THE PARTICIPATION OF CHOLINE IN TRANSMETHYLATION REACTIONS IN NICOTIANA RUSTICA

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Richard Earl Wing

1952

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THE PARTICIPATION OF CHOLINE IN TRANSMETHYLATION REACTIONS IN *NICOTIANA RUSTICA*

By

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A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1952

10/7/52
(28)

ACKNOWLEDGMENT

The author wishes to express sincere gratitude to Dr. Richard Bywaters for his interest and encouragement, which, together with his patience and forbearance during the course of this work has greatly facilitated its completion.

He also wishes to express his appreciation to other members of the Department of Chemistry for their generous aid from time to time; to Dr. E. H. Lucas in the Department of Horticulture for his help in growing the plants and advice in bacteria control, and to Dr. E. B. Doverman and other members of the Department of Bacteriology for their occasional assistance.

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INTRODUCTION

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Transmethylation, the intermolecular transfer of methyl groups within living organisms, since it was first postulated by du Vigneaud (1), has been generally accepted as a metabolic reaction in animals. However, experiments to demonstrate transfer of methyl groups as a metabolic reaction in higher plants have been limited. Kirkwood and Marion (2), have attempted to ascertain the precursors of the methyl group of hordenine, a barley alkaloid, by feeding choline with C^{14} in the methyl group and sodium formate containing C^{14}. Whereas C^{14} from the formate was found in the isolated hordenine, the transfer of the methyl carbon of choline was not observed. However, work carried on by Brown (3), has demonstrated that the methyl group of methionine and also formate carbon may serve as precursors of the methyl group of nicotine in Nicotiana rustica. Since a greater radioactivity in the nicotine was obtained with methionine than with formate when the two were administered at equal molar concentrations and radioactivity, a direct transfer of the methyl group without intermediate oxidation and reduction was postulated.

Because choline as well as methionine has been shown to be a transmethylating agent in animals, it appeared desirable to ascertain whether it might serve as a precursor for the methyl group in nicotine, even though Kirkwood and Marion are of the opinion that it did not participate in the formation of hordinine.

It seemed possible in the work of Kirkwood and Marion that bacteria might destroy choline before it could be absorbed by the plants since no

mention was made in their paper of bacterial control. A second possibility that seemed open for further investigation was the rate of absorption of choline by the plants. If it could be proved that choline was not absorbed by the plants, or was absorbed at a very slow rate, then it would necessarily follow that the choline being fed to the plants could not play an important role in the metabolic processes of the plants. Therefore, before attempting to study the role of choline in plant metabolism, it seemed necessary to carefully examine these two possibilities; to determine, (1) any detrimental effect on choline outside the plant, and (2) the rate of absorption of choline by the plant.

Because of the previous results obtained, it seemed expedient to again use tobacco plants in the same general procedure as that used by Brown (3), with the exception that choline would be used in place of methionine. It also appeared of interest to attempt the isolation of the phospholipids, some of which contain choline, since some idea of the rate of synthesis of these compounds might be obtained. Finally it seemed advisable to isolate the "free" choline if possible, to determine what percent of the choline fed to the plants was actually metabolized by them.

HISTORICAL

HISTORICAL

Late in the nineteenth century, Strecker (4), succeeded in isolating one of the nitrogen bases of the phospholipids. He found this base to be chemically identical with a base he had earlier isolated from bile, and to this compound he gave the name choline.

Since its discovery, a vast amount of information has been compiled (5), concerning its synthesis, physiological role, and its metabolic functions. The greater part of this work has been done in the years between 1935 and 1945.

The functions of choline can be divided into two broad categories. Its first function appears to depend upon the intact molecule (6). This function is independent of its methyl yielding properties and can include its effect in preventing fatty livers and hemorrhagic kidneys in rats and mice (7). Its ability to prevent perosis in chickens and to promote growth in certain mutant strains of a red mold, Neurospora crassa, may also fall into this class. The evidence supporting this "whole" molecule theory lies in the fact that arsenocholine (arsenic replacing the nitrogen), has lipotropic, anti-kidney-hemorrhagic and antiperotic properties (8) (9), but has no labile methyl groups (10). Also, the ethyl homologue of choline has these same physiological characteristics, but does not promote growth with homocystine in the absence of methionine (11).

The second function of choline depends upon its peculiar property of giving up one of its three methyl groups, and in so doing, is transformed

into dimethylethanolamine, the latter having little if any of this labile methyl property.

In investigating the biological reactions of choline, it has been found that it can be oxidized to glycine betaine aldehyde which in turn can be oxidized to glycine betaine (12). This leaves the possibility that choline is first oxidized to betaine before being capable of transmethylation.

Little is known about the use of these methyl groups in plants, but it has been established by Brown (3), that N-methyl groups of nicotine may be formed by transmethylation, and by Fleischer (13), that O-methyl groups of lignin are also partially formed in this same manner.

It has been shown that the enzyme transmethylase (14), and vitamin B_{12} (15), are both necessary for the reaction to occur, however, little is known concerning the mechanism.

The synthesis of choline occurs primarily in plants (16), which is the main source of supply for animals. However, it can also be synthesized to a limited extent in animals. Stetten (17), has demonstrated that glycine, containing nitrogen 15, can act as a precursor of ethanolamine, which in turn can form choline in rats but not in chickens, presumably by a transfer of methyl groups from methionine (18). Though chickens do not have this ability, they can synthesize choline from methylethanolamine (19). Apparently they lack the mechanism for synthesizing methylethanolamine from ethanolamine.

