

METHYL GROUP SYNTHESIS IN PLANT METABOLISM

I. UTILIZATION OF GLYCINE-BETAINE AS A METHYL GROUP PRECURSOR

II. THE BIOSYNTHESIS OF PECTIN METHYL ESTERS

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ABSTRACT

Glycine-betaine and methionine, both of which were labeled in the methyl group with carbon-14, were administered to tobacco and radish plants respectively. Both compounds were shown to be precursors of methyl groups as shown by the incorporation of the carbon-14 into the N-methyl group of the nicotine molecule in the tobacco plant and into the methyl ester group of pectin molecule in the radish roots. The relative radioactivity of the pectin was much greater than that of the nicotine.

The N-methyl group of nicotine was cleaved and the released methyl group was counted for radioactivity as the methyltriethylammonium iodide. Saponification of pectin followed by distillation of the methanol and a subsequent oxidation of methanol to carbon dioxide was carried out in a glass apparatus designed in this laboratory. The carbon dioxide was counted as barium carbonate. Essentially all of the radioactivity of the nicotine was localized in the N-methyl group, whereas about 93 to 95 percent of the radioactivity of pectin was found to be in the methyl ester.

The pectic acid residue which was obtained by the saponification of pectin was shown to contain about one percent of the total radioactivity of the original pectin. The methoxyl content was found to be 8.6 percent of the radish root pectin.

Attempts were made to show the existence of the choline oxidase system in Nicotiana rustica. Homogenates and mitochondrial preparations

of tobacco leaves were used, but no active enzyme system could be demonstrated. C^{14} -methyl choline was administered to tobacco plants and one week later, betaine and choline were extracted from the plants and isolated as the reineckates. The reineckate salt was removed and the betaine and choline were obtained as the chlorides, which were separated by two-dimensional paper chromatography. A radioautograph of the chromatogram revealed two spots corresponding to choline and betaine.

VITA

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INTRODUCTION

In the present study two aspects of the transmethylation reaction, the metabolic transfer of an intact methyl group from one compound to another, were investigated in higher plants. The first was to establish whether the methyl groups of glycine-betaine might be transferred to give the N-methyl group of nicotine and whether choline is oxidized to glycine-betaine¹ before transmethylation can occur. The second was to determine whether transmethylation of the methionine methyl group might result in the formation of an O-methyl of methyl esters in pectin.

Du Vigneaud and his coworkers have shown that transmethylation is a general reaction in animal metabolism (1). Through two lines of research, choline was shown to be one of the naturally occurring sources of methyl groups. One, in experiments with young rats on a methionine-free diet supplemented with homocysteine, choline was shown to support growth (2). Methionine was formed when young rats were fed homocysteine and choline (3). The other approach demonstrated that when choline labeled with deuterium in the methyl groups was fed, creatine and methionine isolated from the rat contained deuterium labeled methyl groups (4). On the other hand, betaine was shown to be more effective than choline in the formation of methionine (5). Whereas choline and also betaine can prevent fatty infiltration of the liver in rats and can cause methylation of homocysteine (3), liver homogenates prepared from chicks, guinea pigs, and rabbits, which do not oxidize choline, are unable to catalyze the formation of

¹ Glycine-betaine will be referred to as betaine henceforth.

methionine from homocysteine and choline (7). Dimethylthetin and propiothetin, sulfur-containing compounds analogous to betaine and alanine-betaine respectively, when incubated with homocysteine and rat liver homogenates yielded methionine (8). The thetins also supported growth of young rats kept on a choline and methionine-free diet supplemented with homocysteine (9). However, when sulfocholine was used instead of choline, rats did not grow (10).

It was shown by Dubnoff that under aerobic conditions, rat liver homogenates and rat liver slices methylated homocysteine to methionine in the presence of choline or betaine. Nevertheless, in the absence of oxygen only betaine served as the methyl donor (7). Further evidence that choline itself does not function as a methyl donor was supplied by experiments with rat liver homogenates incubated with homocysteine and with choline labeled with nitrogen-15. Under aerobic conditions, N¹⁵-dimethylglycine was formed but not N¹⁵-dimethylaminoethanol. If choline were the active methyl donor, N¹⁵-dimethylaminoethanol would have been formed (12).

In 1952, Brown and Byerrum showed that methyl carbon of methionine could serve as a precursor for N-methyl group of nicotine in the tobacco plant, thus, suggesting the occurrence of transmethylation reaction in higher plants (13). Flokstra demonstrated that the methyl carbon of methionine could also serve as a precursor for the methoxyl carbon of lignin in barley (14). By feeding methionine doubly labeled with deuterium and carbon-14 in the methyl group to barley and then isolating the lignin, Dewey showed that the deuterium and carbon-14 of the methoxyl group was in the same ratio as that in the doubly labeled methionine. Therefore, the methyl group of methionine was transferred intact -- a true trans-

methylation reaction -- and the methylation process did not occur by oxidation of the methionine methyl group followed by reduction (15). In the case of barley (16) and castor beans (17), methionine was demonstrated to be a methyl donor in the formation of the alkaloids, hordenine of barley and ricinine of castor beans; choline on the other hand yielded no methyl group. Nevertheless, Wing demonstrated that methyl groups of choline were precursors of the N-methyl group of nicotine (18).

Working with germinating seedlings of chick-pea, Ahmad and Karim showed that methionine, acetone, and methanol can act as precursors of the methyl group of choline (19). In another study it was suggested that small amounts of betaine were found in etiolated seedlings of Hordeum and that the methyl group of betaine should be available for transfer to hordenine, if betaine is the active donor (20). Experiments performed on Beta vulgaris and Atriplex patula by infiltrating choline and betaine aldehyde into the leaves resulted in a significant increase in the betaine content of the tissues. When the leaves were infiltrated with solutions of choline and then kept in an atmosphere of nitrogen, no significant increase in betaine was observed (21).

In view of the above discussion, it seemed interesting to compare the ease with which betaine can offer methyl groups in comparison with choline for the synthesis of nicotine. In order to gain further evidence as to the mechanism of transmethylation and also to explain the fact that choline may yield methyl groups in the tobacco plant but not in etiolated barley seedlings, it was decided to study the enzyme system which might catalyze the oxidation of choline to betaine.

