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PATHWAYS FOR CONVERSION OF THE BETA CARBON OF SERINE TO THE N-METHYL GROUP OF NICOTINE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Stanley Garson Krane

1958

THESIS

PATHWAYS FOR CONVERSION OF THE BETA CARBON OF SERINE TO THE N-METHYL GROUP OF NICOTINE

By

Stanley Carson Krane

A THESIS

Submitted to the College of Science and Arts
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1958

4-1-57
L-8174

ACKNOWLEDGMENTS

The author extends his sincerest thanks to Dr. R. U. Byerrum whose guidance and friendly assistance made this work possible. Thanks are also due other members of the Chemistry Department for their willing advice.

The author also expresses his appreciation to the Department of Chemistry and the National Institutes of Health for their financial aid.

VITA

The author was born February 16, 1937 in New York City. He attended DeWitt Clinton High School in the Bronx and then went on to the City College of New York. The Bachelor of Science Degree was conferred in June of 1957.

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PATHWAYS FOR CONVERSION OF THE BETA CARBON OF SERINE TO THE N-METHYL GROUP OF NICOTINE

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AN ABSTRACT

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Approved R. H. Byerrum

ABSTRACT

Previous research had shown that several substances could function as metabolic precursors of the N-methyl group in nicotine. This study was undertaken in an attempt to elucidate a pathway by which the beta carbon of DL-serine is used for synthesis of N-methyl groups in nicotine. The pathways investigated were (a) incorporation via formaldehyde or an active 1-carbon unit at the oxidation state of formaldehyde, (b) by way of formate or an active 1-carbon unit at the oxidation state of formate, or (c) through transfer to the methyl group of methionine.

Tobacco plants were fed DL-serine-3-C^{14} alone and in combination with relatively large quantities of non-radioactive formaldehyde, sodium formate and DL-methionine, each in a separate experiment. Nicotine was isolated, as the dipicrate, in each case and its radioactivity determined.

By comparing the radioactivities of the nicotine obtained in each experiment it was shown that formaldehyde or an active 1-carbon unit at the oxidation state of formaldehyde probably was an intermediate for the conversion of the beta carbon of serine to the N-methyl group of nicotine. Formate or a 1-carbon unit at the oxidation

state of formate was ruled out as an important intermediate. The results for methionine were erratic and no final conclusion regarding its role as an intermediate for methyl group synthesis from serine's beta carbon was reached.

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INTRODUCTION

During the past decade studies on methylation reactions in plants have been extensively investigated. Particularly the synthesis of the N-methyl group of the alkaloid, nicotine, in tobacco plants, has received a good deal of attention. Reports by Byerrum and several co-workers (1-7) showed that the methyl group of methionine, the carbon of formate, the methyl groups of choline and glycine betaine, the alpha carbon of glycine, the alpha carbon of glycolate, the beta carbon of DL-serine, and the carbon of formaldehyde could all function as metabolic precursors of the N-methyl group in nicotine. It was possible to make comparisons of the extent of incorporation of the above mentioned precursors into nicotine, since the molarity and radioactivity was the same throughout. The methyl groups of methionine, choline, glycine betaine, the beta carbon of serine and the alpha carbon of glycolate were used for methyl group synthesis to about the same extent. The alpha carbon of glycine was utilized about twice as much and formate about one-tenth as much as the first five mentioned. The incorporation of formaldehyde was about twice that of the alpha carbon of glycine; hence it would appear that formaldehyde is used for methyl group

synthesis in nicotine to a greater extent than any of the other precursors studied. However the existence of free formaldehyde has never been demonstrated in plants. An active 1-carbon unit at the oxidation state of formaldehyde has been postulated as the actual methylating agent (7).

