STUDIES ON METHYLATION REACTIONS IN PLANTS: THE ORIGIN OF THE METHYL CARBON OF NICOTINE FORMED BY *NICOTIANA RUSTICA*

By

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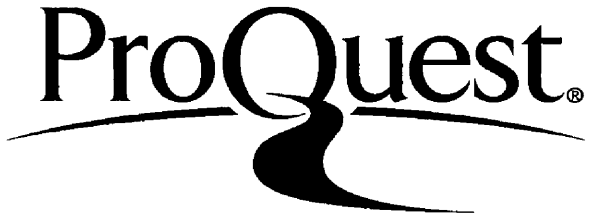

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INTRODUCTION

The investigation described here was undertaken with the dual objective of elucidating some aspects of two distinct problems. The first of these involved the possible role of transmethylation in plant metabolism, and the second concerned pathways in the biosynthesis of alkaloids.

In the course of the past decade a considerable body of evidence has been accumulated to establish the importance of transmethylation in the animal organism (1). In addition, it has been shown that the mould Neurospora crassa can synthesize choline by stepwise methylation of ethanolamine, although the source of the methyl groups is unknown (2). But, while its existence has been postulated (3, 4, 5) almost nothing has been done to establish by direct experimentation whether or not the reaction occurs in the higher plant. In the latter connection, the only evidence has been obtained by means of in vitro experiments. Barrenscheen and von Vályi-Nagy (6) administered methionine to ground wheat germs and found that the synthesis of creatine increased six- to eight-fold in the presence of glycocyamine.

At the commencement of the present study no one, to the writer's knowledge, had attempted to demonstrate transmethylation in the intact plant. Earlier this year such an attempt was reported by Kirkwood and Marion (7) in a study of the formation of the alkaloid hordenine in sprouted barley. They fed C^{14}-methyl choline which has been shown to

act, at least indirectly, as a methyl donor in the animal (8). The hordenine isolated during this experiment possessed negligible radioactivity, and the authors concluded that the N-methyl groups of hordenine do not arise from the choline-methionine system.

The compound chosen for study in our work was nicotine, one of the tobacco alkaloids, which contains an N-methyl group. This compound was selected because of the relative ease of growing tobacco plants, the facility with which nicotine can be isolated and purified, and its comparatively well-known chemistry. Furthermore, it afforded an opportunity of contributing to the second of the two problems referred to above, that of alkaloid biosynthesis. In this field an appreciable amount of work has been published on the physiological aspects (9, 10), but little is known concerning the precursors of the alkaloids or the chemical pathways involved in alkaloid formation. In regard to precursors it appears that the only significant work with nicotine has been that of Klein and Linser (11) and of Dawson (12), who have obtained evidence that proline and pyrrolidonecarboxylic acid act as precursors in tobacco. This evidence was obtained by introducing these compounds into excised shoots and noting an increase in the amount of nicotine formed in the course of the administration period. On the basis of later experiments, however, Dawson (9) has withdrawn his original contention that proline and pyrrolidonecarboxylic acid are nicotine precursors.

In view of the existing state of knowledge in the above-mentioned fields, it appeared promising to study the possible origin of the

N-methyl group of nicotine by transmethylation, using carbon-14 as a radioactive tracer. By feeding to tobacco plants methyl-labelled compounds that have been shown to act as methyl donors in the animal, followed by isolation and degradation of the nicotine synthesized, it was hoped to obtain information concerning the role of transmethylation in alkaloid biogenesis.

EXPERIMENTAL

Synthesis of Radioactive Precursors

The radioactive tracers used in this work were C^{14}-methyl D,L-methionine and C^{14}-formic acid sodium salt. The latter was purchased from Oak Ridge under allocation from the United States Atomic Energy Commission. The methionine was synthesized from C^{14}-methyl iodide which was obtained from Tracerlab, Inc., also under allocation from the Atomic Energy Commission.

The reactions employed in the synthesis of C^{14}-methyl D,L-methionine are summarized below:

(1) D,L-homocystine + $C_6H_5-CH_2Cl$ $\xrightarrow[Na]{NH_3(l)}$ D,L-S-benzylhomocysteine

(2) D,L-S-benzylhomocysteine + $C^{14}H_3I$ $\xrightarrow[Na]{NH_3(l)}$ D,L-methionine

The synthesis of S-benzylhomocysteinewas carried out as described by du Vigneaud and Patterson (13). The methylation step was performed according to the method of Melville, Rachele and Keller (14), but because the C^{14}-methyl iodide was obtained in a glass ampoule a modification of the apparatus was necessary. This is shown in Figure I.

