{14} INTO RAT DNA COMPONENTS

Experiment Number	Compound Isolated: Adenine c.p.m./μM	Diln*	Guanine c.p.m./μM	Diln*	Thymine c.p.m./μM	Diln*	Cytosine c.p.m./μM	Diln*
1	9,770	103	10,090	99	3,075	325	131	7640
2	10,960	91	8,960	112	3,065	325	151	6620
Average	10,365	97	9,525	105	3,068	325	141	7130

* Dilution = specific activity of precursor/specific activity of compound isolated.

DISCUSSION

DISCUSSION

Methylation Studies of Nicotine

The results obtained from the studies using serine-3-C^{14} indicated that the beta carbon of serine can be utilized as a source of the methyl group of nicotine. However, the results obtained from the studies using glycine-1-C^{14} seem to indicate that the carboxyl carbon of glycine was not used for nicotine synthesis under the conditions used. The demethylation of the nicotine isolated from the serine-fed tobacco plants demonstrated that most of the radioactivity in the nicotine was located in the methyl group.

Serine previously had been shown to be a methyl group precursor in animals but this present work is the first evidence of its use in the methylation reactions in higher plants. Weissbach, Klwyn, and Sprinson (32) and Jonsson and Mosher (33) showed that the beta carbon of serine could be utilized by the rat for the synthesis of the choline methyl group. Arnstein and Neuberger (34) also have demonstrated the utilization of the beta carbon of serine for the synthesis of the methionine methyl group. In studies by Arnstein (35), it was found that only the L-serine isomer gave rise to methyl groups in the rat. D,L-serine was used in this present study as it was assumed that both isomers of the amino acid were used in plant metabolism to yield methyl groups. The difference in the use of the two isomers has not been studied in plant metabolism so that clarification of this point must await further investigation.

Previous studies involving various plant products have demonstrated that the methyl groups of methionine (3,4,10), glycine betaine (8), and choline (5), the carbons of formate (3,10), and formaldehyde (36), and the alpha carbons of glycine (11) and glycolate (12) could serve as methyl group precursors. The investigations of nicotine formation in the tobacco plant provide an excellent opportunity to make a comparison of the various compounds fed since the molarity and radioactivity were kept equivalent. The carbon of formaldehyde seems to be utilized as a methyl group precursor to a greater extent than any of the other substances studied. The nicotine isolated from the tobacco plants fed formaldehyde-C^{14} was about twice as radioactive as nicotine from plants fed glycine-2-C^{14}, and almost 4 times as radioactive as from plants fed methionine-methyl-C^{14}. The methyl groups of methionine, choline, and betaine, and the alpha carbon of glycolate appear to enter the methyl group of nicotine to about the same extent, whereas the carbon of formate is incorporated only to about one-tenth the extent of these substances. The beta carbon of serine was incorporated into the methyl group of nicotine to about one-half the extent of the alpha carbon of glycine and 3 to 5 times less than the carbon of formaldehyde. Even if the L-serine isomer were the only form utilized by the tobacco plant, it would still be utilized to a lesser degree than the carbon of formaldehyde.

The above methyl group precursors also have been studied in animal metabolism and seem to show a marked difference in relative importance when compared with the plant methyl group precursors. The methyl groups of glycine betaine and methionine appear to supply most of the methyl

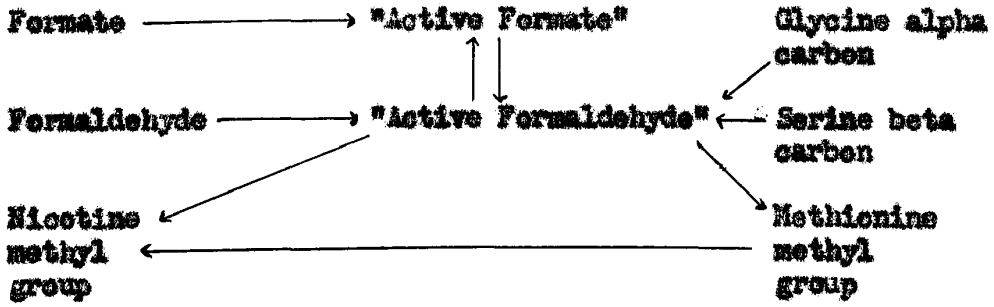
groups by transmethylation in the animal. In de novo synthesis studies in the rat, Arnstein and Neuberger (34) have shown that formate enters the methyl group of methionine at least as rapidly as the beta carbon of serine, and that the beta carbon of serine is utilized to almost 6 times the extent of the alpha carbon of glycine. Stekol et al. (37) have demonstrated that the beta carbon of serine can be used for choline methyl group synthesis to a greater extent than formate or the alpha carbon of glycine. Only a few investigations using formaldehyde (33,38,39) have been made, but they seem to indicate that formaldehyde is utilized at about the same extent or slightly less than formate. A distinct difference therefore is noted in the de novo synthesis of methyl groups in animal and plant metabolism, with serine and formate being more important in the animal and formaldehyde and glycine being the more important compounds for synthesis of N-methyl groups in the plant.