The present study was undertaken in an attempt to elucidate a pathway by which the beta carbon of DL-serine is used for synthesis of N-methyl groups in nicotine. Three possible pathways were investigated. These were (a) incorporation via formaldehyde or the active 1-carbon unit at the oxidation state of formaldehyde, (b) by way of formate or a 1-carbon unit at the oxidation state of formate, or (c) through transfer to the methyl of methionine. The technique used was to feed tobacco plants labeled serine alone and in combination with a relatively large quantity of non-radioactive formaldehyde, formate or methionine, each in a separate experiment. If any of the non-radioactive compounds were intermediates for the conversion of the beta carbon of serine to the N-methyl group of nicotine a decrease in the radioactivity of the nicotine isolated from the plants would be expected; since the radioactivity of the precursor would be diluted by the large pool of non-radioactive intermediate. However if any of the non-radioactive, competing substances were not in the metabolic pathway of the beta carbon of serine no significant decrease in nicotine radioactivity would be expected.

Using this metabolic competion technique the data obtained indicated that formaldehyde or a 1-carbon unit at the oxidation state of formaldehyde was an intermediate for the conversion of the beta carbon of serine to the N-methyl group of nicotine. Formate or a 1-carbon unit at the oxidation state of formate was ruled out as an important intermediate. The results for methionine were erratic and no final conclusion regarding its role as an intermediate for methyl group synthesis from serine's beta carbon was reached.

EXPERIMENTAL AND RESULTS

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Growth and Preparation of the Plants

The tobacco plants used in the experiments were Nicotiana rustica L. var. humilus; a strain with a high nicotine content. The plants were grown, in a green house, from seeds planted in vermiculate (commercially available heat expanded mica). They were watered once a day and fed a nutrient solution twice weekly. The nutrient solution was composed of 1 g. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4, and 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$ all dissolved in 4 liters of tap water. After attaining a height of 0.75 inches the plants were transplanted so as to give each individual more room. The plants were kept in the green house until a height of 6 inches was reached. This took from two to three months.

Upon reaching the desired height the plants were removed from the vermiculate; their roots rinsed with tap water and then clipped, with a scissors, near the stem. These plants were then placed in 125 ml. Erlenmeyer flasks containing 50 ml. of an inorganic nutrient solution; which was a 1:3 dilution of a stock solution whose composition is shown in Table I.

TABLE I

COMPOSITION OF THE STOCK NUTRIENT SOLUTION

Water	1000 ml.	Magnesium sulfate	250 mg.
Calcium nitrate	1 g.	Ammonium sulfate	250 mg.
Potassium chloride	250 mg.	Potassium dihydrogen phosphate	250 mg.
Ferric chloride	2 mg.		

The diluted stock nutrient solution was oxygenated before the plants were placed in it. Each plant had a cotton plug wrapped around the upper part of its stem and inserted in the neck of its flask. The flasks were covered with black paper shields.

New root systems were permitted to develop on these plants; a process which took from 10 to 14 days. Any loss in volume of the original 50 ml. of diluted stock nutrient solution, during this time, was periodically made up with distilled water. When the new roots had grown the plants were fed the metabolic precursors, of the N-methyl group of nicotine, to be described below. During the feeding and afterwards, when the compounds fed were being metabolized, the plants were kept in hoods supplied with artificial lighting. Two 36 inch 30 watt fluorescent tubes were kept lit 12 hours of each day.

Administration and Uptake of Experimental Compounds

In order to increase significantly the metabolic pools of the non-radioactive competing compounds, fed in conjunction

with DL-serine-3-C^{14}, their concentrations were made greater than the concentration of the radioactive serine. It was decided that the non-radioactive competing compound in each experiment be made ten times greater in concentration than the radioactive serine, on a molar basis. Each plant received 4.76×10^{-3} mmol. of radioactive serine and 4.76×10^{-2} mmol. of non-radioactive competing compound; except the control experiment in which the plants received only the 4.76×10^{-3} mmol. of radioactive serine.

The non-radioactive compounds fed along with radioactive serine were DL-methionine, sodium formate, and formaldehyde. Radioactive serine alone was fed as a control experiment. Each combination was made up of 1.5 ml. radioactive serine, 1.0 ml. non-radioactive competing compound (0.0 ml in the control experiment), and sufficient dilute stock nutrient solution to bring the total volume fed to each plant to 3.0 ml.