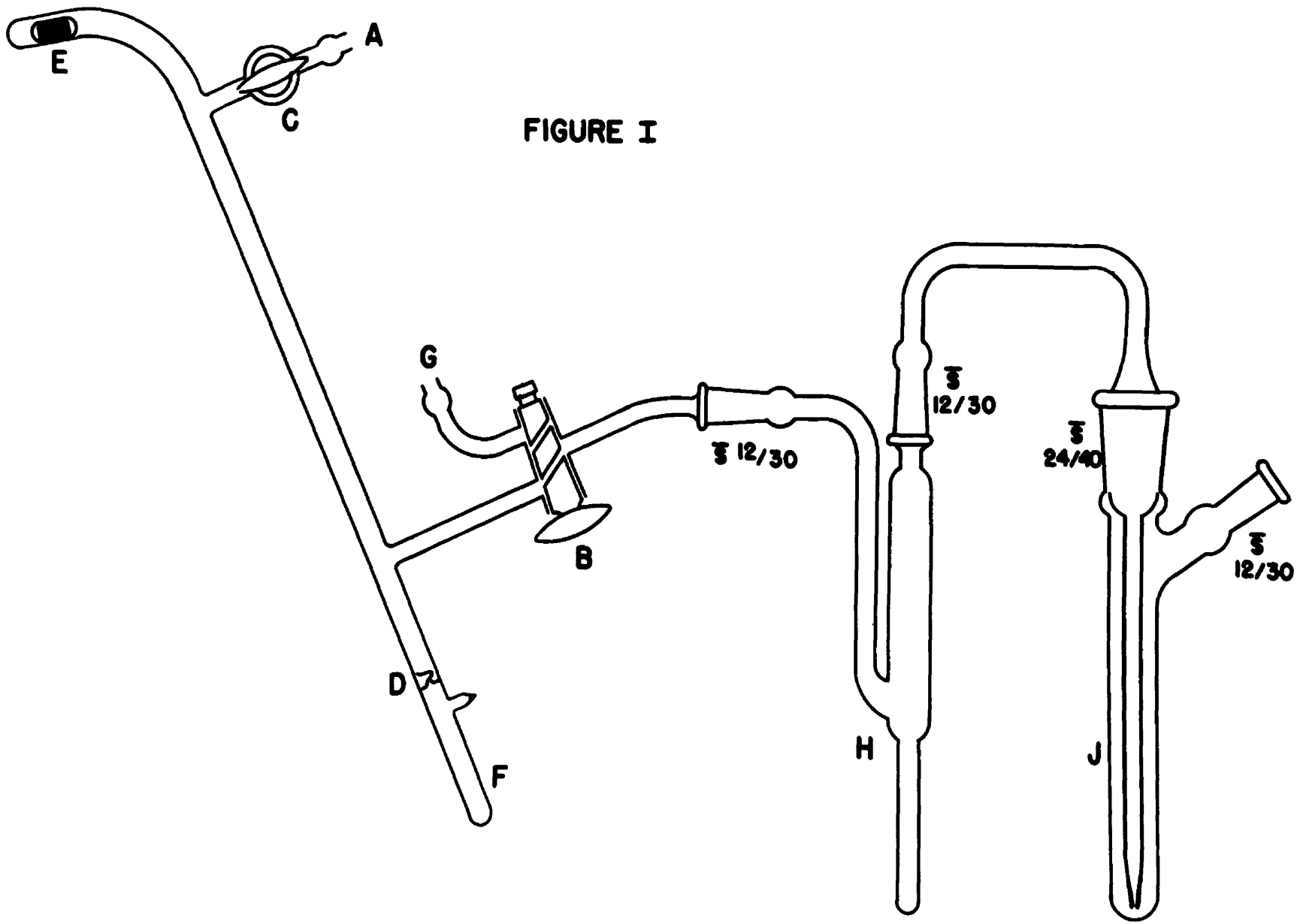

FIGURE I

The equipment was designed so that a portion of the methyl iodide could be retained in a closed tube for use in another synthesis. The trap H and the reaction vessel J shown in the Figure are essentially the same as those used by Melville et al. Trap H contained an appropriate quantity of inactive methyl iodide, calculated to give a yield of 500 mg. of methionine, when combined with the active compound. The apparatus was swept with nitrogen gas, which was dried by first passing it through a soda-lime tower and then through a trap of construction similar to H, cooled in a solid carbon dioxide - methyl cellosolve bath. The ammonia, which served as the reaction medium, was dried by passage of the gas through the soda-lime tower before condensation in vessel J. When it was desired to introduce the methyl iodide, the reaction vessel J and trap H were cooled in CO_2-cellosolve baths, stop-cock B was set as illustrated in the Figure, and the dry nitrogen was admitted at A. The glass-enclosed plummet E was then drawn by a magnet into the long tube and allowed to fall, breaking the sealed capillary D of the glass ampoule F containing the active methyl iodide. The latter was vaporized slowly by heating the ampoule to about 50° in a water bath, and swept through the apparatus in a stream of nitrogen. Some of the vapour condensed in trap H and the rest was carried into the reaction vessel. When the required volume of the active methyl iodide had been swept out of the ampoule, stop-cock C was closed and stop-cock B reversed, confining the remainder of the methyl iodide. The nitrogen was then admitted at G, and the heat transferred

to trap H. The remaining steps in the reaction and purification were carried out as described by Melville and co-workers.