A possible pathway for the synthesis of N-methyl groups in plants might therefore be proposed in light of these studies. Animal studies indicate that glycine enters methyl groups by way of serine, and that possibly formaldehyde, formate, or a "1-carbon unit" condenses with glycine to form serine (34,40,41). However, this does not appear to be the case in the formation of the nicotine methyl group, since neither formaldehyde nor the alpha carbon of glycine seems to be metabolized to a large extent by way of the beta carbon of serine. A suggested idea that the beta carbon of serine may be oxidized to the oxidation state of formaldehyde but not to the oxidation state of formate has been supported by studies by Klein, Weissbach, and Sprinson (42) who demonstrated that

serine is not oxidized to formate to form choline methyl groups in the rat.

Recent proposals of an "active 1-carbon units" by Berg (43) and Kisliuk and Sakami (44) at the oxidation state of formaldehyde appear to be attractive for the utilization of formaldehyde, the alpha carbon of glycine, and the beta carbon of serine for the formation of N-methyl groups in plants. This "active 1-carbon compound" could then be reduced to form methyl groups for transmethylation or the "non-labile" groups of lignin. Elwyn and Sprinson (45) demonstrated a lowered utilization of serine in folic acid deficient rats to lend support to a hydroxymethyl-tetrahydrofolic acid derivative of Kisliuk and Sakami. The addition of homocysteine to rat diets has been shown to increase the incorporation of serine (37) and formate into choline and creatine methyl groups to lend support to a hydroxymethylhomocysteine derivative.

A possible pathway for the formation of N-methyl groups in the plant might therefore be:



The role of the carboxyl carbon of glycine in plant metabolism appears to be about the same as in animal metabolism. Siekevitz and Greenberg (15) demonstrated that the carboxyl carbon of glycine was

oxidized to carbon dioxide and that the carbon dioxide formed was not reduced to formate, whereas the alpha carbon of glycine gave rise to formate. Weisebach, Elwyn and Sprinson (32) also showed that the carboxyl carbon of glycine did not give rise to choline methyl groups in the rat. If the present experiment using glycine-1-C^{14} had been run for a longer period possibly random distribution of radioactivity in the nicotine would have been found as was indicated by Culp (29) in studies with bicarbonate.

Lignin Studies

It was demonstrated in these investigations of methylation in tobacco plants that the methyl carbons of methionine, choline, and betaine, the alpha carbons of glycine and glycolate, the beta carbon of serine, the carbon of formate and formaldehyde, and the carboxyl carbon of glycine can serve as precursors of the methoxyl carbon of lignin. All of the above substances, when fed to tobacco plants, gave rise to radioactivity in the lignin isolated from the plants, but the extent of incorporation varied with the substances. After demethylation of the lignin, it was found that most of the radioactivity of the lignin was located in the methoxyl group of lignin from plants fed D,L-serine-3-C^{14}, D,L-methionine-methyl-C^{14}, glycine-2-C^{14}, formaldehyde-C^{14}, glycolate-2-C^{14}, and formate-C^{14}. The methoxyl groups of the lignin after feeding choline-methyl-C^{14} and betaine-methyl-C^{14} possessed only about one-third to one-fifth of the radioactivity of the lignin, whereas

the methoxyl group of the lignin from plants fed glycine-1-C^{14} contained less than one-thirtieth of the radioactivity of the lignin.