The 3.0 ml. of solution in each experiment were placed in 125 ml. Erlenmeyer flasks which had black paper shields around them. The plants which had generated new roots were placed in these flasks. Each plant was then permitted to absorb the 3.0 ml. of solution. After these initial 3.0 ml. of solution were taken up, an additional 3.0 ml. composed entirely of dilute stock nutrient solution was given each plant. When this was absorbed, 50 ml. of dilute stock nutrient solution was given each plant. The

plants were grown for seven days after feeding, counting the beginning of the first day as the time when the original 3.0 ml. was fed.

When the method of feeding outlined above had previously been used in this laboratory for feeding radioactive acetate and radioactive tryptophan, uptake of the small volumes used was complete in two to three hours (8). However in the present study complete uptake of the initial 3.0 ml. of solution took anywhere from 12 to 24 hours.

One further point of interest was that plants fed the radioactive serine--non-radioactive formaldehyde combination wilted during the seven day growing period following feeding.

Purity of the DL-Serine-3-C^{14}

The labeled serine was purchased from the California Foundation for Biochemical Research (Los Angeles, California). To be certain that no radioactive substances other than DL-serine-C^{14} were present a sample was co-chromatographed with pure non-radioactive DL-serine. The solvent used was butanol--acetic acid--water in a ratio of 4:1:1. The radioactivity on the chromatograms was located with a Farro chromatograph scanner (Farro Scientific Co., Evanston, Illinois) coupled with Nuclear-Chicago Model 1620A ratemeter and a Model AW Esterline Angus graphic ammeter. Only one radioactive spot was found and its R_f value corresponded to that of the non-radioactive serine.

Isolation and Purification of Nicotine

At the end of the seven day growing period following the administration of the solutions described above, nicotine was isolated as the dipicrate from the plants. The plants were removed from the flasks and their roots rinsed with tap water and then blotted with a cheesecloth. The plants were cut into small pieces and dried under a heat lamp at about 80°C for six hours. The dry plant material was ground with a mortar and pestle and placed in a micro-Kjeldahl flask with 20 per cent of its weight of calcium hydroxide. Four drops of G. E. antifoam solution (G. E. Antifoam 60, General Electric Co., Schnectedy, N. Y.) were added and the contents of the flask steam distilled. The distillate was collected in a flask containing 5 ml. of 6 N hydrochloric acid. Steam distillation was continued until no more nicotine was present in the distillate, as was ascertained with silico-tungstic acid, which forms a white precipitate with nicotine. The distillate containing the nicotine hydrochloride was evaporated to dryness *in vacuo*. The residual nicotine hydrochloride was purified by an azlotropic distillation from basic solution; again the distillate was caught in 5 ml. of 6 N hydrochloric acid (9). The purified nicotine hydrochloride was evaporated to dryness *in vacuo* and the residue dissolved in a small volume of methanol and water. An excess of a saturated methanolic solution of picric acid

was added and the nicotine dipicrate was allowed to precipitate. The precipitate was collected on a small sintered glass filter and recrystallized from water. The melting point was 223°-224°C. (uncorrected). One reference records a value of 224°C. (10).

Results

The radioactivity of the nicotine dipicrate isolated in each experiment is presented in Table II. The specific activities were corrected to zero sample thickness (see Appendix for sample calculation). The specific activity of the combinations fed in Run I was 1.72×10^7 cpm/mmol of serine present. In Runs II and III the activity of the serine in the mixtures fed was slightly lower. The maximum specific activities of the nicotine dipicrates isolated in both Runs II and III were corrected to correspond with the activity fed in Run I. The isolated dipicrate was weighed, ground in a small agate mortar, and plated for counting on aluminum planchets having an area of 2.83 sq. cm. All radioactivity measurements were made with a Nuclear-Chicago Model 192 x scaler (Nuclear Instruments and Chemical Corp., Chicago, Illinois) and a Tracerlab Model SC-16 proportional flow counter (Tracerlab Inc., Boston, Mass.).