Preparation of the Tobacco Plants

The tobacco used in these studies was Nicotiana rustica L., var. humilis, a high-nicotine strain. The seeds[1] were planted in flats in the greenhouse and transplanted after about three weeks into small pots, where they were grown until they had attained a height of at least six inches. During this interval the plants were occasionally supplemented with commercial plant food mixture as required. The time necessary for the plants to attain the desired size was from two to three months, the variation presumably being due to seasonal effects.

The plants were prepared for the hydroponic administration of the radioactive materials as follows: The earth was carefully removed from the roots, first by shaking and then by washing under a stream of water. As many as possible of the adhering soil particles were removed in this way. The roots were then immersed in a 0.01% solution of Wyandotte detergent germicide No. 1528[2] for at least one hour, with occasional agitation, to reduce the bacterial population. Following a brief rinse under tap water, the roots of each plant were immersed in 50 ml. of an inorganic nutrient solution in a 125 ml. Erlenmeyer flask.

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[1] The seeds were obtained through the courtesy of Dr. N. A. MacRae of the Canadian Department of Agriculture, Central Experimental Farm, Ottawa.

[2] This material was obtained from the Wyandotte Chemicals Corp., Wyandotte, Mich., through the Michigan State College Department of Horticulture.

The nutrient solution was prepared by diluting the stock solution, the composition of which is shown in Table I, 1:3. The nutrient solution also contained an appropriate quantity of the C^{14}-labelled compound being investigated, as will be described later.

TABLE I

COMPOSITION OF THE NUTRIENT SOLUTION

Water	1 l.	Magnesium sulphate: $MgSO_4$:	250 mg.
Calcium nitrate: $Ca(NO_3)_2$	1 g.	Ammonium sulphate: $(NH_4)_2SO_4$:	250 mg.
Potassium chloride: KCl	250 mg.	Potassium dihydrogen	
Ferric chloride	2 mg.	phosphate: KH_2PO_4:	250 mg.

During the administration of the isotopes it was necessary to grow the plants in a fume hood, to guard against any health hazard arising from the possible liberation of $C^{14}O_2$ through respiration. A source of light was supplied by two 36-inch, 30-watt fluorescent tubes and a 100-watt incandescent bulb, placed about 14 inches above the tops of the plants. The light intensity at the level of the upper leaves was found to be in the range of 200 to 250 foot-candles. The light was left on approximately 12 hours out of 24 while the plants were growing, and distilled water was added as required.

Methylation Experiments with D,L-Methionine

In attempting to establish the origin of the nicotine methyl carbon by transmethylation it appeared logical first to try methyl-labelled methionine as a source of the methyl group. This compound has long been established as a methyl donor in the animal (1), and it has been shown by double labelling with C^{14} and deuterium that the methyl group of methionine is transferred as an entity, without intermediate oxidation and reduction; in other words, a true transmethylation (15).

Uptake of methionine from solution. Before administering the radioactive methionine to the plants, it was necessary to ascertain the best method of incorporating the compound into the plant. It appeared, on an a priori basis, that the most advantageous method, if feasible, would be to allow the plant to absorb the methionine from aqueous solution through the roots, inasmuch as nicotine synthesis has been shown to take place in the roots (16). First, however, a suitable analytical procedure had to be found in order to determine (1) the rate of uptake by the plant, and (2) whether the methionine was destroyed by microörganisms in the medium before there was opportunity for its absorption by the plant. The method of analysis chosen was that of McCarthy and Sullivan (17), a colorimetric method based on the reaction of methionine with nitroprusside to give a red colour.

To test the uptake of methionine three milligrams of the amino acid was administered in the nutrient solution to each of six plants.

At the end of twenty-four hours the plants were removed from the solution and the roots were rinsed into the residual medium, which was then analyzed for methionine. It was found that in the twenty-four hour period 67 to 80% of the methionine was absorbed. No evidence of bacterial growth in the medium was observed during this time, but as a check an experiment was carried out in which the medium was inoculated by the addition of a few root fragments. After twenty-four hours the analysis showed no detectable loss of methionine as compared to uninoculated controls. Even after the seven day administration period subsequently adopted the nutrient solutions did not exhibit the turbidity and slimy consistency or odour characteristic of extensive bacterial growth.

Isolation and purification of nicotine. The procedure used throughout this work for the isolation and purification of nicotine was as follows: The plants to be worked up were removed from the nutrient solution and the roots were rinsed with distilled water, the excess being blotted off with cheesecloth. The plants were then cut into small pieces with scissors and dried under heat lamps as rapidly as possible. Toward the end of the drying period the temperature was maintained at 100° C. for about an hour. When the material had been dried it was ground finely in a mortar, mixed with about one-tenth of its weight of calcium hydroxide, and steam-distilled. The distillate was concentrated, and purification of the alkaloid was accomplished by two successive azeotropic distillations from alkaline medium as described

by Smith (18). This was followed by removal of the water from the acid distillate under reduced pressure, the nicotine crystallizing out as the hydrochloride. This was dissolved in methanol plus a little water, and a saturated methanolic solution of picric acid was added in excess. After the solution had stood for a short time the precipitate of nicotine dipicrate was filtered off, washed with methanol, and recrystallized from hot water. (M. P. 224-5° C.; recorded value 224° C. (19).)