Although S-methyl, N-methyl, and O-methyl group formation in animals and plants have been well known, the possibility of an O-methyl formation in a methyl ester by means of transmethylation has not been postulated to date. Pectin, a methyl ester of polygalacturonic acid, was selected as a desirable compound for this particular study. The selection was based on the abundance of pectic substances in plants, the ease of its isolation, and the rapid rate of its formation. Since a fast-growing plant was desirable, it was decided to use radish roots.

The term pectin was derived from the Greek " $\pi\eta\chi\tau\omicron\varsigma$ ", meaning to congeal or solidify (22). Because of a great heterogeneity in the literature with regard to what is meant by "pectin", the term as used in this work will adhere to the Revised Nomenclature of the Pectic Substances adopted in 1944 (23). Wide variations in nomenclature probably resulted from different preparative methods which yielded products of diverse compositions.

In 1913, Fellenberg discovered the methyl ester group in pectic substances (26). Four years later, Suarez (24) reported the presence of uronic acid in pectin, and Ehrlich (25) postulated that galacturonic acid was the basic building block of pectic substances. In the ensuing years, various structures for pectic acid were presented. Some of these are: the octa-uronic structure of Smolenski (made up of four digalacturonic acids) (27), the cyclic structure (a basic unit made up of four galacturonic acid residues linked in a ring with arabinose and galactose as side chains) of Nanji, Paton, and Ling (28), and the tetragalacturonic acid structure of Ehrlich (29). Although most of the earlier workers were in favor of the cyclic structure, Morell, Baur, and Link in 1934

indicated by means of a modified end-group assay that pectic acid was a linear polymer (30).

Studies of physical properties of pectic substances and nitropectin (pectin nitrated with nitric acid) (32, 33), and of periodic acid oxidation of polygalacturonic acid (34), showed a close resemblance to cellulose. Exhaustive methylation of pectic acid followed by hydrolysis indicated that pectic acid consists of pyranose galacturonic acid residues linked through 1,4- α -glycosidic linkages (35).

Several enzymes found in plants may degrade pectin. Three pectic enzymes generally recognized by the early workers were (36): protopectinase, an enzyme which hydrolyzed protopectin to soluble pectin; pectinase, an enzyme which hydrolyzed pectic compounds to galacturonic and smaller polygalacturonic acids; and pectase, an esterase which hydrolyzed the methyl ester groups of pectin and pectinic acids. In 1945, there was evidence that pectinase could catalyze the hydrolysis of only those glycosidic bonds adjacent to free carboxyl groups (37). McCready and Seegmiller, in 1954 (38), divided the pectic enzymes into two classes -- those having esterase activity and those catalyzing the hydrolysis of glycosidic linkages. They have shown that purified citrus pectinesterase does not hydrolyze the ester groups of di- and trigalacturonic acids nor the half esters of digalacturonic acid, but attacks esters with Degree of Polymerization Number of ten and greater. Purified fungal polygalacturonase was shown to hydrolyze glycosidic bonds between uronic acids containing adjacent free carboxyl groups in all polygalacturonic acids. However, glycosidic bonds between uronic acids, one of which is esterified, were not hydrolyzed. Such results tend to indicate the possible importance

of the pectinesterase over the polygalacturonase with respect to the formation of the polymethyl esters of large pectic compounds. Two questions still remain in the methyl-ester formation of pectin: whether the methyl group replaces the proton of the carboxyl group of the galacturonic acid residues, or whether methyl ether is first formed with a galactose residue and then the methylene group is oxidized to form the ester. In view of the great significance of one-carbon precursors in both animal and plant metabolism, the possible origin of the O-methyl in the formation of the polyesters appeared worthy of investigation.

EXPERIMENTAL

Synthesis of C¹⁴-methyl betaine

In the studies in which betaine was utilized as a possible methyl group precursor for the N-methyl group of nicotine, it was first necessary to synthesize C¹⁴-methyl betaine. This synthesis was carried out as described by Ferger and du Vigneaud (39). In 32 ml. of 75 percent ethanol (v/v), 16.8 mM of sodium dimethylglycinate was dissolved and then the mixture cooled to -10°C. One millicurie of C¹⁴-methyl iodide (specific activity of one millicurie per millimole) obtained from Tracerlab, Inc., under allocation from the Atomic Energy Commission, was then added. Five minutes later, 15.6 mM of inactive methyl iodide was added. The reaction vessel was then stoppered and heated at 70° for eighty-five minutes. The resulting mixture was then evaporated to a small volume in vacuo and a freshly prepared saturated aqueous ammonium reineckate solution was added. After acidifying the solution to Congo Red paper with hydrochloric acid, the mixture was allowed to cool overnight in the refrigerator. The resulting betaine reineckate was isolated by filtration, dissolved in 160 ml. of 0.1 N ammonium hydroxide solution, and then shaken with excess silver oxide until the reineckate color (red) disappeared. The silver reineckate was collected on a filter and washed. The combined filtrate and washing was heated to 60° and aerated four hours to remove the ammonia. After concentrating the solution to about 80 ml., it was acidified with hydrochloric acid and the small quantity of silver chloride which formed was filtered off. The precipitate was washed with a small

quantity of water and the filtrate and washings were combined, evaporated to dryness, and dissolved in a minimum volume of boiling ethanol. This solution was filtered and then allowed to cool overnight in the refrigerator to obtain crystals of betaine chloride. After collecting the crystals on a filter and evaporating the filtrate to about 50 ml., the second crop of betaine chloride was collected. The combined product was recrystallized twice from ethanol: 1.33 g. of betaine chloride was obtained. (Anal. Calc. for $C_5H_{12}O_2NCl$: Cl, 23.08%. Found: Cl, 23.20%.) (40).