TABLE II

RADIOACTIVITY OF THE NICOTINE DIPICRATE

Combination Fed	Run	Number of Plants	Dry Wt. of Plants (gms.)	Wt. of Dipicrate Isolated (mgs.)	Specific Activity of Nicotine Dipicrate (cpm/mmol) $x10^{-4}$
Serine (control)	1	4	1.5	34.6	1.4
	2	4	1.4	23.2	3.2
	3	4	1.4	19.3	0.6
Serine-- Methionine	1	2	0.5	8.1	0.08
	2	4	1.6	8.3	3.6
	3	4	1.9	37.6	0.17
Serine-- Formate	1	2	0.7	3.8	0.76
	2	4	1.8	21.3	0.59
	3	5	2.1	55.4	0.22
Serine-- Formaldehyde	1	2	0.6	4.2	0.089
	2	4	1.9	29.3	0.101
	3	5	2.2	43.3	0.048

TABLE III

COMPARISON OF THE INCORPORATION OF THE BETA CARBON OF SERINE IN NICOTINE DEPENDING ON COMPETING COMPOUND PRESENT

$$\left(\frac{\text{Specific Activity Nicotine Dipicrate in Control}}{\text{Specific Activity Nicotine Dipicrate in Competition Experiment}}\right)$$

Run	Serine-Methionine	Serine-Formate	Serine-Formaldehyde
1	18	2	16
2	0.9	5	32
3	4	3	13
Ave.*	8 ± 9	3 ± 2	20 ± 10

* $\pm$ values are the standard deviations from the mean.

DISCUSSION

DISCUSSION

The nicotine dipicrate isolated was radioactive; this indicated that in each of the experiments the beta carbon of DL-serine was utilized for synthesis of N-methyl groups. It was assumed that essentially all of the radioactivity of the nicotine was in the N-methyl group. This had been indicated in previous experiments (7). An examination of Table III shows that the ratios presented therein are inversely proportional to the extent to which the beta carbon of serine was converted to the methyl group. A large ratio would be indicative of a compound which competed to a large extent, in methyl group synthesis, with serine. Such a compound would be expected to act as an intermediate for the conversion of serine's beta carbon to the nicotine molecule. From Table III it can be seen that the largest ratio was obtained for the serine-formaldehyde combination, the smallest for serine-formate and intermediate between these two was the serine-methionine combination. Hence these data seem to indicate that for the conversion of serine to methyl groups, formaldehyde probably is an intermediate, formate probably is not and methionine may or may not be an intermediate. It is important to note that the range of values varied considerably in the serine-methionine

runs. In fact if each of the three runs for the serine-methionine combination were examined individually instead of as an average three different interpretions could be arrived at.

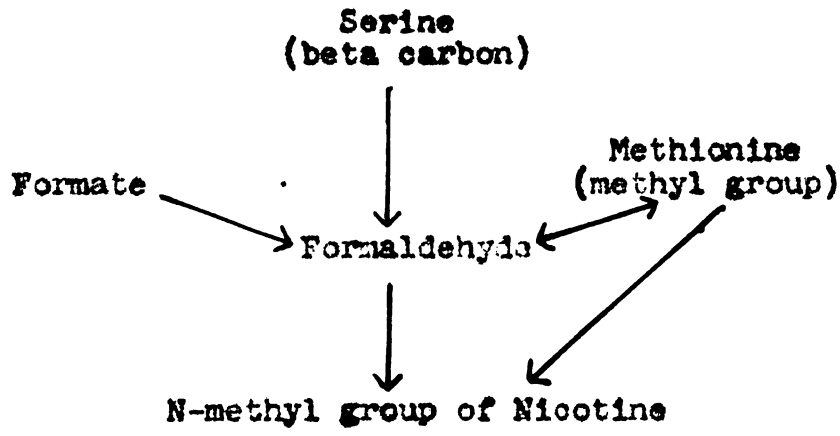
Even formate lowered the incorporation of serine into nicotine to some extent; though not very much when compared to formaldehyde. This can be explained on the basis that formate itself can be used to a slight extent for methyl group synthesis (1). However the bulk of formate probably does not arise from the beta carbon of serine in tobacco plants.