The three natural occuring derivatives of choline are acetylcholine, lecithin, and sphingomyelin. The first, acetylcholine, is a chemical

necessary in nerve stimulus. Its formation and reverse reaction are catalyzed respectively by choline acetylase and cholinesterase (20).

Lecithin and spingomyelin are two of the three main classes of phospholipids. The choline in the phospholipid molecule is held by an ester linkage to phosphoric acid, and it has been proved, by using choline containing P^{32}, that the choline of the phospholipid can come from free choline (21). The function of these choline containing phospholipids is as yet obscure.

The phospholipid concentration in plants has been reported to be very small, making its isolation difficult. In view of the uncertainty concerning choline metabolism in higher plants, the intention of this experiment was, (1) determine if choline is taken in by the plants and if it is destroyed by soil bacteria, and (2) to isolate lecithin, phospholipids, and unmetabolized choline, and determine the radioactivity of each in an attempt to determine if transfer of the methyl group or other metabolism of choline has occurred.

EXPERIMENTAL

EXPERIMENTAL

Quantitative Determination of Choline Uptake in Plants and Destruction of Choline by Bacteria

Growth of plants. The tobacco plants, Nicotiana rustica L., var. humilis, which are grown commercially for their high nicotine content, were used for all phases of these experiments. The plants were grown from seeds,[1] which, from the time they were planted, were ready for transplanting in about three weeks. The plants, about one-half inch high, were removed from the flats in which they were planted, and transplanted to three inch pots. The pots were watered once a day, and at least once a week were fed a solution of Plant Marvel fertilizer. This insured the presence of the necessary ions for optimum growth and appeared to keep the plants from budding before they were treated experimentally. They were grown to a height of at least six inches, which took from two to three months, depending upon the environmental conditions.

The plants were prepared for the hydroponic administration of choline as follows: After removal from the pots, the roots were washed free of soil without destroying any more of the small roots than was necessary. The roots were soaked in a 0.1 per cent Wyandotte detergent germicide[2] for one-half hour, removed from the detergent and rinsed well with distilled water. Following the rinse, the roots of each plant were

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

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

immersed in 50 ml. of an inorganic nutrient solution in a 125 ml. Erlenmeyer flask. The nutrient solution was prepared by diluting 1:8 the stock solution, the composition of which is shown in Table I. All the weights are calculated as the anhydrous salt and only C.P. grade chemicals were used. Where any other material was added to the nutrient solution, a sufficient description will be given under a discussion of that part of the experiment.

TABLE I

COMPOSITION OF THE NUTRIENT SOLUTION

Water	1,000 ml.	Magnesium sulfate: $MgSO_4$	250 mgs.
Calcium nitrate: $Ca(NO_3)_2$	1 gm.	Ammonium sulfate: $(NH_4)_2SO_4$	250 mgs.
Potassium chloride: KCl	250 mgs.	Potassium dihydrogen phosphate: KH_2PO_4	250 mgs.
Ferric chloride: $FeCl_3$	5 mgs.		

Since one part of the experiment consisted of growing the plants in a nutrient solution containing radioactive choline, it was deemed advisable to grow the plants for all phases in a hood under artificial lighting. This would help to eliminate any possible health hazard arising from plant respiration during the metabolism of the radioactive material and would help to standardize conditions for the other parts of the experiment.

A source of light in the hood was supplied by two 36 inch, 30 watt fluorescent tubes and a 100 watt incandescent bulb, placed about 14 inches above the top of the plants. The light intensity at the level of the upper leaves was found to be in the range of 200-250 foot-candles. The

light was left on approximately 12 hours out of 24, while the plants were growing, and nutrient solution was added as required.

Choline absorption by plants: This part of the experiment was performed for the purpose of determining if choline could be absorbed by the plants, and if so to gain some idea of the absorption rate. A second purpose was to study the rate of choline metabolism by microorganisms outside the plant.

In order to determine the choline uptake in the tobacco plants, it was first necessary to procure a method for the quantitative determination of small amounts of choline. One of the most widely accepted methods at this time is that of Glick (22). This consisted essentially of precipitating the choline from a basic solution with Reinecke salt, dissolving in a known volume of acetone, and reading the optical density of the red acetone solution with a spectrophotometer. The amount of choline recovered was then read directly from a previously prepared standard curve, obtained by plotting known weights of choline vs. optical density of the choline reinecknte- acetone solutions. The latter, of course was obtained by precipitating the known weights of choline with Reinecke salt and dissolving the precipitate in acetone.

To prepare the standard curve, choline chloride was first recrystalized three times from a 1:3 mixture of absolute ethanol and calcium chloride dried Skellysolve B (b.p. 67-68°C.). A standard solution of the choline was then made up, containing one mg. of choline/ml. water. Then, to four, 125 ml. Erlenmeyer flasks to which previously had been added 50 ml. of the plant nutrient solution, were added 2, 4, 6, and 8 ml. of the standard

choline chloride solution respectively. The flasks were then diluted to 110 ml. with distilled water to make the total volume comparable to the total volume in the choline determination discussed on the following pages. To these four flasks was now added 12 ml. of a methanol solution of Reinecke salt (2 gm. Reinecke salt/100 ml. methanol). The flasks after cooling for two hours in a refrigerator at 10° C. were separately removed and immediately filtered with suction through a small sintered-glass funnel of medium porosity. The precipitate was washed with about two ml. of n-propanol and dried on the funnel using suction. The precipitate was then dissolved in as small an amount of acetone as possible, and drawn by means of gentle suction into a small test tube previously calibrated to ten ml. The funnel was carefully rinsed several times with small amounts of acetone and the rinse drawn into the test tube until the volume approached the ten ml. calibration mark. The solution in the test tube was made up to the ten ml. volume, stoppered, and shaken to insure uniform color. The optical density of the solution was determined directly by use of a Beckman model B spectrophotometer. After repeating this procedure for all four flasks and duplicates of each for control, the data was plotted on graph paper to obtain the standard curve.