The O-methyl group is unusual in the sense that it does not exist in animal metabolism. It was first shown by Byerrum and Flekstra (46) that the O-methyl group of barley lignin could arise from the methyl carbon of methionine, and the carbon of formate. The methionine methyl group was demonstrated to be incorperated to a much greater extent than the formate. Dubeck and Kirkwood (6) found that the methyl group of methionine could be utilized as a source of the O-methyl group of ricinine, an alkaloid from the castor bean, but they failed to show that either the choline methyl groups or formate could give rise to the methyl groups of this alkaloid. Sribney and Kirkwood (47) also have demonstrated that the methyl group of methionine could serve as a precursor of the methylenedioxy groups of protopine in Dicentra hybrids. Subsequent studies by Byerrum, Flekstra, Dewey, and Ball (10) have shown that the methyl group of methionine is transferred intact to give rise to the O-methyl group of lignin in barley and tobacco plants, an instance of transmethylation in higher plants. Transmethylation in higher plants had been shown earlier involving the methionine methyl group and the nicotine methyl group (9). Studies by Stone (48) in which labeled carbon dioxide was fed to wheat plants for a short time indicate that the methylation of lignin is an irreversible process, the methyl groups being of a "non-labile" type. He demonstrated that the total radioactivity acquired by the syringaldehyde portion of lignin remained constant throughout the growth of the plant, indicating that lignin was

an end-product in the plant and methylation was a reaction in the synthesis of lignin.

The results obtained in the present studies differ from the results obtained in previous plant methylation studies (3,5,8,11,12,36) mentioned in the discussion of serine incorporation into the N-methyl group of nicotine. The beta carbon of serine appears to be incorporated to the greatest extent into the O-methyl groups of tobacco lignin when compared with the other precursors. It is incorporated to nearly twice the extent of the carbon of formaldehyde, about 4 times the extent of the alpha carbon of glycine and glycolate, and the methyl group of methionine, and about 10 times the extent of the carbon of formate. If only the L-serine is utilized by the plant then serine would be an even better methyl group precursor when compared to the other substances.

The reason for the low recovery of the total radioactivity after demethylation of the lignin from plants fed choline and betaine is not understood at present and is under investigation. The results obtained from the demethylation of the lignin from plants fed glycine-1-C^{14} seem to indicate that the carboxyl carbon of glycine enters the lignin molecule randomly much as carbon dioxide enters the nicotine molecule as demonstrated by Culp (29).

The important role which seems to be assigned to the beta carbon of serine would indicate that in higher plants the O-methyl group is formed at least partially by a different pathway than is the N-methyl group of nicotine. This suggests that possibly the beta carbon of serine is metabolized through more than one pathway. The possibility

of a "1-carbon unit" (43,44) similar to that proposed in the previous discussion seems to be attractive here also, but since the beta carbon of serine is incorporated to a greater extent than the carbon of formaldehyde there may be still another pathway. As mentioned previously, the methylation of lignin seems to be an irreversible process which might lend support to the idea of the involvement of the oxygen of the serine hydroxyl group. Perhaps the serine condenses with the ring structure of lignin and then the carbon linkage between the alpha and beta carbons is cleaved, followed by reduction to yield the methoxyl group. A study using doubly labeled serine with O^{18} in the hydroxyl group and C^{14} in the beta position would be useful in proving this hypothesis.

These studies also indicate that the carbons of formaldehyde and formate, and the alpha carbons of glycine and glycolate might be utilized for methyl group synthesis by way of the beta carbon of serine as suggested in animal metabolism (14,39). This again differs from the results obtained from the methylation studies of the N-methyl group of nicotine. These investigations showed that the carbon of formaldehyde and the alpha carbon of glycine were incorporated to a greater extent than was the beta carbon of serine, which suggest that they were not metabolized by way of serine.

Incorporation of Formaldehyde into Thymine and Purines

The present study has shown that the carbon of formaldehyde can serve as a precursor of thymine and the purines, adenine and guanine.

The low radioactivity found in cytosine lends support to the assumption that all the radioactivity of thymine was located in the methyl group. Studies using formate-C^{14}, serine-3-C^{14}, and glycine-2-C^{14} (17,19) have demonstrated that upon degradation of thymine most of the radioactivity was located in the methyl group, and in each case the thymine ring isotope level corresponded to the isotope level of the cytosine ring.