Administration of C^{14}-methyl D,L-methionine. In preliminary experiments with the administration of radio-methionine the tobacco plants were allowed to grow in contact with the isotope for a period of twenty-four hours. Two milligrams of methionine, containing 10^5 counts per minute[3], was given to each plant. On working up the plant material, however, only very low activity (about 25 c.p.m. over background at infinite thickness) could be demonstrated in the nicotine picrate. The time in contact with the isotope was, therefore, extended to seven days, using the same quantity of administered methionine. The nicotine picrate isolated after this longer interval was found to possess considerably greater activity, having a maximum specific activity, calculated for zero sample thickness[4], of the order of 4,000 c.p.m. per millimole. One recrystallization was found sufficient to bring the

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[3] This was determined by counting an infinitely thin layer. All counts were made on a Nuclear Instrument & Chemical Corp. end-window counter on the top shelf of the counter assembly. The overall efficiency was 7%.

[4] For calculations, see Appendix I.

activity to a constant value. The seven-day administration period was used in all subsequent experiments.

In order to establish whether or not the radioactivity in the nicotine was localized in the methyl carbon it was necessary to cleave the methyl group from the nicotine molecule, and obtain it in the form of a solid compound suitable for counting. The first method tried for the removal of the methyl group was that of Laiblin (20), who reported having isolated methylamine after complete oxidation of nicotine by alkaline permanganate. Several attempts to duplicate this result, using one gram of nicotine, proved unsuccessful. As the quantities available in this work were much smaller, it was not considered of practical value to continue with this method, and it was abandoned.

Greater success was encountered using a procedure originally described by Herzig and Meyer (21) and later adapted by Pregl (22) as a general micro-method for methyl- and ethyl-imino determinations. This technique involves heating nicotine with hydriodic acid to form the ternary ammonium salt and then splitting off methyl iodide at a higher temperature. In the Pregl method the methyl iodide is swept through alcoholic silver nitrate and the resulting precipitate of silver iodide is weighed, but in the present work it was necessary, of course, to recover the methyl radical. Simmonds and co-workers (23), who demethylated tissue proteins with hydriodic acid, reacted methyl iodide with trimethylamine to form tetramethylammonium iodide, a solid. This principle was modified in our work by the substitution of triethylamine,