To determine the choline absorption by the tobacco plants, four plants were drawn and prepared for the hydroponic administration of choline in the manner previously outlined, without, however, soaking them in the detergent germicide. After removal of about a dozen small roots, the plants were placed in four separate 125 ml. Erlenmeyer flasks containing 50 ml. of the nutrient and also five ml. of the standard choline chloride.

Four more Erlenmeyer flasks were also prepared to contain the same amount of nutrient and choline chloride. Into two of these flasks were placed a dozen of the small roots previously removed from the plants; a half dozen to each flask. The other two Erlenmeyer flasks which served as a control contained only the nutrient solution and five ml. of the standard choline chloride.

To one half the flasks containing the plants, to one of the two containing the roots, and to one of the two controls was added three drops of the 0.1 per cent Wyandotte detergent germicide. All eight of the flasks were placed in the hood and allowed to remain for 48 hours, with the lights controlled as previously outlined. During the 48 hours, most of the nutrient solution was taken up by the plants, but no change in volume was noted in the two flasks containing the roots, or in the two controls.

At the end of the 48 hours, the plants were carefully removed from the flasks. At the same time the plant roots were washed thoroughly with a fine stream from a washbottle and the washings added to the flasks. Following this, all eight of the solutions remaining in the Erlenmeyer flasks were separately filtered through Whatman number 42 filter paper to remove dirt, roots, and any particles larger than colloidal size. The filters were rinsed with a wash bottle and the rinse added to the filtrate. The separate filtrates were adjusted to approximately equal volume of about 110 ml., corresponding to the volume used in preparing the standard curve. Each was then analyzed for choline using the method previously described. The results are shown in Table II, Part I.

These results show that the plants are capable of taking up over 95 per cent of the five milligrams of choline fed to them in 48 hours. The results of the root controls, however, indicate that microorganisms destroy a large amount of the choline in the 48 hour period. The Wyandotte detergent germicide used seems to have little, if any, effect in control of the choline destroying bacteria under these conditions. However, Lucas (25), states that the detergent is germicidal as far as most bacteria are concerned and is not detrimental to the plants when they are soaked in a moderately strong solution before being placed in the nutrient.

To further verify the hypothesis that bacteria destroyed the choline, two more samples were run, using fine roots from a plant, as was done before, together with the nutrient solution and five milliliters of the standard choline solution. Two drops of toluene were used this time as a preservative. After 48 hours, under the same conditions as before, the amount of choline remaining in the nutrient solution was determined. The results in Table II, Part B, show a recovery of choline approximately equal to that of the controls, indicating no destruction of the choline.

It was not possible to find in the literature any information concerning the effect of toluene on growing plants, therefore, another means of controlling bacterial growth had to be found. A decision was made to try aureomycin, since it had been used as a germicide by the Horticulture Department (28) prior to this experiment, and appeared to have no effect on plant growth. Their results indicated a drop in bacteria count without detrimental effect to the plants.

To test the ability of aureomycin to control the bacteria plants, small roots, and controls, (two samples of each), were prepared in the same manner as before with the exception that the roots of the plants were soaked for one-half hour in a 0.1 per cent Wyandotte detergent germicide. The roots were rinsed thoroughly with distilled water before placing the plants in the nutrient solution containing five milligrams of choline chloride. This time, however, the nutrient solution was prepared to contain a 1:100,000 dilution of aureomycin. After 48 hours in the hood, the residual choline chloride was analyzed as before. The results are given in Table II, Part B. It can be seen that the recovery of choline is, within experimental error, the same for the flasks containing roots as for the control flasks, again showing a complete uptake of choline by the plants.

It seemed of interest to determine how much of an effect the aureomycin had in reducing the bacteria count in the nutrient solution. To determine this, two plants were prepared for growth in the nutrient solution containing five milligrams choline. One of the plants was placed in the nutrient solution with no attempt to control the bacteria, while the other was soaked in the germicide and placed in the nutrient containing the 1:100,000 aureomycin. Both were grown for four days in the hood. At the end of this time, one cc. of solution was removed from each, using aseptic technique. These were diluted 1:100, 1:1000, 1:10,000 and 1:100,000, plated out on sterile nutrient agar plates, incubated for 48 hours at room temperature and counted. The plant solution without bacteria control gave a count of 8.8×10^6 organisms/cc. while the plant solution with the aureomycin added gave a count of 2×10^5 organisms/cc., which is equal to a 96 per cent reduction in count using the aureomycin.