Preparation of the tobacco plants

Seeds of Nicotiana rustica L., var. humilis², a high nicotine strain, were planted in flats containing vermiculite³ in the greenhouse. Tap water was applied to keep the vermiculite moist, and a nutrient solution containing 1 g. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4 , 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$ dissolved in four liters of tap water was applied twice a week. Within three weeks, the plants were transplanted allowing two to three inches between plants. Probably due to seasonal changes, the plants required about two to three months to attain the desired height of six inches.

For the hydroponic administration of the radioactive material, the plants were prepared as follows: the vermiculite was washed off under a

² The seeds were obtained through the courtesy of Dr. N. A. MacRae of the Canadian Department of Agriculture, Central Experimental Farm, Ottawa.

³ Vermiculite is a commercially available heat expanded mica.

⁴ This germicide was obtained from the Wyandotte Chemicals Corp., Wyandotte, Mich., through the Michigan State University Department of Horticulture.

stream of water; the roots were soaked one hour in a 0.01 percent solution of Wyandotte detergent germicide No. 1528⁴ to reduce the bacterial population; the roots were washed with water; and the plant was placed in a 125 ml. Erlenmeyer flask containing 0.5 ml. of aureomycin (1:1000), the Cl⁴-methyl betaine, and 50 ml. of an inorganic nutrient medium. This solution was prepared by diluting with three parts of water, one part of a stock solution which had the following composition: water, 2 l.; Ca(NO₃)₂·4H₂O, 2.61 g.; KCl, 500 mg.; FeCl₃·6H₂O, 5.6 mg.; MgSO₄·7H₂O, 756 mg.; (NH₄)₂SO₄, 500 mg.; KH₂PO₄, 500 mg. The aureomycin was added to prevent the growth of root microorganisms. Oxygen was bubbled through the nutrient solution twice a day for two minutes to prevent wilting of the plants and also to provide aeration for the roots. Nutrient solution was added as required to keep the volume constant.

The plants were grown in the hood illuminated by two 36-inch, 30-watt fluorescent tubes and a 100-watt incandescent bulb placed about fourteen inches above the tops of the plants. At this level of the plants, the light intensity was 200 to 250 foot-candles. The lights were left on twelve hours every day, from eight o'clock in the morning to eight o'clock in the evening.

Uptake of betaine from solution

It was demonstrated by Wing (18) that tobacco plants can absorb choline from aqueous solution through the roots; therefore, it seemed feasible that betaine might also be absorbed through the roots. However, it was necessary to ascertain the extent of uptake of betaine by the plant and the destruction of betaine by microorganisms outside the plant.

Twelve 125 ml. Erlenmeyer flasks were set up, each containing 25 ml. inorganic nutrient medium, 0.5 ml. aureomycin solution (1:1000), and 2.06 mg. betaine chloride. Plants, the roots of which were soaked in 0.01 percent solution of Wyandotte detergent germicide for one hour, were placed into four of the flasks; about six root fragments, into another four; and the remaining four flasks served as controls. Seventy-two hours later, the roots of the plants and the root fragments were washed, the washings added to their respective flasks and the solution in each flask filtered into separate 50 ml. suction flasks. The filtrates were evaporated to dryness in vacuo at 50 to 60° and transferred quantitatively into 15 ml. centrifuge tubes with enough water to make a volume of 3.0 ml.

Betaine was determined by the periodide method of Reifer (41) in the following manner. After dissolving 0.3 g. sodium chloride in the 3.0 ml. solution, 0.5 ml. concentrated phosphoric acid was added, followed by 1 ml. potassium triiodide (2.5 g. iodine, 3.75 g. potassium iodide, and 10 g. sodium chloride in 100 ml. water). The mixture was stirred and then kept for three hours in a salt-ice bath at -5 to -10°C. Three layers were obtained after centrifuging for five minutes at 2500 revolutions per minute in a clinical centrifuge. The top layer, a solution of potassium triiodide, was removed by suction without disturbing the second layer, which was phosphoric acid. The sides of the tube were washed three times with 3 ml. portions of ice water without centrifuging, and the phosphoric acid layer was removed at the last washing. The bottom layer containing the betaine periodide was then dissolved in 3 ml. of 95 percent ethanol and titrated with 0.05 N sodium thiosulfate solution using a starch indicator. The amount of betaine present in the unknown was ascertained

by reference to a standard curve in which milliliters of 0.05 N sodium thiosulfate had been plotted against a known quantity of betaine chloride recrystallized three times from ethanol. The plants absorbed 98.5 percent of the betaine chloride originally added during the 72-hour period, whereas the loss of betaine chloride due to microorganisms was negligible as judged from the fact that no loss of betaine occurred in flasks inoculated with root fragments as compared to the controls.

Administration of C^{14} -methyl betaine and the isolation of nicotine

Preliminary feeding experiment showed that 2.06 mg. betaine chloride having a radioactivity of 0.5×10^5 counts per minute administered per plant was not radioactive enough to get a significant count in the nicotine that was isolated from the plants. Consequently, betaine chloride having an activity of 1.92×10^5 counts per minute per 2.06 mg. was administered to each plant. Seven days later, the roots of the plants were washed with distilled water and then blotted with cheesecloth to remove the excess water. The plants were then cut into small pieces with scissors, dried at a temperature below 80° under an infra-red lamp, ground in a mortar, mixed with one-tenth of their weight of calcium hydroxide, and then steam-distilled. The distillate was collected -- until no precipitate formed with silicotungstic acid (12 g. per 100 ml. water) -- in a flask containing hydrochloric acid and then it was concentrated in vacuo. Following the procedure of Smith (42), the solution was made alkaline and the nicotine was purified by two successive azeotropic distillations into hydrochloric acid. The acid distillate was evaporated to dryness in vacuo and the resulting nicotine hydrochloride was dissolved

in a small volume of water. An equal volume of saturated methanol solution of picric acid was added, and the mixture was kept in the refrigerator for half an hour. The nicotine dipicrate which precipitated was collected on a filter, washed with cold water, and then recrystallized from hot water (m.p. 224-225°; recorded value 224°) (43). After grinding the nicotine dipicrate in a mortar, it was plated on an aluminum counting disc (2.83 sq. cm.). Counting for radioactivity was done with a windowless flow counter from Tracer Lab. and a Scaling Unit from Nuclear Corp. using a gas mixture of helium (99.05 percent) and isobutane (0.95 percent). By using the formula shown in Appendix I, the maximum specific activity per millimole of the nicotine dipicrate was calculated. The results shown in the second column of Table I indicate that the nicotine from C¹⁴-methyl betaine fed plants was radioactive and suggest that betaine is a precursor for some portion of the nicotine molecule.