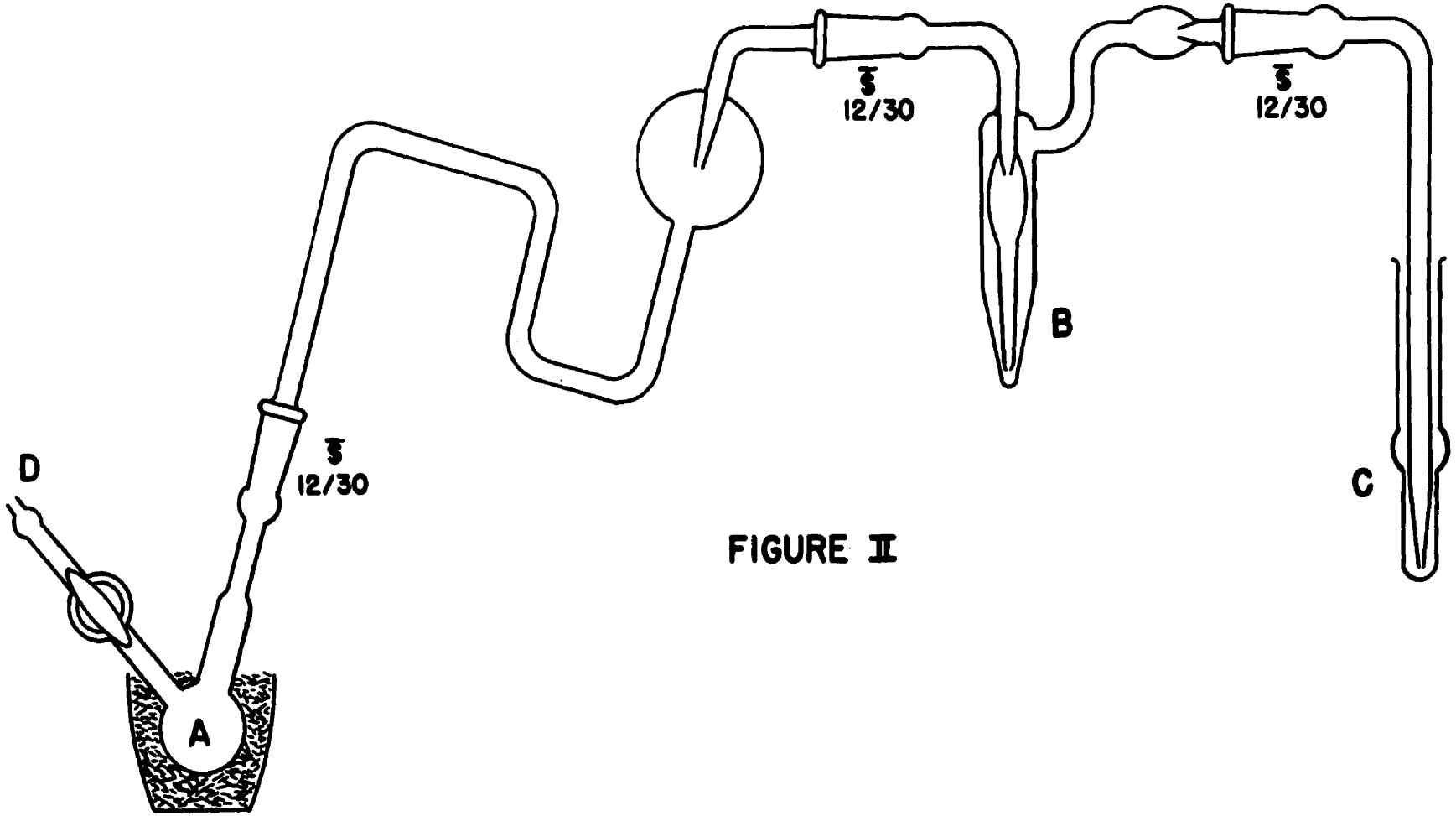

FIGURE II

which we found to react quantitatively with methyl iodide to form methyl triethylammonium iodide, and which has the advantage of much lower volatility as compared to trimethylamine.

The demethylation apparatus used, a modified form of that described by Pregl, as shown in Figure II. The material to be demethylated (the equivalent of 50 mg. of nicotine was used) is placed in the reaction vessel A, together with 45 mg. of ammonium iodide, two drops of 5% gold chloride solution and 3 ml. of hydriodic acid (specific gravity 1.7). The gas-washing vessel B contains 1.5 ml. of the $CdSO_4$-$Na_2S_2O_3$ solution described by Pregl, to remove iodine and hydrogen iodide. The receiver C contains a 5% ethanolic solution of triethylamine, and is cooled in a CO_2-methyl cellosolve bath.

It was not found practical to use the nicotine picrate as such for demethylation, as difficulty was encountered in getting it into solution. The picrate was, therefore, dissolved in dilute sodium hydroxide and the nicotine was recovered by azeotropic distillation as before. The acid distillate was then concentrated, the concentration being completed in the reaction flask A, which was disconnected from the remainder of the demethylation apparatus.

After the addition of the other reactants as described above, the flask A was reconnected and heated[5] in the cupric oxide bath. A slow

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[5] Heating with hydriodic acid causes a gradual etching of the glass, eventually rendering it fragile. It is not advisable to use the vessel more than six or seven times.

stream of nitrogen was passed in through the side-arm D, and the bath temperature was raised to 200° C. in 20 to 25 minutes. By this time some condensed hydriodic acid had collected in the U-tube trap. The temperature was then raised slowly to 350-60° C., and held there for 45 minutes. The heat was removed and the sweeping continued until the reaction flask had cooled. The receiving tube was then taken off and the delivery tip rinsed with a small amount of ethanol. The alcoholic solution was shaken, corked tightly, and allowed to stand over-night at room temperature. In the morning most of the alcohol was removed by heating, and the last of the solvent and the excess amine were evaporated in a vacuum desiccator. A white crystalline residue of methyl triethylammonium iodide was recovered in 55-70% of theoretical yield, based on nicotine. (Anal. Calcd. for $C_7H_{18}NI$: I, 52.20. Found: I, 52.31.)

This quaternary compound was counted under the same conditions as the nicotine picrate and found to possess activity. The maximum specific activity was calculated on a millimole basis using the formula shown in Appendix I. The factor 'b' used was the same as that obtained from the picrate curve; this procedure introduces some error but the magnitude of the error probably is low (24). The maximum specific activities of the nicotine picrate and methyl triethylammonium iodide samples obtained in three experimental runs are shown in Table II.

TABLE II

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

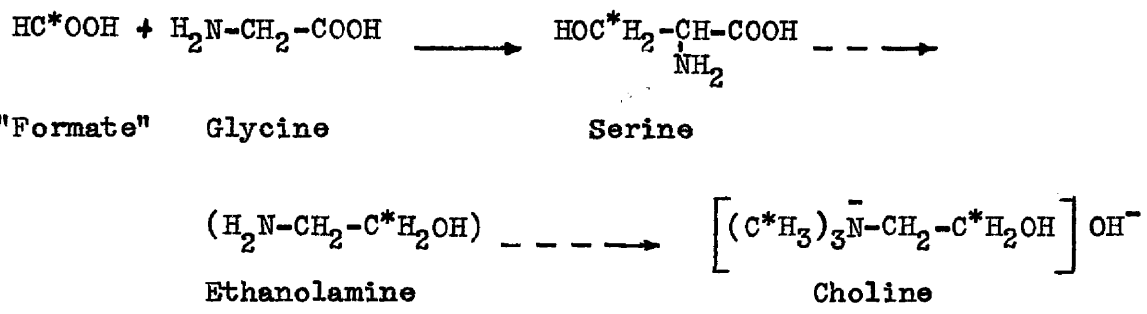
Experiment No.	Maximum specific activity (counts per minute per millimole)	
	Nicotine Dipicrate	Quaternary Iodide
1. (39 plants)	4.28×10^3	4.42×10^3
2. (40 plants)	4.24×10^3	3.92×10^3
3. (39 plants)	7.53×10^3	7.60×10^3