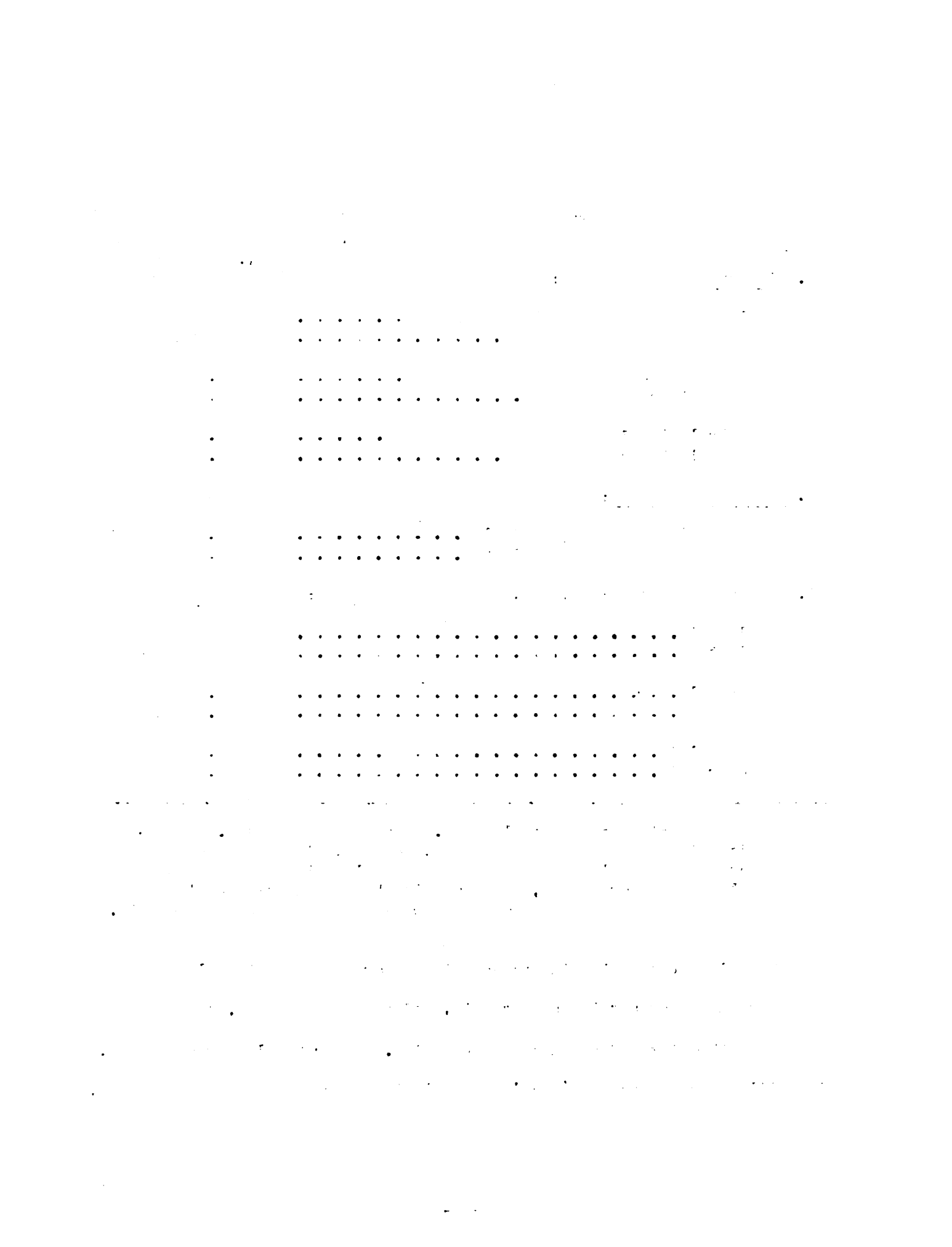
TABLE II

CHOLINE CHLORIDE RECOVERY IN PLANTS AND CONTROLS AFTER 48 HOURS
(5 mg. choline added in each case)

	Mg. Choline Chloride Recovered
1. Without germicide added:	
Plants with 3 drops detergent added	0
Plants without detergent	0
Roots with 3 drops detergent added	0.98
Roots without detergent	2.12
Control with 3 drops detergent added	4.75*
Control without detergent	4.70
2. With toluene added:	
Roots plus toluene (Sample 1)	4.56
(Sample 2)	4.88
3. With aureomycin (1:100,000), two samples of each:	
Plant 1	0
Plant 2	0
Roots 1	4.40
Roots 2	4.48
Control 1	4.54
Control 2	4.68

* In no sample was the total 5 mgs. of choline recovered. No explanation could be given for this, however, since the recovery was approximately the same in the control as in the presence of toluene and aureomycin, the inability to recover the total choline added was not thought to be due to the action of bacteria.

The plate containing the aureomycin appeared to contain only four different types or strains of bacteria, and one type of mold, as could be judged by the size and shape of the colonies. Since 2×10^6 organisms/cc. is still a sizable number (equal to the limit of Grade A pasteurized milk),



it is probable that the organism or organisms causing the destruction of choline were destroyed by the aureomycin.

The evidence presented in this phase of the experiments shows that in all probability, choline is destroyed by certain soil and air bacteria, and that by the use of proper methods this destruction can be greatly reduced.

Because of this evidence, the plants during the hydroponic administration of choline were grown in a nutrient solution containing aureomycin, after first being soaked in the Wyandotte detergent germicide.

Growth of Plants on Radioactive Choline Chloride

With isolation of nicotine, phospholipids, and unmetabolized choline:

Tobacco plants were grown in a nutrient solution containing radioactive choline for one week. Following this, an attempt was made to isolate the plant phospholipids, nicotine, and free choline chloride from the plants.

Two separate trials were made. The first trial consisted of growing the plants on the radioactive material and isolating the three fractions mentioned above, in an attempt to study the role of choline in the plant. The second trial, which consisted principally of the same thing, was done to confirm the results obtained in the original trial. However, due to difficulties encountered in the first trial, several modifications were made in the second. To help clarify the situation in the following parts of the paper where the procedure was different for the two trials, reference will be made to Trial 1, and Trial 2, with the understanding that Trial 1 is the original and Trial 2 the second trial intended to confirm the results of the original Trial.