TABLE I

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER
C¹⁴-METHYL BETAINE ADMINISTRATION

Experiment No.	Maximum Specific Activity (counts per minute per millimole)	
	Nicotine Dipicrate	Quaternary Iodide
1. (26 plants)	1.97 x 10 ³	2.05 x 10 ³
2. (30 plants)	1.61 x 10 ³	1.78 x 10 ³

Demethylation of nicotine

Since the nicotine isolated from C^{14} -methyl betaine fed plants was radioactive, it was of interest to ascertain the amount of the total radioactivity located in the N-methyl group. The nicotine N-methyl group was, therefore, cleaved according to the procedure of Pregl (44) as modified by Brown (45). This method consisted essentially of treating the nicotine with ammonium iodide and hydriodic acid to form the quaternary ammonium salt and then splitting off methyl iodide at 350 to 360° in the presence of gold chloride. An ethanol solution of triethylamine, cooled in a CO_2 -methylcellosolve bath, was used to absorb the liberated methyl iodide. After standing overnight and then evaporating off the ethanol and excess triethylamine, the methyltriethylammonium iodide which had formed was obtained as a white solid. This was counted in the same way as the nicotine dipicrate, and the maximum specific activity per millimole of the quaternary iodide was also calculated by using the formula shown in the appendix. The results are shown in Table I.

From the figures illustrated in Table I, it can be seen that, within experimental error, all the radioactivity of the nicotine was recovered in the quaternary iodide. It is, therefore, evident that the radioactivity of the nicotine after feeding C^{14} -methyl betaine was localized in the methyl group. These results indicate that the methyl groups of betaine are precursors for the N-methyl group of nicotine.

Feeding C¹⁴-methyl choline

In an attempt to ascertain whether or not choline might first be oxidized to betaine before it could yield its methyl groups for nicotine synthesis, it was decided to feed radioactive choline to tobacco plants and after a suitable period of time to try to isolate radioactive betaine from these plants.

Each of fourteen plants -- prepared by cutting off the roots and allowing the regeneration of new roots in the synthetic nutrient medium for a period of one week prior to feeding -- was administered 2.0 mg. of choline chloride having an activity of about 10^5 counts per minute. The choline used in these experiments was isolated as the reineckate from plants which had previously been fed C¹⁴-methyl choline by Wing (18). The plants were grown in a manner similar to that described earlier.

One week after feeding the radioactive choline, the roots of each plant were removed from the nutrient solution in which they were growing, washed with water and then blotted with cheesecloth. The plants were then cut into small pieces with scissors and dropped into 500 ml. of boiling 0.02 N hydrochloric acid solution to inactivate the enzymes. After cooling, the mixture was ground in a Waring Blendor for 15 minutes and then filtered through an extraction thimble. The thimble containing the residue was transferred to a Soxhlet extractor and was extracted for seven and one-half hours using the acidic filtrate obtained above as the extraction solvent. Twenty-five milliliters of 1 N sodium hydroxide solution was added to the extract thus obtained and this solution was then concentrated in vacuo at 50° to about 200 ml. The nicotine was removed during this concentration as an azeotropic mixture with water. In order to remove

some of the interfering substances such as chlorophyll, leaf pigments, and phospholipids, the cooled concentrated extract was washed with petroleum ether several times. The aqueous layer was collected and filtered through a thimble filter. After washing the residue with water, the washings were combined with the filtrate and concentrated to about 25 ml. An equal volume of freshly prepared saturated aqueous ammonium reineckate solution was added, and the reineckate suspension was cooled in an ice-bath for thirty minutes. The reineckate crystals were collected on a filter, dissolved in acetone, and the pink color removed as silver reineckate by adding excess silver nitrate solution. After centrifugation, the clear solution was decanted and acidified with hydrochloric acid. The silver chloride suspension was centrifuged and the solution was decanted and then evaporated to dryness in vacuo. Since the residue had a greenish tinge, it was dissolved in 1 ml. of water, and the precipitation with ammonium reineckate was repeated to obtain a white crystalline residue which was a mixture of choline and betaine chlorides.

Detection of C^{14} -methyl betaine

A very small amount of water was added to the crystalline residue described above and the solution was applied -- three applications of five lambda each -- on one corner of a Whatman Number 1 filter paper. The mixture of chlorides was then separated by using the principle of two-dimensional ascending chromatography. The first solvent system was n-propanol:1 N acetic acid = 3:1 (21); and the second, n-butanol:ethanol:acetic acid:water = 9:1:1:2. A radioautograph was taken by placing a

Kodak X-ray film over the developed chromatogram for three days and then developing the film in Kodak developer D-19. There were two spots on the film which corresponded to those of betaine and choline. The R_f values for a standard solution of betaine and choline were: solvent 1, betaine 0.36, choline 0.46; and solvent 2, betaine 0.23, choline 0.25. Dragendorff reagent (55) was used to identify choline and betaine on the chromatogram.

These results are an indication that choline was oxidized to betaine in the plant, although it is possible that the methyl groups of choline were transferred to a precursor of betaine to give the radioactive betaine isolated.

Preparation of the radish plants

Before any feeding of isotopic compounds to the radish plant could be carried out, a suitable method of raising the plant was required. Since the radish plant cannot withstand the shock of transplanting in its third week of growth, it could not be raised in flats containing soil or vermiculite. Consequently, attempts were made to culture the plants in water in the greenhouse. The Comet variety⁵ was used for this purpose.