It can be seen from these figures that the recovery of the nicotine activity as the methyl group of the quaternary iodide ranged from about 93 to 103%, indicating that, within experimental error, all the activity is localized in the methyl carbon of the nicotine. It is not clear what caused the relatively high values obtained in Experiment 3, as the treatment was kept as uniform as possible in all the experiments. The explanation may lie, again, in seasonal influences, as the plants were grown at different times of the year.

Methylation Experiments with Formate

On the basis of recent experiments formate, or some one-carbon compound which has been designated as "formate", appears to have an important role in methylation reactions. du Vigneaud and his co-workers, in experiments with rats using C^{14}, have recently shown that formate can act as a precursor of the methyl carbon of choline (25). In view of the

findings of Sakami (26) and of Weissbach, Elwyn and Sprinson (27), it is probable that the conversion takes place by way of serine, as shown schematically below:



It is probable that other intermediates, as yet unknown, also enter into this transformation. Choline can transfer its methyl carbon, through betaine, to methionine (8). On the other hand, methionine can supply a methyl carbon to sarcosine (28), and Mackenzie (28) has shown that the methyl group of sarcosine can be oxidized to formate by rat liver homogenates. Most of this formate is subsequently oxidized further to carbon dioxide, but it is conceivable that part of it enters the formate pool of the organism and is reconverted into the methyl carbon of methionine by the reactions shown above, if conditions exist under which synthesis of the methyl group is taking place.

Under normal conditions in the plant the labile methyl groups must be synthesized from simpler compounds, as an exogenous source of labile methyls is unavailable. If the situation is analogous to that in the animal, formate administered to a plant would be incorporated in part into labile methyl groups and into various methylated compounds. That

such is the case has been shown by Kirkwood and Marion (7), who fed C^{14}-formate to sprouting barley and isolated choline and hordenine containing C^{14} in the methyl groups.

Formate may be involved in methylation reactions in two ways: (1) by conversion first to labile methyl groups, which are then transferred as such, or (2) by acting as a more direct precursor, possibly arising from "labile" methyl groups by oxidation. It was felt that it might be possible to compare the formation of the nicotine methyl carbon from formate with that from methionine, and thus obtain some indication as to which is the more direct precursor. There are obviously several complications in any such experiment; it is necessary to assume that the rates of uptake are about the same, that the size of the pools in the plant remains constant, and that there is no variation in the ratio of nicotine formed during the period of the experiment to that previously stored in the plant. It can be seen from Table II that there is in reality a variation in one or more of these factors, the specific activity of the nicotine in Experiment 3 being about 75% higher than that in the other two experiments. It follows, therefore, that any change in the activity of the nicotine subsequent to the administration of formate, as compared to that following the administration of methionine, would have to be of the order of several hundred per cent before any inferences as to pathways could be drawn.

Uptake of formate from solution. As in the experiments with methionine, it was desirable to gain some idea of the rate of uptake

of formate from solution by the plants. Unfortunately, however, no micromethod for formate could be found which was conducive to results as accurate as were obtainable in the methionine experiments. The procedure finally adopted was that of Grant (29), in which mercuric chloride is reduced by formate to mercurous chloride; the latter is in turn reacted with a phosphomolybdic- phosphotungstic acid to produce a blue colour. In applying this procedure to formate in the plant nutrient solutions it was found that the ammonium salt in the medium caused precipitation on the addition of the mercuric chloride reagent; hence it was necessary to remove the ammonia by first making the solution alkaline and boiling. An additional complication was the progressive development of turbidity after formation of the blue colour, followed by the separation of a gelatinous precipitate; even the most exhaustive washing of the mercurous chloride failed to prevent this. Nevertheless, it was found possible to obtain at least semi-quantitative analyses on formate in the nutrient medium. Over a forty-eight hour period tobacco plants were found to absorb formate from a solution of one milligram of the sodium salt in 50 ml. of the nutrient medium; a quantity of the order of half the total present was taken up in this time, the amount varying somewhat with plant size. No loss by bacterial action, in flasks inoculated with plant root fragments, could be observed in the interval. In subsequent experiments, as in the case of those with methionine, no signs of extensive bacterial growth were observed during a seven-day administration period, and a microscopic examination of the filtrate from the residual medium at the end of this

time confirmed this observation. To check further the uptake of formate by the plants a determination was made during one of the runs by drying and counting an aliquot of the nutrient solution. In less than three days from the start of the run, only about one per cent of the administered radioactivity could be accounted for in this way. Thus by this time, at most, virtually all of the formate had been absorbed by the plant.