It has been well known from investigations with pigeons that formate or biological precursors of formate such as the beta carbon of serine, the alpha carbon of glycine, and the methyl group of choline could give rise to the 2 and 8 carbons of uric acid. Formate has been shown by Totter, Velkin, and Carter (17) to give rise to the methyl group of thymine in the chick and rat. The methyl group of methionine formerly was found to serve as a rather ineffective thymine precursor by Brown (18), but recently it was found to be readily converted to the 2 and 8 carbons of purines by Sims and Johnsen (18). Elwyn and Sprinson (19) have shown recently that the beta carbon of serine and the alpha carbon of glycine could serve as precursors of the methyl group of thymine, demonstrating that serine was incorporated to a greater extent than was glycine. The beta carbon of serine also was shown to enter the 2 and 8 carbons of the purines.

Recent work reported by Herrmann, Fairley, and Byerrum (49) dealt with a comparison of the carbon of formate and the methyl group of methionine as precursors of the methyl group of thymine and the ureide carbons of the purine. Earlier studies using formate (17) and methionine (18) had failed to provide a comparison of the respective

utilizations for these processes. Herrmann et al. (49) found that formate was a more effective precursor of the thymine methyl group and the ureide carbons of adenine and guanine than was the methyl group of methionine. However when a ratio of the radioactivity of adenine to the radioactivity of thymine was considered, studies using formate gave a ratio of about 5 to 1, whereas studies using methionine gave a ratio of about 1.4 to 1. These ratios indicate the methyl group of methionine would enter the methyl group of thymine more preferentially than does formate when compared to the entrance into the ureide carbons of the purines. The work of Elwyn and Sprinson (19) gave a corresponding ratio of 1.5 to 1 for the beta carbon of serine indicating that the methyl group of methionine and the beta carbon of serine may form a common intermediate before giving rise to the ureide carbons of the purines and the methyl group of thymine. The present study using formaldehyde gave an adenine to thymine activity ratio of about 3 to 1.

The dilution value, that is the specific activity of the precursor/specific activity of the compound isolated, obtained in the present study was compared to the dilution values obtained from the formate and methionine study (49). They indicated that formaldehyde is incorporated into the purines to about the same extent as formate, and about 9 times the extent of the methyl group of methionine. However when compared with respect to the incorporation into thymine, formaldehyde was used to twice the extent of formate, and 4 times the extent of methionine.

These results seem to indicate that formaldehyde has an intermediate role as a thymine precursor when compared to serine, methionine,

and formate. Elwyn and Sprinson (19) demonstrated that during the conversion of the beta carbon of serine to the thymine methyl group the hydrogens on the beta carbon accompany the carbon. Whereas in the conversion to the ureide carbons of the purines extensive labilization of the hydrogens took place, indicating two distinct pathways for the two processes. In studies of the synthesis of serine by Kisliuk and Sakami (44) and by Mitoma and Greenberg (39), it was suggested that an active intermediate compound, possibly a tetrahydrofolic acid derivative, at the oxidation state of formaldehyde existed which could give rise to serine or to the methyl groups of thymine and choline. Berg (43) also has proposed that hydroxymethylhomocysteine was an important "active 1-carbon intermediate". Kisliuk and Sakami (44) also postulated an "active 1-carbon compound" at the oxidation state of formate which might form the ureide carbons of the purines or be reduced to form the "active hydroxymethyltetrahydrofolic acid" at the oxidation state of formaldehyde. These postulations would help to explain the difference in the incorporation of formaldehyde and formate into the methyl group of thymine. Recently Greenberg (50) also has presented a scheme for the interrelationship of formaldehyde, the beta carbon of serine, formate, and the purine ureide carbons utilizing the "active 1-carbon compound", a derivative of tetrahydrofolic acid as proposed by Kisliuk and Sakami (44). Wyatt and Cohen (51) have reported the occurrence of 5-hydroxymethylcytosine in phage deoxyribonucleic acid and proposed a possible pathway for the formation of thymine by way of the 5-hydroxymethylcytosine which seems to be attractive in light of the results with formaldehyde.

SUMMARY

SUMMARY

1. The administration of serine-3-C^{14} to tobacco plants resulted in the formation of radioactive nicotine. Most of the radioactivity was shown to be centered in the methyl group.

2. When compared with other methyl group precursors previously fed to tobacco plants, the beta-carbon of serine seems to be incorperated into the nicotine molecule in a lesser amount than formaldehyde and the alpha-carbon of glycine, but at about the same extent as methionine, choline, betaine, and glycolate. Possible mechanisms for the formation of the N-methyl group are discussed.

3. After the administration of glycine-1-C^{14} to tobacco plants, the nicotine dipicrate isolated possessed no radioactivity. It is therefore assumed that the carboxyl carbon of glycine does not enter into nicotine synthesis under the conditions used.