The seeds were soaked in water for about ten minutes and then spread upon a wet cheesecloth supported by a half-inch-square-mesh wire screen coated liberally with paraffin. This was placed in a five-inch-deep glass container, painted black on the outside, so that the seeds were about a half inch below the rim of the container. Water was then added to the level of the seeds. In order to encourage the roots to grow straight down, the water-level was lowered gradually with growth. On the third day, the water was replaced with 50 percent nutrient solution of Hoagland⁶ (46). Solution 1 was prepared as follows: 1 ml. of M potassium nitrate, 2 ml. of M magnesium sulfate, and water were mixed to make 1 liter of solution. A supplementary solution was prepared as follows: 2.86 g. boric acid, 1.15 g. manganous chloride, 124 mg. zinc sulfate, 51 mg. copper sulfate, and 20 mg. molybdic acid were dissolved in 1 liter of water. A 0.5 percent iron tartrate solution was also made. The nutrient solution was then prepared by adding 1 ml. of supplementary solution and 1 ml. of iron tartrate solution to each liter of solution 1.

⁵ The seeds were obtained from the Ferry-Morse Seed Co., #39476; Detroit, Michigan.

⁶ This solution was made from "Baker's Analyzed" reagent grade chemicals.

On the fifth day, the plants were transferred to a porcelain-coated pan⁷ containing the nutrient solution of Hoagland. The pan was covered with a piece of paraffin-coated cardboard in which holes had been punched. The holes were seven-eighths of an inch in diameter and their centers were two inches apart. The cardboard in turn had a layer of non-absorbent cotton over it. The radish plants were placed in the holes in such a manner that the roots were partially immersed in the nutrient solution in the pan. The cotton above the cardboard was used as a support for the plants. Five milliliters of the "iron" solution was administered twice a week, and the level of the nutrient solution was maintained at the base of the tuberos portion of the root. Under such conditions, a three-week-old plant had a bulb about half-inch in diameter and an exuberant root system; the radish was considered ready for the feeding of C¹⁴-methyl methionine.

Uptake of C¹⁴-methyl methionine from solution

Although it was shown that tobacco plants absorbed methionine through the roots (13), it was not known whether radish plants would behave similarly. Consequently, an experiment was carried out as follows: twelve 100 ml. short-neck graduates -- each containing 40 ml. Hoagland solution, 0.5 ml. aureomycin (1:1000), and 2.0 mg. DL-methionine -- were divided into three groups of four. The first group contained plants; the second contained root fragments; and the third was used as control. Each graduate was wrapped with paper to keep out direct sunlight.

⁷ This pan was 10 $\frac{1}{2}$ " x 14 $\frac{1}{2}$ " x 5"

A week later, the roots of the plants and the root fragments were washed with water, and the washings were added to their respective graduates. Each solution was then filtered, concentrated in vacuo at 40° to a small volume, and then made to a volume of 10 ml. For each determination, a 5 ml. aliquot was taken and the methionine was determined according to the method of McCarthy and Sullivan (47). The uptake of methionine by the plants was complete. There was essentially no loss of methionine due to microorganisms in the second group containing root fragments, nor was there any loss in the control.

Administration of C¹⁴-methyl methionine and isolation of pectin

Three-week-old plants were prepared as described earlier, and each plant was fed 2.0 mg. methionine having an activity of 6.09×10^4 counts per minute. The plants were harvested after a growth period of nine days. The leaves were cut off and discarded. The roots were washed, blotted with cheesecloth, then cut into thin slices and dropped into boiling 95 percent ethanol -- the volume calculated to give a final concentration of 70 to 80 percent ethanol upon an assumed 95 percent moisture content of the radish. Boiling was continued for five minutes to inactivate the enzymes and then the extract was allowed to stand overnight. The pink-colored ethanol extract was separated from the radish root slices by filtration through four-layers of cheesecloth. The residue was returned to the original beaker, washed with 95 percent ethanol, and the mixture was again filtered through the original cheesecloth. Enough 95 percent ethanol was then added to cover the residue. The ethanol was filtered off the following day and the radish root residue was thoroughly dried

in the oven at 100°C.

The isolation and purification of pectin was carried out essentially as described by Kertesz (48). After grinding the dried radish root residue in a mortar, 60 ml. water was added to every gram of residue. This mixture was put on a shaker for two hours, and was then centrifuged fifteen minutes at a maximum relative centrifugal force of 1400 x g. The supernatant was kept in the cold room (4-6°C.), and the residue was extracted with 0.05 N hydrochloric acid (25-30 ml. per gram of dried residue) at 80° for two hours and the mixture then centrifuged. The residue resulting from the last centrifugation was extracted three more times in a similar manner. The supernatants were combined, filtered (Whatman No. 1), and made to 80 percent with ethanol (v/v). The crude pectin which precipitated in the alcohol solution was then filtered by gravity through a fluted filter paper (Whatman No. 12). The crude pectin was "dissolved" in water at 80°, cooled to 25°, and then reprecipitated from a 55 percent ethanol solution. This method of purification was repeated two more times. The resulting product was then washed with 95 percent ethanol followed by diethyl ether. The purified pectin was air-dried to remove excess ether and finally dried over barium oxide in a vacuum dessicator. The yield of pectin along with the radioactivity of the methionine fed per plant is presented in Table II. After grinding the pectin in a mortar and plating on an aluminum counting disc, it was counted with an end-window counter. The activity was then corrected to counts per minute per milligram of pectin and also to counts per minute per 120 mg. of pectin at infinite thickness by using the formula shown in Appendix I. The latter means of expressing radioactivity was used because

this was the weight of pectin which was saponified in experiments to be described below. The results are listed in Table II. It is evident from the data presented that after feeding C^{14} -methyl methionine to radish plants there was a high incorporation of radioactivity into the pectin of the roots.

Pectin doubly-labeled with deuterium and carbon-14

Doubly-labeled methionine was prepared by dissolving 144 mg. deuterio-methyl methionine with 16 mg. C^{14} -methyl methionine in enough water to make a solution of 200 ml. A total of 5.0 ml., equivalent to 2.4×10^4 counts per minute, was administered in two doses -- the first and the third day of feeding -- to each three-week-old plant. After a feeding period of twelve days, the plants were harvested and the pectin isolated as described above. Since a solid derivative of the doubly-labeled methanol was desired, the second and third purification of the pectin was carried out in 50 percent acetone solution to eliminate any occlusion of the ethanol. The resulting pectin was counted in the same way as the pectin described earlier. The specific activity is presented in Table II. Although the radioactivity of the pectin in this experiment was lower than in the other two, it will be noted in Table II that the radioactivity of the methionine administered was also less. If one takes this fact into account it may be seen that approximately the same extent of incorporation occurred in the third experiment as in the other two.

