# THE ROLE OF THE ALPHA CARBON OF GLYCINE IN METHYLATION STUDIES IN TOBACCO PLANTS

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# THE ROLE OF THE ALPHA CARBON OF GLYCINE IN METHYLATION STUDIES IN TOBACCO PLANTS

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# INTRODUCTION

# INTRODUCTION

In 1940 it was demonstrated by du Vigneaud and his collaborators (1) that transmethylation, the intermolecular transfer of methyl groups, was a reaction in the metabolism of animals. In 1951 Brown and Byerrum (2) performed experiments which suggested that transmethylation might also occur in higher plants. These studies showed that the methyl carbon of methionine could serve as a precursor for the methyl carbon of nicotine in tobacco plants and it was postulated that a direct transfer of a methyl group had occurred. In addition it was demonstrated by Flokstra (3) that the methyl carbon of methionine could also serve as a precursor for the methoxyl carbon of lignin in barley. In an attempt to answer the question of whether the methyl group of methionine was transferred directly or was oxidized and subsequently reduced, Byerrum and Dewey (4) fed methionine doubly labeled with deuterium and carbon -14 in the methyl group to barley, and found the same deuterium to carbon -14 ratio in the methoxyl groups of the isolated lignin as was in the doubly labeled methionine. During the same period additional compounds were shown to contribute to the methyl groups of lignin. Formate (2) (3) was found to form the methyl carbon of nicotine and methoxyl carbon of lignin, although the rate of incorporation of formate was only about one tenth that of methionine methyls. Ming (5) was successful in demonstrating the use of choline labeled with carbon -14 in the methyl groups as a precursor of the methyl carbon of nicotine. However, Kirkwood and co-workers (6) (7) have failed in attempts to use choline for the methyl carbon of the alkaloids, hordenine of barley, and ricinine

of castor beans, although methionine was demonstrated to result in the labeling of the methyl carbon of both alkaloids.

A number of different compounds have been shown to be methyl carbon precursors in animals, these are the alpha carbon of glycine (8), the methyl carbon of choline (9), the methyl carbon of methionine (10), sodium formate (11), 2-carbon of the imidazole ring of histidine (12), the beta carbon of serine (8), the methyl carbon of glycine betaine (13), and the methyl carbon of acetone ($1^4$). The alpha and beta carbon atoms of serine have been shown by Siekevitz and Greenberg (15) to originate from the alpha carbon of glycine, in that glycine is oxidatively deaminated to glyoxylic acid which was futher oxidized to formate and carbon dioxide. The carbon dioxide which came from the carboxyl group was not reduced to formate. These findings were recently confirmed by Weinhouse (16), who had originally shown the formation of formate from the alpha carbon of glycine, glycolic acid, and glyoxylic acid.

With all this interest in the alpha carbon of glycine as a methyl precursor centered on animal tissue, the possibility of it acting as a methyl carbon precursor in higher plants seemed very logical. The study undertaken here was an attempt to demonstrate the use of the alpha carbon of glycine as a precursor for the methyl carbon of nicotine in tobacco plants, using carbon-$14$ as a tracer. The results of the experimental work definitely show that the alpha carbon of glycine can form the methyl carbon of nicotine, at about the same rate as methionine and choline, and about ten times as fast as formate.

EXPERIMENTAL

EXPERIMENTAL

## Growth of Plants

The tobacco plants used in these experiments were of a high nicotine strain, Nicotiana rustica L., var. humilis. The seeds were planted in flats containing vermiculite,[1] and were transplanted after a period of two or three weeks into flats, allowing about two inches between each plant. The plants were watered each day and were fed a commercial inorganic nutrient solution twice a week. The period of growth in the greenhouse varied with seasonal conditions, but two to three months were usually required to obtain the desired plant height of about six inches. Budding and flowering were noted in some cases during the experimental growth period, but for the most part were absent.

To prepare the plants for hydroponic administration of the glycine they were first removed from the flats and the vermiculite was removed from the roots as completely as possible. They were then washed carefully to free them of the most of the remaining extraneous material and to avoid damage to the roots. The roots were then soaked in a 0.01 percent Nyandotte detergent germicide for an hour, removed from the detergent, and washed well with distilled water. After the rinsing, each plant was immersed in 50 ml. of an inorganic nutrient solution in a 125 ml. Erlenmeyer flask. The nutrient solution was prepared by diluting the stock nutrient solution 1:5. The composition of the stock

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[1] A commercial brand of heat expanded mica.

solution is shown in Table I. All the weights are of the anhydrous salts, and only C.P. grade chemicals were used. A description will be given later of any other materials added to the nutrient solution.

TABLE I

COMPOSITION OF THE NUTRIENT SOLUTION

| | | | |
|---|---|---|---|
| Water | 1,000 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 $KH_2PO_4$ | 250 mg. |
| Ferric chloride $FeCl_3$ | 2 mg. | | |

All the experiments were carried out in a special fume hood to avoid any health hazard that might arise from the use of radioactive material.