4. Lignin isolated from tobacco plants fed serine-3-C^{14}, formaldehyde-C^{14}, methionine-methyl-C^{14}, glycine-2-C^{14}, glycine-1-C^{14}, choline-methyl-C^{14}, glycine betaine-methyl-C^{14}, glycolate-2-C^{14}, and formate-C^{14} has been shown to possess radioactivity. Most if not all of the radioactivity was shown to be located in the methoxyl group except after feeding choline, betaine, and glycine-1-C^{14}. The results with choline and betaine feeding are unexplained at present, and the carboxyl carbon of glycine appears to result in random distribution of the carbon in the lignin molecule.

5. A comparison of results indicates that the beta carbon of serine is the best methoxyl carbon precursor studied and formaldehyde is second best. Methionine, the alpha carbons of glycine and glycolate are incorporated at about the same extent. Formate appears to be incorporated better into O-methyl groups than into N-methyl groups when compared to the other methyl group precursors. The significance of the results obtained are discussed in view of possible pathways.

6. Radioactive formaldehyde, when injected intraperitoneally into rats, has been shown to yield radioactive purines and pyrimidines isolated from deoxyribonucleic acid. The purines, adenine and guanine, possessed a large amount of activity, as did the pyrimidine, thymine, but cytosine showed little activity. Most of the radioactivity of thymine appears to be located in the methyl group. When compared with methionine and formate, formaldehyde appears to be intermediate in the formation of the thymine methyl group and purine ureide carbons, but seems to be a good source for both of these carbons.

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APPENDIX

APPENDIX I

The formula used in correcting the observed count of nicotine dipicrate and methyltriethylammonium iodide 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 counts (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 = 116.3 c.p.m., W = 60.6 mg., M = 620,

T = 21.4 mg/cm.^{2}, b = 0.290.

$$A_m = \frac{116.3 \times 620}{60.6 \times .290} = 4.1 \times 10^3 \text{ c.p.m./mM}$$

APPENDIX II

A) The formula used in correcting the observed counts to zero sample thickness for lignin samples was:

$$A_m = \frac{C_o \cdot 60}{b \cdot W}$$

where A_m = maximum specific activity (counts/minute/60 mg. of lignin)

C_o = observed counts (counts/minute/sample weight of lignin)

W = mg. of sample counted

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

Sample calculation:

C_o = 1445 c.p.m., W = 40 mg., T = 14.13 mg/cm.^{2}, b = 0.492

$$A_m = \frac{1445 \times 60}{.492 \times 40} = 4410$$

B) The formula used to correct the observed counts for the methyltriethylammonium iodide to zero sample thickness was:

$$A_m = \frac{C_o \times I \times 100}{b \times W \times 95}$$

where A_m = maximum specific activity (counts/minute/total mg. of iodide obtained from 60 mg. of lignin)

C_o = observed counts (counts/minute)

I = mg. of methyltriethylammonium iodide obtained from demethylation of 60 mg. of lignin

W = mg. of methyltriethylammonium iodide counted

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

$\frac{100}{95}$ = correction factor, based on 95% recovery of methoxyl groups from vanillin (27).

Sample calculation:

C_o = 2740 c.p.m., I = 31.1 mg., W = 21.7 mg., T = 7.67 mg./cm.^{2},

b = 0.95

$A_m = \frac{2740 \times 31.1 \times 100}{0.95 \times 21.7 \times 95}$ = 4350 c.p.m./ weight of iodide obtained from 60 mg. of lignin.

APPENDIX III

The formula used to correct the observed counts of purines and pyrimidines to specific activity.

$$S = \frac{C_o \times a_M \times 1000 \text{ ml}}{A_s \times 1 \times 10^6}$$

where S = specific activity (counts/minute/micromole)

C_o = observed counts (counts/minute/ml. of sample)

a_M = molar absorbancy index (molar extinction coefficient)

A_s = absorbancy of sample counted

$1/1 \times 10^6$ = factor to convert moles to micromoles

Sample calculation: Thymine

C_o = 112 c.p.m./ml. of sample, $A_M = 7 \times 10^3$ (260mu, pH 8.25)

A_s = 0.257

$$S = \frac{112 \times 7 \times 10^3 \times 1000}{.257 \times 1 \times 10^6} = 3050 \text{ c.p.m./}\mu M$$