Determination of the methoxyl (methyl ester) group of pectin

Since the methyl ester of pectin was shown to be readily hydrolyzable

TABLE II

ADMINISTRATION OF ISOTOPICALLY LABELED METHIONINE
AND THE ISOLATION OF PECTIN FROM RADISH ROOTS

Experiment No.	Methionine Labeled With	Activity Fed Per Plant	Radish Roots		Pectin Isolated (mg.)
			Fresh Weight (gram)	Dry Weight (gram)	
1. (32 plants)	C ¹⁴ -methyl	6.09 x 10 ⁴	89.5	4.2	305.2
2. (38 plants)	C ¹⁴ -methyl	6.09 x 10 ⁴	198	7.4	802.8
3. (40 plants)	C ¹⁴ D ₃ -methyl	2.40 x 10 ⁴	368	13.5	1662.8

LOCATION OF RADIOACTIVITY IN PECTIN
AFTER METHIONINE ADMINISTRATION

Experiment No.	Maximum Specific Activity Counts Per Minute Per			Percent Activity in Methoxyl Group of Pectin
	1 mg. Pectin	120 mg. Pectin	MeOH from 120 mg. Pectin (Counted as BaCO ₃)	
1.	42.8	5140	4790	93.2
2.	32.3	3880	3680	94.8
3.	7.95	954	908	95.2



FIGURE I

(49) and since methanol does not form an azeotropic mixture with water, it was decided to make use of those properties in determining the methoxyl content of pectin. Nevertheless, since the liberated methanol could not be counted as such, a solid derivative had to be made. Experience showed that counting carbon-14 in the form of barium carbonate had several advantages over the use of solid derivatives of methanol. With such considerations, an apparatus was designed as shown in Fig. 1 for converting the liberated methanol to barium carbonate.

In chamber I, containing 8.4 ml. of water, 120 mg. of pectin was dispersed by warming and then stirring with a piece of soft iron (encased in glass, 5 x 25 mm.) by means of a magnet. A small amount of antifoam⁸ was added and the resulting mixture was frozen in a CO₂-methylcellosolve bath. This was followed with 0.2 ml. methanol -- used as carrier for the C¹⁴-methanol -- and 3.6 ml. of 1 N sodium hydroxide solution. This mixture was immediately frozen, and the container then attached to the apparatus while keeping it in the cooling bath. In chamber II was placed a piece of soft iron; chamber III, 100 mg. of potassium iodate; chamber IIIA, 25 ml. of combustion mixture saturated with potassium iodate (50); chamber IV, 8 ml. of 1 N sodium hydroxide solution (51); and chamber V, 20 ml. of 40 percent sodium hydroxide solution. Chamber VI, kept in CO₂-methylcellosolve bath, acted as a trap to remove water. The stopcocks were turned as shown in Fig. I and the system was evacuated with a Cenco-Pressovac 4 pump. Stopcock E was then closed and CO₂-free air was then admitted into the system by turning stopcock A several times. After re-evacuating the system,

⁸ This is a silicone product, Dow Corning Antifoam A, obtained from the Dow Corning Corp., Midland, Mich.

stopcocks A, B, D, and E were closed.

Chamber I was allowed to thaw, and it was gradually heated to 80° using a water-bath. This chamber was then cooled in an ice-bath with constant stirring. The stirring was to insure complete saponification of the pectin and also to bring about the complete mixing of the carrier with the radioactive methanol. After reheating chamber I to 80°, chamber II was warmed with a microburner and stopcock A was carefully opened to initiate the distillation. When the initial rapid distillation was over, chamber II was cooled with the CO₂-methylcellosolve bath while chamber I was being stirred with a magnetic stirrer as shown in Fig. I. Distillation was continued at 20-25° until about 8 ml. was distilled into chamber II. Careful manipulation of stopcock A was required to prevent bumping. Chamber II was thawed, followed by a gradual heating to 80° -- to effect fractionation of methanol from water -- while chamber III was warmed with a micro-burner. Then stopcock B was carefully opened. After the initial rapid distillation, the solution in chamber II was stirred with the magnetic stirrer. When chamber III was cooled to about 25°, the lower half of it was chilled with the CO₂-methylcellosolve bath. In order to prevent bumping and to aid fractionation, stopcock B was carefully regulated until 0.5-1 ml. of distillate was collected in chamber III. The level of the CO₂-methylcellosolve bath was then raised to the neck of chamber III, stopcock B was closed, and about 10 ml. of the combustion mixture was allowed to drip into chamber III. Stopcock A was turned to admit CO₂-free air, and stopcock B was carefully turned to equalize the pressure in chambers II and III and closed immediately. Stopcock C was opened and then stopcock D was turned to allow carbon-dioxide to escape from Chamber III into

chamber IV. The CO₂-methylcellosolve bath was replaced with an ice-bath in order to thaw chamber III at a slow rate.