Artificial lighting was used in all the experimental work. The source of light consisted of two 36 inch, 30 watt fluorescent tubes and a 100 watt incandescent bulb. These lights were placed about 14 inches above the top of the plants, and were found to give a light intensity in the range of 200 - 250 foot candles at the top of the leaves. The lights were left on for twelve hours each day and nutrient solution was added to the flasks when needed. Oxygen was bubbled through the solution twice a day for two minutes, once when the lights were turned on and once when the lights were turned off.

It seemed advisable to run all the plants in the same manner so that the metabolic rates and thus the rate of synthesis of nicotine would be relatively constant.

## Uptake of Glycine by the Plants.

Before metabolic studies with glycine could be undertaken it was necessary to ascertain the absorption rate of glycine by the roots, and to determine whether or not glycine was destroyed by microorganisms.

Since glycine was the only material present in the original solution that would react with ninhydrin, the ninhydrin method of Hardy and MacLean (17) was used for its analysis. A standard curve was made by using 5 ml. of various standard glycine solutions, 0.3 ml. of pyridine, and 1 ml. of 1 percent ninhydrin solution. These solutions were pipetted into large pyrex tubes graduated at 50 ml., placed in a boiling water bath for fifteen minutes, then cooled in cold water and diluted to 50 ml. with distilled water. The color intensity was measured with a Kellige electric colorimeter as compared with a blank prepared under the same conditions as above with the exception that distilled water was used in place of the glycine solution. Optical density was plotted against concentration and a straight line curve was obtained, with a slight deviation at values less than 0.05 mg. per 5 ml. of glycine standard.

Four plants were prepared as described previously, using 50 ml. of nutrient solution, 2 ml. of glycine standard (1 mg./ml.) and 3 drops of 0.1 percent Wyandotte detergent germicide in each 125 ml. Erlenmeyer flask. Root fragments were placed in four other flasks prepared in the same manner, and four control flasks containing just the three solutions were prepared. The 12 flasks were placed in the hood for forty-eight hours with lights and oxygen controlled as previously stated.

At the end of the forty-eight hours the flasks were taken from the hood, and the plants were carefully removed from the flasks. A fine stream of distilled water from a wash bottle was used to cleanse the roots of the plants, the washings being added to the respective flasks. The contents of all the flasks were filtered through Whatman number 42 paper and were diluted to 50 ml. Five ml. aliquots were taken from each flask and run by the previously outlined ninhydrin method. It was found that in all flasks the glycine content was very low. This was interpreted as indicating destruction by microorganisms. Results similar to these were obtained by King (5) when choline was fed; so it was decided to follow his procedure in the use of aureomycin to control the action of microorganisms.

The 12 flasks were set up as before with the exception that in place of the detergent, 0.5 ml. of 1:1000 solution of aureomycin was used. At the end of forty-eight hours, the contents of two flasks of each set were filtered, each diluted to 50 ml. and analyzed using the ninhydrin method. An uptake of 52 percent of the original glycine was indicated in that period by the growing plants.

After a four day period, the remaining flasks were treated in the previously described manner and it was indicated that an absorption of 70 percent of the glycine had occurred. No decrease in the original amount of glycine was indicated in the analysis of the flasks containing root fragments, or the control flasks, indicating that destruction by bacteria was eliminated. Aureomycin in the same concentration as above was used in all subsequent experiments.

The apparent slow absorption rate of glycine prompted the use of a longer growing period of ten days for the plants. Brown (2) and Ming (5) in previous work in this laboratory used a seven day administration period; so one group of plants was also grown for seven days for comparison of nicotine synthesis during the two different growing periods.

## Administration of Radioactive Glycine

The molar quantity and radioactivity of glycine administered to each plant was calculated to be equal to the methionine and formate previously fed in methylation studies. One milligram of the glycine, containing $10^5$ counts per minute, was given to each plant.*

The plants were prepared for hydroponic administration as outlined previously. Two groups of plants were grown for ten days, and the third group was grown for a week.

## Isolation and Purification of Nicotine

After the growing period the plants were removed from the flasks, and the roots were washed with distilled water, the excess blotted off with a cheese cloth. The plants were subsequently cut up into small pieces and dried under infrared lamps as rapidly as possible. The temperature was kept at 60° C. for an hour near the end of the drying period.

The dried 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 gave no

* Obtained from Tracerlab Inc., Boston, Massachusetts.

precipitate with silicotungstic acid, indicating that the alkaloids were no longer coming over in the distillate. The distillate was collected in 5 ml. of 6N hydrochloric acid, and was concentrated in vacuo. Two successive azeotropic distillations from alkaline medium were carried out to purify the alkaloid as described by Smith (15), each time the distillate being collected in a small amount of dilute hydrochloric acid. 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 standing a short time, the nicotine dipicrate which precipitated, was filtered off, washed with methanol, and recrystallized from hot water.