Preparation of radioactive choline: The choline chloride used in this experiment was synthesized by Brown, from methyl iodide and dimethylethanolamine. Immediately before use, it was recrystallized from a 5:1 anhydrous Skellysolve B-absolute ethanol mixture. The recrystallized product was then dissolved in water and diluted, to prepare a standard solution containing one milligram of choline chloride per milliliter of water. This standard solution was counted on a Nuclear Corporation Scaler and a count of 7.6×10^6 counts/mg./min. at infinite thinness was obtained.

Preparation and growth of plants on radioactive choline: Twenty-seven plants were grown and prepared for the hydroponic administration of radioactive choline by the method previously outlined. The Erlenmeyer flasks were prepared by adding to them, 50 milliliters of the nutrient solution containing 1:100,000 aureomycin and two cc. (2 mg.) of the radioactive choline chloride. This amount of choline chloride was equal to 1.5×10^5 counts/min. at infinite thinness.

The plants were placed in the flasks and grown for one week in the hood, adding more nutrient solution without the aureomycin when necessary.

One serious difficulty encountered in Trial 1, was a wilting of the plants during the week in which they were grown in the hood. By the end of the fourth day, three of the plants had a decided wilt, with the leaves drooping clear down to touch the Erlenmeyer flasks. Each day, an increasing number displayed a limp appearance, until by the end of the seventh day, fifteen of the twenty-seven plants were limp and flaccid. The three plants to first show wilt were by now completely dead. The three dead

plants were discarded from the experiment, but the rest were of necessity used, since there would have been an insufficient amount of starting material for the isolations if all the wilted plants had been discarded.

Before starting Trial 2, a discussion of the problem with Lucas (23), led to the suggestion that the trouble might lie in an insufficient oxygen content in the nutrient solution for plant respiration to take place. It was thought that this might possibly be remedied by bubbling air or oxygen into the nutrient solution twice a day during the week in which the plants were to be grown in it. This was carried out in Trial 2, by passing a very fine stream of oxygen into the nutrient solution for one minute twice a day. Once then the lights were turned on in the morning and once just before turning them off at night.

At the end of the third day of Trial 2, a slight wilt was noted in three plants. By the following morning, however, all symptoms of limpness had disappeared. With the exception of the third day, no wilt was noted during the week in which the plants were grown.

One other observation of significance was the production of root hairs during the growing period. Though root hairs, resembling mold hyphae in appearance, were produced on the roots of the plants in Trial 1, the production was much more pronounced in Trial 2. Also, there appeared to be an increase in the growth of the plants in Trial 2, which was not evident in Trial 1, though no actual measurements were taken to determine whether or not the observation was correct.

At the end of the seven day growing period, the plants were removed and cut into small portions. Drying was accomplished as quickly as

possible with the aid of infra-red heat lamps, without raising the temperature above 80 degrees centigrade. After completion of the drying, the residue was finely ground with a mortar and pestle, and placed in three Soxhlet extraction thimbles, in preparation for extraction.

Isolation of phospholipid: In attempting the isolation of the phospholipids, a modified method of Smith and Chibnall (24), was used. This consists of an ether extraction using Soxhlet extractors, evaporation of the extract to near dryness, and precipitation of the phospholipids by the addition of two volumes of acetone and one or two drops of saturated mercuric chloride. The mercuric chloride was found by later workers to aid in the precipitation.

In Trial 1, this method proved to be quite unsatisfactory due to the large amount of chlorophyll and leaf pigments that were also extracted. When evaporation was attempted, the pigments precipitated before the volume was decreased enough to precipitate the phospholipids. Another difficulty was the color of the solution, which upon concentration, became so dark that when the acetone was added, it was impossible to determine if a precipitate formed. Centrifugation disclosed a small amount of material in the bottom of the centrifuge tube. However, this later proved to be insoluble in ether, and since this is not characteristic of phosphatides, it was assumed that the precipitate was actually very small solid particles washed through the Soxhlet thimbles during the extraction.

In Trial 2, a modification of this method was made by first extracting with acetone to remove most of the plant pigments. After a 12 hour extraction, the acetone extract was evaporated to dryness and tested for

radioactivity, which, with the monitor, yielded approximately 1000 counts per minute at a distance of about one-half inch.

It was suspected that this activity might be due to extracted choline chloride. To further investigate this the residue was taken up in ethyl ether in which it was completely soluble, and the ether solution washed with water. The ether layer still contained all the chlorophyll, leaving it so darkly pigmented that it remained opaque even to strong light. The water layer became a dark orange in color, probably due to extracting one or more of the carotenes from the ether layer. The two layers were separated and washed twice, the water layer with ether and the ether layer with water. The ether washes were added to the original ether layer and the water washes added to the water layer. Both layers were evaporated to dryness, and again tested for radioactivity. The water extracted residue now had an activity of 1000 counts per minute, which was probably due to either the orange pigment or to choline chloride. The ether extracted residue, however, still gave an activity of 500 counts per minute. Since the choline would be more soluble in water than in ether due to its polar characteristics, the activity in the ether layer was probably due to some unknown substance. This leaves a possible transfer of methyl groups to one or more of the plant pigments, or to some other compound extracted by acetone and soluble in the ether layer.

A further investigation of this acetone extracted material would be a highly interesting problem for the future, since the plant pigments are fairly easy to separate in a chromatographic column.