Administration of C^{14}-formate sodium salt. In administering radioactive formate to the tobacco plants it was desirable, for purposes of comparison, to maintain the conditions of treatment as similar to those of the methionine experiments as possible. It was not feasible, of course, to maintain constant the previous treatment of the plants, because of seasonal variations in light, temperature, and other factors. Dilution of a sample of C^{14}-formate was carried out so that 0.91 mg. of the sodium salt, containing 10^5 counts per minute was given to each plant; the molar quantity and the total counts per minute were thus the same as that of the methionine. The plants were maintained under the same growing conditions as described previously for the seven-day period. At the end of this time they were worked up as before, and nicotine was again isolated as the dipicrate. After counting, the nicotine was recovered and demethylated as in the methionine experiments and the resulting quaternary iodide was counted. Table III shows the values obtained in two runs of plants.

TABLE III

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER FORMATE ADMINISTRATION

Experiment No.	Maximum specific activity (counts per minute per millimole)	
	Nicotine Dipicrate	Quaternary Iodide
1. (25 plants)	9.6×10^2	9.3×10^2
2. (29 plants)	4.4×10^2	4.1×10^2

It is evident from these results that there is some incorporation of the formate carbon into nicotine, and that most if not all of the activity is in the methyl carbon. The low count in this case introduces relatively larger errors, and it is not possible to compare the specific activities of the nicotine and the quaternary iodide with the degree of accuracy obtainable in the methionine experiments.

A comparison of the values recorded in Table III with those of Table II reveals that the former are considerably lower. The lowest specific activity obtained for the methyl carbon in the methionine experiments (II,2) is over four times as high as the higher obtained after formate administration (III,1), whereas at the other extreme the value II, 3 is nearly nineteen times in excess of III,2. It appears, therefore, that when C^{14}-formate is fed to N. rustica the specific activity of the nicotine methyl carbon isolated is about one order of magnitude lower than that which results after feeding C^{14}-methyl methionine, under conditions maintained as nearly as possible constant.

DISCUSSION

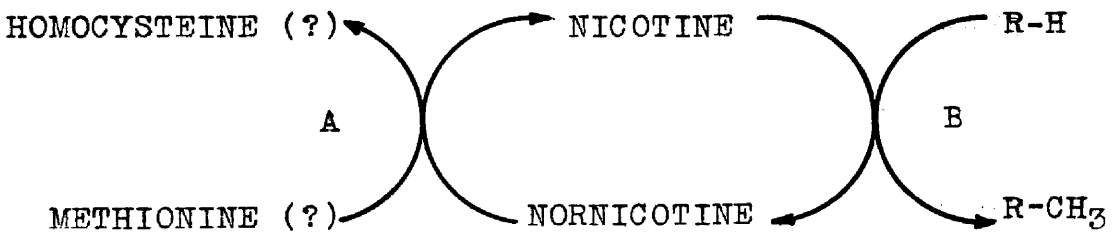
The results of these investigations show that the methyl carbon of methionine can act as a precursor of the methyl carbon of nicotine *in vivo*, and that to an appreciably lesser extent formate can also act as a precursor. The data presented constitute the first direct chemical evidence that transmethylation takes place in higher plants. The lower specific activities obtained using formate serve as an indication that the methionine methyl group is not oxidized to formate and then reduced during the transfer of the methyl carbon to nicotine. If such an oxidation and reduction does occur, the administration of formate should have resulted in a specific activity for the nicotine methyl at least as high as that obtained following the feeding of methionine. It can be inferred from our results that the function of formate in transmethylation is, alternatively, that of a precursor in the biosynthesis of labile methyl groups such as that of methionine. It is probable, as pointed out earlier, that formate is converted to the labile methyl by way of serine in some manner as yet incompletely understood.

The final proof that the methionine methyl group is transferred as an entity to nicotine must await the completion of experiments involving double-labelling with carbon-14 and deuterium. These experiments are now being conducted in this laboratory, and the results will be forthcoming at a later date.

It is somewhat difficult to reconcile the results of our experiments with those of Kirkwood and Marion (7), who obtained considerable incorporation of formate carbon into the methyl groups of hordenine but only negligible incorporation from the methyl carbon of choline. However Wing (30), in preliminary experiments in this laboratory, has found evidence that choline is subject to quite rapid destruction by bacterial action in the plant nutrient solution, and as the above authors do not refer to any precautions taken to restrict bacterial growth it is possible that much of the choline was destroyed before it could be absorbed by the barley. It would appear that further investigation is indicated, possibly involving a direct comparison of the effects of choline and methionine under sterile conditions, in the same species.