The nicotine dipicrate was ground finely in a mortar, plated on tared aluminum disks and weighed. These preparations were counted on the top shelf of the counter assembly. The counts per minute per millimole at infinite thickness (see appendix 1) are shown in Table II. The column labeled nicotine dipicrate shows a definite radioactivity in the nicotine molecule.

## Demethylation

It was desirable to establish whether or not the radio-activity of the alpha carbon of glycine was localized in the methyl carbon of nicotine. The demethylation of the nicotine was therefore undertaken. The procedure followed was essentially that of Fregl (19) as modified by Simmonds (9) and Brown (2). The apparatus used was the modification

of Brown's. Using this procedure the methyl group is isolated as methyltriethylammonium iodide, a white crystalline compound suitable for counting.

Since the nicotine dipicrate was found to be quite insoluble and unsuitable for demethylation, the nicotine was recovered by dissolving it in sodium hydroxide and isolating the nicotine by azeotropic distillation through a Widmer column. The distillate was concentrated to dryness in vacuo, the last part done in the flask from the demethylation apparatus.

The reaction flask containing the nicotine hydrochloride was attached to the remaining demethylation apparatus and the following were added on the basis of 50 mg. of nicotine; 45 mg. of ammonium iodide, two drops of 5 percent gold chloride, and 3 ml. of hydriodic acid. The gas washing apparatus contained 0.75 ml. of 5 percent cadium sulfate, and 0.75 ml. of 5 percent sodium thiosulfate to remove iodine and hydrogen iodide. The receiver contained a 5 percent ethanolic solution of triethylamine, cooled in a carbon dioxide-methyl cellosolve bath. A stream of nitrogen was bubbled through the reaction train constantly.

The flask was imbedded in a copper oxide bath and was heated to 200° C. in 20-25 minutes. The temperature was then slowly raised to 350 - 360° C. and held there for 45 minutes. The stream of nitrogen was continued until the apparatus had cooled. The delivery tube was rinsed with ethanol into the receiver, which was then stoppered, shaken, and allowed to stand over night at room temperature. After that most of the ethanol and excess triethyl amine was evaporated over an infrared lamp.

The last of the ethanol and triethyl amine were evaporated in a vacuum dessicator. The methyltriethylammonium iodide recovered was a white crystalline compound.

The quaternary compound was dissolved in a small amount of ethanol and was plated on tared aluminum counting plates. The excess ethanol was evaporated over an infrared lamp, and then the plates were weighed to acquire the weight of the plated compound. These plates were counted as mentioned previously, and the results reported as counts per minute per millimole in Table II.

## Results

TABLE II

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER ALPHA CARBON - 14 OF GLYCINE ADMINISTRATION

| Trial No. | Growth Period Days | No. of Plants | Maximum Specific Activity (counts per minute per millimole) Nicotine Dipicrate | Methyltriethyl-ammonium Iodide |
|---|---|---|---|---|
| 1 | 10 | 30 | $1.36 \times 10^4$ | $1.02 \times 10^4$ |
| 2 | 10 | 29 | $9.02 \times 10^3$ | $6.75 \times 10^3$ |
| 3 | 7 | 30 | $8.52 \times 10^3$ | $8.50 \times 10^3$ |

These results show that the alpha carbon of glycine is incorporated into the methyl carbon of nicotine. It is indicated that most of the radioactivity of the nicotine after feeding glycine labeled with carbon -14 in the alpha carbon was located in the methyl group. Since 100 percent recovery of counts from the methyl carbon was not obtained in all three runs it would appear that some of the glycine was used in nicotine ring synthesis.

# DISCUSSION

## DISCUSSION

These investigations of methylation in tobacco plants show that the alpha carbon of glycine can serve as a precursor of the methyl carbon of nicotine *in vivo*. Demethylation of the nicotine from the plants grown for ten days resulted in 75 percent recovery of the radioactivity, whereas demethylation of the nicotine from the seven day growth period gave almost 100 percent recovery of the radioactivity from the nicotine methyl groups. This difference in recovery of radioactivity may not be significant, but it may be plausible that the alpha carbon of glycine could be used in the ring synthesis of normicotine or other precursors. Since the glycine was absorbed more slowly than methionine and formate, the longer growing period of ten days seemed to be the best from the standpoint of a better comparison of the rate of incorporation of the compounds in nicotine synthesis.