Using a micro-burner, chamber III was carefully heated to drive the carbon-dioxide into chamber IV. Heating was continued for about five minutes after an "apparent" boiling, at which time the evolution of oxygen from the solid potassium iodate had ceased. Chamber IV was then connected to an aspirator and stopcocks A and B were opened to flush out the system with CO₂-free air. The contents of chamber IV were quantitatively transferred to a 50 ml. centrifuge tube using 10 ml. of boiled distilled water. Fifteen milliliters of a saturated barium hydroxide solution was added and after standing for three to four hours, the suspension of barium carbonate was centrifuged for five minutes at the maximum speed in an International Clinical Centrifuge. The residue was washed twice with 20 ml. of boiled distilled water, transferred to a tared filter stick of medium porosity, and finally dried at 100°C. The barium carbonate was ground in a small mortar and then plated on an aluminum disc (2.83 sq. cm.) by making a slurry using absolute ethanol. A hot-plate carefully regulated with a powerstat was used to evaporate the ethanol after plating. The Tracerlab End-window Counter was used to determine the activity of the barium carbonate. The maximum specific activity was calculated by using the formula shown in Appendix I. The fraction of maximum specific activity, f , was obtained from the self-absorption correction curve of Calvin, et al (52). The results are given in Table II and are expressed as counts per minute obtained from 120 mg. of pectin. It is interesting to note that as the yield of pectin increased -- attributable to seasonal differences -- from Experiment 1 to 3, the maximum specific activity

decreased respectively. Therefore, it seems that as the rate of pectin formation increased, there was a corresponding dilution in the activity of the pectin -- probably due to the decreasing pool-size of the C^{14} -methyl groups. Or another possibility is that the pool of pectin before feeding began was slightly greater in the third experiment. Nevertheless, even with these possible factors, the radioactivity in the methoxyl group of pectin remained relatively constant.

Isolation and determination of pectic acid obtained from pectin

The sodium pectinate, which was left in chamber I following the methoxyl determination of pectin, was acidified with hydrochloric acid. This mixture was centrifuged and the supernatant was decanted. The residue was dissolved in 1 N sodium hydroxide solution, heated to 60°, filtered, acidified with hydrochloric acid, and then centrifuged. After discarding the supernatant, the resulting pectic acid was dispersed in water by warming and then reprecipitated by making the solution to 60 percent (v/v) with ethanol. The pectic acid, which was precipitated as a flocculent white precipitate, was dispersed in 95 percent ethanol and then centrifuged. The residue was redispersed in 95 percent ethanol, made to 50 percent diethyl ether (v/v), filtered, washed with diethyl ether, air-dried, and then dried over barium oxide in a vacuum dessicator.

When the pectic acid was counted in the same way as the pectin, it was found to have some radioactivity. By using the formula shown in Appendix I, the maximum specific activities of the samples were calculated to be 0.15-0.74 counts per minute per milligram, which roughly would be about 1 percent of the radioactivity of the original pectin. In this

case, "f" was obtained from the self-absorption correction curve for pectin. It is assumed a priori that calculations based on such values of "f" do not introduce appreciable error in the final activities of the pectic acid. The total activity of each sample of pectic acid was not presented in Table II since pectic acid preparations were not obtained quantitatively from pectin. Nevertheless, the low activity in the pectic acid residue indicates that some of the Cl^{14} -methyl group was incorporated into the pectic acid molecule. According to the method of preparation, it is highly improbable that the activity is due to unhydrolyzed methyl esters. It is possible that some of the methyl groups of methionine were oxidized to CO_2 which was then used in the photosynthesis of the uronic acid portion of the pectin.

Preparation of deuteriomethyl-3,5-dinitrobenzoate for deuterium analysis

By employing the apparatus shown in Fig. I and the procedure described for the saponification of the methyl ester of pectin, 500 mg. of pectin dispersed in 16 ml. of water was hydrolyzed with 9 ml. of 1 N sodium hydroxide solution. About 10 ml. of distillate was collected and transferred to a large test tube (1" x 8"). One-half gram of anhydrous sodium acetate and 10 ml. of 40 percent sodium hydroxide solution were dissolved in the aqueous methanol solution and the mixture was chilled to 0° in an ice-bath.

In order to prepare the deuteriomethyl-3,5-dinitrobenzoate, the method of Lipscomb and Baker (54) was modified. About 3 g. of freshly prepared 3,5-dinitrobenzoyl chloride (53) was dissolved in 30 ml. of anhydrous diethyl ether and then added to the aqueous methanol solution.

After the mixture was kept in an ice-bath for half an hour with occasional shaking, it was transferred to a separatory funnel. The ether layer was removed and dried in a stream of air, and the residue was recrystallized from methanol three times to yield deuteriomethyl-3,5-dinitrobenzoate (m.p. 106-107°; lit. 107°). The deuterium-containing methyl-3,5-dinitrobenzoate will be analyzed for deuterium by a commercial analytical laboratory. The analysis has not as yet been completed and therefore it is not possible to state with certainty that the transfer of a methyl group from methionine to give the methyl esters of pectin was a trans-methylation reaction.

Methoxyl (methyl ester) content of radish root pectin

Following the procedures described earlier, 120 mg. of pectin was saponified (without using any methanol as carrier) and the percent methoxyl group (methyl ester) in radish root pectin was determined. The yield of barium carbonate obtained from the oxidation of the methanol from the saponification was 65.7 mg. By using the formula shown in Appendix II, the methoxyl content of radish pectin was found to be 8.6 percent.

DISCUSSION

Studies of betaine as a methyl donor

The results obtained from the administration of C^{14} -methyl betaine to tobacco plants indicated that, within experimental error, all the radioactivity of the nicotine was localized in the methyl group. Thus, it was suggested that the methyl groups of betaine are precursors of the N-methyl group of nicotine. Since the betaine used in this work had one of the three N-methyl groups labeled with carbon-14 and since betaine yields only one of its three methyl groups in transmethylation, the probability of yielding the C^{14} -methyl group in a methyl group transfer was one out of three. When this probability was taken into consideration, betaine was incorporated to a slightly greater extent than methionine (45) into the N-methyl group of nicotine.

When betaine was compared with choline (18) as a methyl group precursor for the N-methyl group of nicotine in Nicotiana rustica, it was found that the rate of incorporation of the methyl group from betaine was slightly higher than that from choline. Nevertheless, it was also demonstrated (59) that although betaine was a precursor for the N-methyl groups of the alkaloids tyramine and hordenine in the barley plant, it failed as a methyl donor in the young castor bean. In addition, when C^{14} -methyl betaine was administered to 11-day-old barley plants (59), it was found that the choline, which was later isolated from the plant, possessed radioactivity. The relatively low specific activity of the choline appeared more in the magnitude of a transmethylation reaction than a

direct reduction of the betaine to choline. The metabolic significance of such a reaction is not known since choline is not a methyl donor in the barley seedlings.