Dawson (4) has shown by means of grafting experiments that in species which produce nornicotine, such as N. glutinosa and N. glauca, this alkaloid is produced not in the roots, but in the leaves, by demethylation of nicotine translocated from the roots. On the basis of these findings he has advanced the plausible hypothesis that a transmethylation reaction takes place in the leaf, in which nicotine serves as the methyl donor and some as yet unknown compound or compounds as the acceptor. To explain the fact that many species, e.g. N. tabacum and N. rustica, contain little or no nornicotine he suggests that such species have lost the enzymatic system responsible for the transmethylation.

The data presented in this paper, however, constitute strong evidence that, in N. rustica at least, a transmethylating system is operative during the primary synthesis of nicotine. As it is probable that the enzyme systems involved in these two transmethylation reactions would be very closely related if not actually identical, it appears to the writer unlikely that one system would be lost in the course of natural selection and the other retained. For this reason it seems desirable to advance an alternative hypothesis, which is based on the assumption that nornicotine, once formed in the leaf, can be remethylated at the expense of methionine, or some other labile methyl donor. This is illustrated diagrammatically below. If transmethylation involving nicotine as a methyl donor



takes place in the leaf, it is reasonable to postulate a second transmethylation at this site in which nornicotine acts as a methyl acceptor. Although the investigations of Dawson (16) indicate that nicotine can be completely synthesized only in the root they do not, as James (10) has pointed out, exclude the possibility that the leaves, too, may form nicotine if provided with suitable precursors. Even aside from this

consideration, there is no evidence to suggest that the root is the only organ where methylation can occur. If the reactions in the leaf are as represented in the diagram it is no longer necessary to postulate the loss of reaction B in a species such as N. rustica; it is necessary only that the ratio of reaction rate A to reaction rate B differ from one species to another. In N. rustica, for example, if B were a slow reaction and A relatively rapid, the nornicotine formed would soon be remethylated through the methionine system and hence would not accumulate. Conversely, in nornicotine-producing species, the accumulation of nornicotine could be explained if A were a very slow reaction and B a more rapid one. In this case nornicotine would be formed faster than it could be remethylated and would tend to accumulate in the leaf.

The situation obtaining in N. glauca introduces further complexities. It has been found that certain strains of this species store some nicotine as well as nornicotine, whereas others do not possess nicotine in detectable amounts (4). Dawson (4) has explained this difference on the basis of loss of the transmethylating mechanism in the former case. While this appears to be the simplest explanation, it could also be explained by assuming that in the strains which accumulate no nicotine reaction B is sufficiently rapid to demethylate all the nicotine arising from primary synthesis plus the small amount being supplied through reaction A. We believe, as does Dawson (9), that the limiting factor in reaction A is most likely to be the supply of the hypothetical methyl acceptor, which is presumably supplied through translocation. The accumulation of nicotine in other strains of N. glauca

could, therefore, be explained by a reduction in the rate of reaction B caused by a more limited supply of the acceptor. Leaves of N. glauca grown on plants grafted to the high-nicotine producing N. tabacum have been observed to be capable of considerably greater rates of nornicotine synthesis (4). We have only to assume here that a greater supply of the methyl acceptor is produced concurrently with the nicotine in the roots and transported to the enzyme system in the leaves. This is not inconsistent with the slow rate of reaction B previously postulated for N. tabacum, as only the rate relative to reaction A was then being considered. The limiting factor in reaction A may well be the availability of methionine or some related compound as a methyl donor in the leaf.

It should be emphasized that the above scheme is almost entirely hypothetical. We consider it probable that the differences in nicotine and nornicotine concentrations between species and strains are due to variations in reaction rates, and ultimately to the availability of the various reactants, but Dawson's hypothesis remains a possibility and cannot by any means be ruled out on the strength of existing evidence. It is apparent, of course, that much more experimental work will be required before any final conclusion can be reached. The first thing to be determined should be whether or not the methyl group of nicotine is, in reality, labile. This information should be obtainable by studying the fate of C^{14}-methyl nicotine introduced into an intact plant. If nicotine can be shown in this way to act as a methyl donor, the first

positive evidence for a biological function of the alkaloids will have been obtained. An extension of these tracer techniques might also elucidate the other inter-relationships which exist among the tobacco alkaloids.

SUMMARY

1. Nicotine isolated from Nicotiana rustica L., var. humilis, previously fed methionine containing carbon-14 in the methyl group, has been shown to possess radioactivity. By means of degradation experiments this activity has been found to be localized in the methyl carbon.

2. After administration of C^{14}-formate to this species, nicotine of appreciably lower specific activity has been obtained. Most, if not all of the activity has also been shown to reside in the methyl carbon.

3. On the basis of these results it is postulated that formate is first converted into labile methyl groups, such as that of methionine, and that these are transferred to nicotine, in a manner analogous to transmethylation reactions already demonstrated in the animal.

4. The significance of these findings as related to certain aspects of alkaloid biogenesis is discussed.

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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 calculation:

Nicotine picrate -- C_o = 142 c.p.m., W = 26.4 mg., M = 620,

T = 9.0 mg./cm.^{2}

$$A_m = \frac{142 \times 620}{26.4 \times 0.465} = 7.17 \times 10^3 \text{ c.p.m./mM.}$$