Since the molar quantities and counts of the glycine, methionine, and formate administered to the plants were equal, a comparison can be made of the rates of incorporation into nicotine methyl groups. The alpha carbon of glycine was incorporated at a rate slightly greater than the highest rate for methionine (2), and 2 or 3 times the lowest value obtained for the methionine. Comparison with the formate is even more significant in that the alpha carbon of glycine is incorporated at a rate 10 to 20 times that found for formate. The alpha carbon of glycine goes into nicotine at a rate slightly greater than that obtained for choline (5).

These results with plants differ sharply with those results obtained from methylation studies in animals. Methionine has been shown to be a

much faster methylating agent than the alpha carbon of glycine, the values ranging from 4 times in the synthesis of creatine (23) to 16 times in the synthesis of choline. Formate was incorporated at about the same rate as was the alpha carbon of glycine.

There appear to be several possibilities for the mechanism of methylation by the alpha carbon of glycine in plants: 1) the glycine is oxidized to formate which is then reduced to form the methyl group or 2) the glycine is hydrolytically deaminated to glycolic acid or oxidatively deaminated to glyoxylic acid and is reduced to a methyl group without going to formate or 3) the nitrogen of glycine is incorporated into the nicotine ring and the alpha carbon goes along to form the methyl group. The rather rapid rate of incorporation as compared to that of formate seems to rule out the first proposal. Tolbert and Burris (22) recently found an enzyme in green leaves that will oxidize glycolic acid to glyoxylic acid. However, this enzyme was not found in the roots, and it was shown by Dawson (20) that nicotine synthesis takes place in the roots. On the other hand, it has been shown by James (21) that nicotine can be synthesized in the leaves if the suitable precursor is available. Glycine has also been shown to form the 4-carbon, 5-carbon, and 7-nitrogen of purines (25), and the alpha carbon and nitrogen form part of the pyrrole rings in the porphyrin synthesis (24). These studies give little indication of which of the above proposals (2 or 3) might be correct.

Transmethylation studies (3) in animals indicate that glycine is oxidized to formate and upon reduction forms the beta carbon of serine.

Vitamin $B_{12}$ seems to be essential in the transformation from glycine to serine, and folic acid is required for the methylation reaction.

Formal proposal of a mechanism by which glycine reacts in methylation must await studies using glycine doubly labeled with carbon - $14$ and deuterium in the alpha position, and serine labeled with carbon - $14$ in the beta carbon. It would also be of interest to ascertain whether the alpha carbon of glycine forms the methoxyl carbon of lignin in tobacco.

SUMMARY

SUMMARY

1. Glycine labeled with carbon - $14$ in the alpha carbon was administered to a high nicotine strain of tobacco, Nicotiana rustica L., var. humilis. The nicotine isolated from the tobacco plants was found to possess radioactivity. Demethylation experiments showed that practically all this activity was located in the methyl carbon.

2. A comparison of the rates of incorporation into nicotine of the alpha carbon of glycine, methionine, formate and choline, indicates that the alpha carbon of glycine is incorporated at about the same rate as methionine and choline, and about 10 times faster than formate.

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## REFERENCES


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APPENDIX I

## APPENDIX I

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 calculations:

Nicotine dipicrate—$C_o$ = 366 c.p.m., $W$ = 60 mg., $M$ = 620.

T = 21.2 mg/cm.$^2$

$$A_m = \frac{366 \times 620}{60 \times .293} = 1.29 \times 10^4 \text{ c.p.m./mM}$$

# 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 for two years in the U.S. Navy Medical Corps, 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 College 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, he resumed his studies at Michigan State College in the Fall of 1952 as a Special Graduate Research Assistant under an Atomic Energy Commission Grant.

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AN ABSTRACT

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The alpha carbon of glycine has been shown in previous experiments to be a methyl carbon precursor in animal metabolism, but no similar experiments have been performed with higher plants. This study was an attempt to demonstrate the role of the alpha carbon of glycine in the methylation processes of higher plants. A high nicotine strain of tobacco was used as the experimental plant. The rate of absorption of the glycine seemed to be slower than the rates observed for methionine, formate, and choline in earlier experiments. When the plants were fed 2 mg. of glycine, an uptake of 52 percent in two days, and 70 percent in four days was noted. Destruction of the glycine by microorganisms in the nutrient solution was better controlled by aureomycin than by a Wyandotte detergent germicide.

Radioactive glycine, labeled in the alpha carbon, was fed to the plants and the nicotine, isolated as nicotine dipicrate, was found to possess radioactivity. Demethylation of the nicotine, and isolation of the methyl group as methyltriethylammonium iodide, showed that the majority of the radioactivity of the nicotine molecule was located in the methyl carbon.

Comparison of the rates of incorporation of various precursors into the nicotine methyl group showed that the alpha carbon of glycine was incorporated at about the same rate as the methyl groups of methionine and choline, and about 10 times the rate of formate.

The results indicate that the alpha carbon of glycine can serve as a methyl carbon precursor and that it does not go by the route of formation of free formate.