If choline had to be oxidized to betaine before it could be a methyl donor, then the in vivo oxidation of choline to betaine could have occurred through a choline oxidase system. Consequently, Cl^{14} -methyl choline was administered to tobacco plants. The resulting choline and betaine which were isolated from the plants were separated by a two-dimensional chromatography. When a radioautography of the chromatogram was taken by using an X-ray film, two spots corresponding to those of choline and betaine were found when the film was developed. It is possible that the spot corresponding to the radioactive betaine was formed by a transmethylation of the Cl^{14} -methyl group from choline to a precursor of betaine. Nevertheless, such a path for the formation of betaine seemed unlikely. Therefore, these results would seem to indicate that choline was probably oxidized to betaine.

Since the betaine content of leaves infiltrated with choline and betaine aldehyde was increased (21), it might be assumed that an enzymic oxidation of choline to betaine could occur stepwise with betaine aldehyde as the intermediate. That choline was not a methyl group precursor in the sprouting barley (16) and in the seedlings of Ricinus communis grown in darkness (17) could then be due to the lack of enzyme system which could oxidize choline to betaine.

Preliminary experiments by Cromwell and Rennie (58) showed that homogenates of roots of Beta vulgaris brought about the conversion of choline to betaine. Nevertheless, a later report indicated that such a

conversion was brought about by bacterial systems (21).

In this laboratory, work with homogenates and mitochondria from leaves of Nicotiana rustica was also conducted to attempt to discover a choline oxidase system. Various cofactors for choline oxidase were tried in order to follow the enzymic oxidation of choline by manometric method. Some inhibitors of polyphenol oxidases were also used in an attempt to suppress the uptake of oxygen by the endogenous substances. Nevertheless, no enzymic oxidation of choline by these preparations could be demonstrated.

Studies on the biosynthesis of pectin

In the present study, evidence has been presented which indicates that the methyl esters of pectin are synthesized by a transmethylation reaction in which the methyl group of methionine is transferred to a galacturonic acid unit to yield the ester. This is the first instance reported in which a methyl ester has been formed by a transmethylation reaction.

A study was made on the rate of incorporation of the methyl group of C¹⁴-methyl and C¹⁴-deuteriomethyl methionine into the molecule of radish pectin. About 93 to 95 percent of the activity of the pectin was located in the methyl ester. The dilution factor -- the molar ratio of the activities of the methionine as to that of the methanol -- was about 330. This was about a five times greater incorporation than was obtained for the N-methyl formation of nicotine using methionine as the precursor (45). Such a high incorporation of methyl groups into the pectin molecule tends to indicate that the reaction was a true transmethylation. Nevertheless, an analysis of the deuterium to carbon-14 ratio in the methyl

ester as compared with the deuterium to carbon-14 ratio in the methionine methyl group must be made as a final proof. These experiments are in progress.

The possibility of methyl ether formation with the sixth carbon of a galactose residue and a subsequent oxidation to an ester does not seem likely. Otherwise, it would seem that the residual pectic acid obtained by saponification of the pectin would have contained an appreciably greater radioactivity due to some ether groups. Nevertheless, one could argue that the rate determining step was the formation of an ether, and that the subsequent oxidation of the ether to an ester occurred instantly. Inasmuch as galactose is not considered part of a pectin molecule (6, 33, 57) and since a parallel organic reaction in which an ether is oxidized to an ester is not known, such an argument does not seem tenable. Consequently, the formation of the methyl esters of pectin probably occurred by a direct esterification of the polygalacturonic acid, or by the polymerization of galacturonic acids after esterification. The latter possibility would be unlikely in view of the fact that very little galacturonic acid has been detected in fresh plant tissues (11). On the other hand, 15.4 percent of the total pectic substances (on dry matter basis) (56) in radish was characterized as pectic acid. Therefore, it seemed most probable that there was an enzyme system which could catalyze the substitution of the proton with a methyl group to form the methyl esters of polygalacturonic acids. Such a mechanism suggests that the transfer of the methyl group was effected as the carbonium ion and that the in vivo transmethylation reactions resulting in N-methyl and C-methyl groups were, likewise, due to the carbonium ion transfer.

SUMMARY

1. After the administration of C^{14} -methyl betaine to tobacco plants, radioactive nicotine was isolated. Essentially all of the radioactivity was shown to be in the methyl group.

2. Tobacco plants when fed choline labeled in the methyl group with carbon- 14 produced radioactive betaine. Radioautography of a two-dimensional chromatogram revealed the radioactivity of the betaine.

3. Several attempts were made to follow the enzymic oxidation of choline, but no activity could be demonstrated with homogenates and mitochondria.

4. Radish plants produced radioactive pectin when C^{14} -methyl and C^{14} -deuteriomethyl methionine were administered. Almost all of the radioactivity was localized in the methyl group of the ester. A direct transfer of the methyl group from a thioether to an ester was postulated. That transmethylation involves the transfer of the methyl group as a carbonium ion was also postulated.

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APPENDIX I

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

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

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

C_o = observed count (counts/minute), less background

M = molecular weight of compound

W = weight of sample counted

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

Sample calculation:

$C_o = 55.8$, $W = 59.8$ mg., $M = 620$, $T = 21.1$ mg./cm.², $f = 0.295$

$$A_m = \frac{55.8 \times 620}{59.8 \times 0.295} = 1.97 \times 10^3 \text{ counts/minute/mM at infinite thinness.}$$

APPENDIX II

The formula used in calculating the methoxyl content of pectin was:

$$M = \frac{B \times W_m \times 100}{W_b \times P}$$

where M = percent of methoxyl in pectin

B = weight of barium carbonate

W_m = formula weight of methoxyl (OCH_3)

W_b = molecular weight of barium carbonate

P = weight of pectin

Sample calculations:

B = 65.7 mg., W_m = 31.0, W_b = 197, P = 120 mg.

$$M = \frac{65.7 \times 31.0 \times 100}{197 \times 120} = 8.6 \text{ percent methoxyl in pectin.}$$