The acetone was removed from the thimbles by evaporation and the ether extraction and subsequent concentration repeated as before. This time evaporation was continued to a volume of about five milliliters in a centrifuge tube. The solution remained almost colorless and with no visible precipitation. The addition of two volumes of acetone and two drops of mercuric chloride yielded no precipitate of phospholipids. It seemed possible that the amount of phospholipid could be so small that it still remained soluble, therefore, the mixture was cooled to a -10 degrees Centigrade to decrease the solubility. This resulted in the formation of a very fine precipitate which after standing at a -10 degrees Centigrade for several days, settled into two distinct layers. The bottom layer was observed to be of a clear, colorless, gel-like substance, and the top layer of an opaque mass of finely divided particles. The total depth of the two layers in the pointed centrifuge tube did not measure more than 0.3 centimeters indicating a very small amount of material. Each layer was separately drawn off with a micropipette, placed on an aluminum disk and counted. The clear, colorless layer, when placed on the disk, had the characteristics of a colorless, heavy oil. This fraction showed no activity at all.

The top layer of the centrifuge tube, weighing about 20 milligrams, was also placed on a disk and counted. This layer showed an activity of 18 counts per minute above background.

A qualitative analysis was made to determine the presence of choline containing phospholipids. A procedure devised by Entenman, Taurog and Chaikoff (25), for the determination of choline in phospholipids was used.

This method, a micro technique capable of determining less than one milligram of choline, consists essentially of a hydrolysis with a saturated solution of barium hydroxide and evaporation to near dryness on a steam bath. This was followed by the addition of a small amount of dilute HCl to form the more soluble barium chloride. The water layer was then washed with ether to remove hydrolyzed fatty acids. Following this, a dilute HCl solution of Reinecke salt was added to precipitate any hydrolyzed choline chloride. At this point a slight modification of their procedure was made. Reinecke salt was added to only one-half the water fraction. To the other half was added ammonium molybdate, which was heated on a steam bath to detect the presence of phosphate.

Both tests yielded negative results. This indicated the absence of phosphatides, or such a small concentration as to be undetectible by this method.

Isolation of choline chloride. It seemed of interest to isolate any choline chloride still remaining in the plants to determine how much was metabolized by the plants during the week in which they were grown on the radioactive choline chloride.

Following the ether extraction of the ground plants, the Soxhlet thimbles were removed and dried. These were then replaced in the Soxhlet apparatus and extracted with water for 12 hours. The water extract was placed in a liter flask and to the flask was also added the residue from the acetone and ether extractions, to recover any choline or nicotine remaining in this residue.

The water extract was made basic with $CaOH_2$ and distilled to concentrate. The distillate containing nicotine, was caught in a receiving flask. This distillation was continued until all the nicotine was distilled off. Complete removal of the nicotine was indicated by the absence of a precipitate upon the addition of silicotungstic acid to the distillate as it came from the condenser. This distillate was set aside for isolation and purification of the nicotine following the isolation of the choline chloride.

The concentration of the water extract was continued to a volume of approximately 100 milliliters. This was filtered with difficulty through a fluted filter, and the filter rinsed thoroughly with a fine stream of distilled water. To the basic filtrate now having a volume of about 200 cc., was added 25 cc. Reinecke salt (2 gm./100 ml. methanol) to precipitate the choline chloride. The filtrate was placed in a refrigerator at 10 degrees Centigrade for four hours and filtered through a sintered glass funnel of medium porosity. The precipitate was washed with four, two cc. portions of cold n-propanol and then two cc. of ether. The precipitate was dissolved and washed through the filter funnel with acetone using gentle suction and as small an amount of acetone as possible. The acetone solution was transferred to a 100 milliliter beaker and evaporated to dryness. The dried residue was washed as before with the same amount of n-propanol and ether. The residue was transferred to the sintered glass funnel and dried by suction. It was again dissolved in acetone and washed through the funnel into a tared weigh bottle, evaporated to near dryness and completely dried in vacuo. Aliquots were made of the

dried residue, and from these aliquots, the total number of counts at infinite thinness in Trial 1, and Trial 2 was determined. From both the weight of the precipitate and the number of counts recovered, the percent of choline chloride unmetabolized by the plants was determined.

Precipitation of the choline with Reinecke salt from a basic solution is thought to eliminate the co-precipitation of most compounds precipitable with this reagent. If, however, some other compound or compounds are precipitated with the choline, they would add little, if any, to the radioactivity of the choline reinecknate, though they might add slightly to the total weight of the salt.

The results of this determination are shown in Table III.

TABLE III

CHOLINE CHLORIDE RECOVERY FROM PLANTS

Trial No.	Wt. Choline Reinecknate	Total Counts Recovered	Total Counts Fed Plants	Percent Recovery
1	112.4 mgs.	7.9×10^5	4.1×10^6	19.3
2	102.0 mgs.	6.9×10^5	4.1×10^6	16.8

No attempt was made to determine if this method of isolation of the choline chloride is quantitative, and without doubt it is not. However, this does mean that if the precipitate is pure choline reinecknate, or if the impurity is non-radioactive, then no more choline could have been used by the plants than the difference between 100 percent and the percent recovered in Table III. Therefore, the plants are not likely to

have metabolized more than 85 percent of the choline taken up by them during the week in which they were grown in the nutrient solution.

Isolation and purification of nicotine: The distillate containing the nicotine was concentrated, and purification of the alkaloid was accomplished by two successive azeotropic distillations through a Widmer column from an alkaline medium as described by Smith (26). The distillate was caught in an HCl solution to form the non-volatile nicotine hydrochloride. Water was removed from the acid distillate under reduced pressure, the nicotine crystallising out as the hydrochloride. The precipitate was dissolved in methanol with a little water added, and a saturated methanolic solution of picric acid was added in excess. After standing for a short time the precipitated nicotine dipicrate was filtered off, washed with methanol, and recrystallized from hot water. (m.p. 216.5-218°C.; recorded value 218°C. (27)). The crystallized nicotine dipicrate was transferred to an aluminum counting plate and counted in the scaler. The counts/min./millimole at infinite thinness (see Appendix I) are shown in Table IV.