The problem of methyl group synthesis has been investigated in animal metabolism with regard to the relationships of the compounds studied in the present work. In 1940 du Vigneaud (11) showed that the methyl group of methionine can be used for synthesis of methyl groups in choline. Formate and formaldehyde also can act as methyl precursors in choline (12); both being utilised to about the same extent. Jonsson and Mosher (13) demonstrated that the beta carbon of serine and the carbon of formaldehyde could be used to synthesize labile methyl groups in rats. At the same time Weissbach, Elwyn and Sprinson (14) reported similar results for the alpha carbon of glycine and the beta carbon of serine; they also found that the carboxyl carbon of glycine was not used for methyl group synthesis. The alpha carbon of glycine was not used as much as the beta

carbon of serine. This same group of workers (15) also showed that the beta carbon of serine was not oxidized to formate; this is in agreement with the results obtained in the present study. It seems that in animal metabolism methionine and glycine betaine account for most of the methyl group synthesis that takes place (16, 17), whereas in plants the alpha carbon of glycine and formaldehyde appear to play the most important roles.

Byerrum et al. (7) proposed that formaldehyde, the alpha carbon of glycine and the beta carbon of serine might give rise to a common active 1-carbon unit at the oxidation state of formaldehyde. Berg (18) and Kisliuk and Sakami (19) had previously postulated the same thing in their studies of animal metabolism. The biologically active 1-carbon unit appears to be a derivative of folic acid (20). The work of Kisliuk and Sakami (19) has given experimental support for this. An attempt has been made by Brown (21) to isolate radioactive folic acid from tobacco plants after feeding radioactive formaldehyde. The results were inconclusive but it appeared that a radioactive compound resembling folic acid had been isolated.

The following scheme is postulated for N-methyl group synthesis in tobacco plants on the basis of the results reported in this study.



Methionine is visualized as yielding methyl groups either by direct transmethylation or by oxidation to formaldehyde followed by reduction. Serine has been postulated to give rise to the methyl group of either nicotine or methionine by way of formaldehyde; although evidence for the latter pathway was inconclusive from the data presented here. Since the nature of the active 1-carbon unit is not known it has been excluded from the scheme.

One further point that must be mentioned is that Arnstein (22) showed, in animal metabolism, that only the L-isomer of serine was used for methyl group synthesis. The work reported here used DL-serine; therefore, the difference of the two forms in plant metabolism should be investigated.

The possibility of serine's beta carbon yielding methyl groups via transfer to methionine (either directly or by way of formaldehyde) should be reinvestigated because of the dubious results obtained. Other experiments using

different combinations of compounds (e.g. a series with radioactive formaldehyde and various non-radioactive competing compounds) might help to clarify the problem.

SUMMARY

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1. Tobacco plants were fed DL-serine-3-C^{14} alone and in combination with relatively large quantities of non-radioactive formaldehyde, sodium formate and DL-methionine, each in a separate experiment. Nicotine was isolated, as the dipicrate, in each case and its radioactivity determined.

2. By comparing the radioactivities of the nicotine obtained in each experiment it was shown that formaldehyde or an active 1-carbon unit at the oxidation state of formaldehyde probably was an intermediate for the conversion of the beta carbon of serine to the N-methyl group of nicotine. Formate or a 1-carbon unit at the oxidation state of formate was ruled out as an important intermediate. The results for methionine were erratic and no final conclusion regarding its role as an intermediate for methyl group synthesis from serine's beta carbon was reached.

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APPENDIX

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

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

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

C_o = observed count (counts/minute)

M = molecular weight of compound

W = weight of sample counted

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

Sample calculation:

C_o = 236.8 c.p.m., W = 34.6 mg., M = 620

T = 12.2 mg./sq. cm., b = 0.400

$$A_m = \frac{236.8 \times 620}{34.6 \times 0.400} = 1.06 \times 10^4 \text{ c.p.m/mmol.}$$

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