Demethylation of nicotine: The nicotine was demethylated to determine what percentage of the radioactivity of the nicotine was located in the N-methyl group of the alkaloid. This procedure for demethylation, given in detail by Pregl and Grant (28), consists of treating the sample with HI and NH_4I at a high temperature. Gold chloride is used as a catalyst and the product formed is CH_3I.

Because the dipicrate has been shown to be quite insoluble, the nicotine was recovered from the dipicrate by distillation from a basic solution through a Widmer column into HCl. The nicotine chloride was concentrated and dried in vacuo,, taken up in a small amount of water, transferred to the distillation flask of the demethylation apparatus, and again concentrated and dried before proceeding with the demethylation.

The methyl iodide formed during the demethylation was allowed to react with triethylamine to form triethylmethylammonium iodide. Evaporation of the excess triethylamine was accomplished on an infra-red heat lamp leaving the solid triethylmethylammonium iodide. This was ground with a small mortar and pestle, dried, placed on a disk and counted. Precautions were necessary to keep free of contact with moisture because the compound was quite hygroscopic.

The counts/min./millimole at infinite thinness are recorded in Table IV.

TABLE IV

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

Trial No.	Maximum Specific Activity (counts per minute per millimole)		
	Nicotine Dipicrate	Quaternary Iodide	Percent Recovery
1 (27 plants)	6.14×10^2	8.40×10^2	88
2 "	1.48×10^3	1.41×10^3	96

A comparison of the radioactivity found in the nicotine from the two trials would at first appear to be of great variance. However,

considering the factors involved that are beyond the experimenter's control, such as seasonal and temperature variations during the early life of the plants, and the amount of nicotine synthesized in the plant before feeding the radioactive material, the results obtained are well within reason. The higher count of the second trial may be at least partially accounted for by the wilting plants of Trial 1, since after wilting, the metabolic balances in the plant must surely be upset and retarded. And, in turn, the processes for nicotine synthesis must also be retarded to a noticeable degree.

The percent recovery for Trial 1, (Table IV) is subject to a much greater error than that of Trial 2, due to the smaller count of Trial 1. For example, an increase of one count per minute of the triethylmethylammonium iodide in Trial 1, when actually counted in the scaler, would increase the percent recovery to 98 percent, after conversion to counts/min./millimole at infinite thinness. This is a total increase of four percent.

For Trial 2, however, a similar increase of one count per minute would only increase the percent recovery to 96.8, or a total increase of 1.8 percent. This means that the percent recovery in Trial 2 is about three times as accurate as Trial 1. Within experimental error, then, the recovery approaches 100 percent. This confirms the results of Brown, that the radioactivity is essentially in the N-methyl group of the nicotine molecule.

DISCUSSION

DISCUSSION

The results of these investigations show that the methyl carbons of choline can act as a precursor of the methyl carbon of nicotine *in vivo*. However, no evidence was obtained as to the mechanism by which this takes place. It is probable that the reaction is one of the generally accepted transmethylation reactions in which the whole methyl group is transferred to the substrate. This, nevertheless, is an assumption. It could possibly be some type of an oxidation-reduction reaction so that only the carbon atom of the methyl group is transferred. The final proof that the 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.

That choline can act as a methyl donor in plants is not in accordance with the results of Kirkwood and Marion, who are of the opinion that choline does not act as a precursor of the methyl groups of hordenine. Their opinion is derived from the fact that radioactivity was found to a much greater extent in hordenine when barley plants were fed radioactive sodium formate than when fed radioactive choline. Thus the formation of the methyl groups on hordenine would appear to be formed from an oxidation-reduction process rather than a transmethylation. The above authors, however, do not refer to any precautions taken to restrict bacterial growth, nor do they mention any preliminary investigations to determine if the choline could be destroyed by soil microorganisms.

With the information obtained through investigations conducted in this laboratory, then, it seems plausible that the choline was, to a great extent, destroyed before the barley plants had an opportunity to absorb it. This, of course, would account for their negligible results.

In the light of this evidence, it would seem of interest to repeat the work of Kirkwood and Burton, using efficient germicidal control, to see if the results obtained would still agree with their original work.

The amount of radioactivity found in the nicotine from this experiment is extremely small compared to the original amount that was fed to the plants. From this evidence, it might be considered that the rate of transmethylation from choline is rather slow, however, it is more probable that the rate of synthesis of nornicotine is the controlling factor. Nicotine is not thought to be metabolized in the tobacco plant, therefore, that which is synthesized must remain in the plant since the plant has no apparent excretory system. Inasmuch as the largest concentration of nicotine found in the adult plant is no more than eight percent of the dry weight, its synthesis cannot proceed at a very rapid rate. This might account for the relatively low activity found in the radioactive nicotine.

The negative results obtained in the attempted isolation of the phospholipids, though disappointing, was not completely unexpected, since the phospholipid content of most plants is quite small. Chibnall and Smith (24), found that 20 kilograms fresh weight tissue of cocksfoot yielded only two to four grams of crude phospholipid. If a direct comparison can be made to the tobacco plants used in this experiment, the

800 grams fresh weight tissue of tobacco would have yielded approximately 100 milligrams of phosphatides. However, Chibnall and Jordan (29), found that in experimental work with the growing bean plant Phaseolus multiflorus, the rapidly growing tissues are very low in phosphatide, and that the material accumulates as the tissue matures. Since the tobacco plants used in this experiment were young and rapidly growing, this probably accounts for the lack of phosphatides in the attempted isolation.

Reasonably good correlation is shown between the rate of transfer of the methyl group from methionine and choline. Brown (5), in his work with radioactive methionine, fed 40 plants a total of 4×10^6 counts per minute in the methyl carbon. He recovered in his nicotine dipicrate a maximum specific activity of 8.4×10^3 counts per minute per millimole, which was the average of three trials.

In this experiment, 27 plants were fed a total of 4.1×10^6 counts per minute, and a maximum specific activity of 1.5×10^3 counts per minute per millimole was found in the nicotine dipicrate.

In comparing this study with that of Brown (5), then, the total number of counts fed the plants was of the same order of magnitude, while the number of counts recovered in the nicotine dipicrate of this experiment is about 18 percent of that recovered by Brown. It would first appear then, that the rate of transmethylation of choline is about 18 percent that of methionine. However, it must be remembered that for every radioactive methionine molecule there is only one methyl group, which can be radioactive. With choline however, for every radioactive molecule there are three methyl groups and only one of these is radioactive.

This would mean that if the same number of radioactive methionine molecules as choline molecules were to transmethylate, the number of radioactive methyl groups transferred from choline would be only one-third the number transferred from methionine.

With this in mind, the count recovered in the nicotine dipicrate of this experiment should have been 33 percent that recovered by Brown if the rate of transmethylation was the same in both cases. Therefore, the 28 percent found experimentally, divided by the 33 percent theoretical gives for choline a transmethylation rate of approximately 85 percent of that for methionine. It would seem then, from these results, that the rate of transmethylation with choline is approximately the same as that with methionine. However, this is only a tentative assumption, and omits further investigation before any definite statement can be made.

SUMMARY

1. Nicotine isolated from Nicotiana rustica L., var. humilis, previously fed choline 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 located largely in the methyl carbon.

2. Choline has been found to be destroyed by soil and air microorganisms, which can be controlled by the use of germicides.

3. Tobacco plants, growing in a water solution, were found to need an extra amount of oxygen bubbled into the solution to keep them alive and healthy.

4. The phospholipid content of rapidly growing tobacco plants has been found to be very small as has been found for other plants by previous workers.

5. Not more than 65 percent of the choline fed to, and taken up by, tobacco plants was found to be metabolized by the plants after a week's growth.

6. Reasonable correlation between the rate of transmethylation of choline and methionine has been found, though this still awaits further proof.

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APPENDIX

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

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

where A_m = max. sp. activity (counts/min./mM.)

C_o = observed count (counts/min.), minus background count

M = molecular weight of compound

W = weight of sample counted

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

Sample calculation:

C_o = 61.1 W = 20.8 mgs. M = 248 b = .32

$$A_m = \frac{61.1 \times 248}{20.8 \times .32} = 1.41 \times 10^3 \text{ counts/min./mM at inf. thinness.}$$

THE PARTICIPATION OF CHOLINE IN TRANSMETHYLATION REACTIONS IN *NICOTIANA RUSTICA*

By

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

Year 1952

Approved R. U. Byerrum

THE PARTICIPATION OF CHOLINE IN TRANSMETHYLATION REACTIONS IN NICOTIANA RUSTICA

By

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Choline, (trimethylethanolamine) has been demonstrated in animals to have transmethylating properties of the same type as methionine and glycine betaines. In this reaction, choline gives up one of its three methyl groups to form dimethylethanolamine and a free methyl group. The latter is used in the synthesis of other biological compounds needing a methyl group. Though an extensive investigation of this reaction has been done with animals, relatively little has been done with plants. Canadian workers Kirkwood and Marion, after an investigation of this reaction in plants, are of the opinion that choline is not a transmethylating agent. However, due to the similarity of the metabolic reactions in plants and animals, it was decided to further investigate this reaction. Two possibilities apparently not investigated by these workers were: (1) that no absorption occurred during the hydroponic administration of choline, and (2) that soil bacteria destroyed the choline before the plants had a chance to absorb it. The results of an investigation of these two possibilities proved that choline could be absorbed by tobacco plants but that choline was destroyed by bacteria. It was further found that by the use of aureomycin and a detergent germicide, the bacteria count could be controlled so that destruction of the choline did not occur.

These results led to a further investigation to determine if choline, after being absorbed by the plants, could function as a transmethylating

agent. Tobacco plants were grown in an inorganic nutrient solution containing radioactive choline, the activity located in one of the three methyl groups. Isolations of nicotine, to determine if transmethylation has taken place, phospholipids since some contain choline, and unmetabolized choline, were attempted. Isolation of phospholipids yielded negative results which was not unexpected since the concentration of phosphotides in plants is very small and the amount of starting material was limited. The unmetabolized choline isolated gave evidence that not more than 85 per cent of the choline fed to the plants was used by them during the growing period on the radioactive material.

The isolated nicotine was found to be radioactive indicating a transmethylation had taken place. The nicotine was demethylated to form methyl iodide. This was reacted with triethylamine to form triethylmethylammonium iodide. A comparison of the maximum specific activity (counts/min./millimole) of the nicotine and quaternary ammonium iodide showed that most if not all the activity of the nicotine was located in its methyl group.

These results, then, show that choline can act as a precursor of the methyl group of nicotine. However, no evidence was found to determine if the methyl group was transferred as an entity or if it is an oxidation-reduction reaction in which the carbon atom alone is transferred.