A STUDY OF PYRUVATE METABOLISM IN THE TOBACCO PLANT

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Maria E. Frontera-Aymat

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AN ABSTRACT

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

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The present study was undertaken in an attempt to prove whether or not the beta-carbon of pyruvate might contribute to the one-carbon pool and then be a precursor of methyl groups in the intact tobacco plant.

Pyruvate-3-C^{14} was fed to three groups of tobacco plants. The nicotine isolated from them was found to be radioactive. The nicotine was demethylated and it was found that the radioactivity in the N-methyl group was not greater than would be expected if the carbons of the nicotine molecule were randomly labeled.

From the results obtained in the present study it appears that the beta-carbon of pyruvate does not contribute to an appreciable extent to the one-carbon pool in metabolism.

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INTRODUCTION

The path of carbon during photosynthesis has been a subject of much interest during the last few years. After carbon-14 became available it was possible to follow carbon through the various routes that it takes in the plant on the way from CO_2 to sugar and other plant materials. The method of study used by Calvin et al.(1) has been to feed a plant some labeled CO_2 in the light and allow only a very short time for photosynthesis. By examining the compounds present in the plant after exposures to radioactive carbon dioxide for various times, it has been possible to elucidate the first few reactions in the path of carbon during photosynthesis.

Methyl groups are present in various plant products. It is interesting to notice that no labeling has been found in these groups when plants have been allowed to photosynthesize in the presence of labeled CO_2 for periods up to 30 minutes (2). In view of this finding and in an attempt to determine how CO_2 goes into methyl groups, carbon-14 labeled intermediates in the CO_2-methyl pathway have been fed to intact plants. Using methionine-methyl-C-14, Byerrum and Brown (3) demonstrated that the methyl carbon of methionine could be incorporated into the N-methyl group of

nicotine and a direct transfer of the methyl group was postulated. Flokstra (4) also demonstrated the incorporation of the methyl carbon of methionine into the methoxyl carbon of lignin in barley. During the same period formate (5) was found to form the methyl carbon of nicotine and the methoxyl group of lignin. In an attempt to determine whether the methyl group of methionine might be transferred by oxidation and subsequent reduction or directly as a methyl group, Dewey et al. (6) fed methionine doubly labeled with carbon-14 and deuterium to barley. Direct transmethylation has been shown to occur since it was observed that the same deuterium to carbon-14 ratio was present in the methyl group of lignin as occurred in the methyl group of methionine.

A number of different compounds have been shown to be methyl precursors in higher plants. Kirkwood and Marion (7, 10) showed the incorporation of formate into the methyl groups of choline and hordenine of barley although they were unable to demonstrate the transfer of choline methyls to hordenine. However, Byerrum and Wing (8) demonstrated that the methyl carbons of choline could be transferred to give the methyl group in nicotine in Nicotiana rustica. Kirkwood et al. (9) were also able to demonstrate that methionine can donate methyl groups to the alkaloid ricinine of castor beans. Sato (11) observed that the methyl group of methionine could give rise to the methoxyl group of pectin in radish plants. Glycine betaine serves as a source of labile methyls in the

barley plant, as judged by the transfer of its methyls to the alkaloids N-methyltyramine and hordenine (12). Sato (11) showed the incorporation of the methyl groups of glycine betaine into the nicotine N-methyl group. Byerrum, Hamill and Ball (13) have also demonstrated that the alpha-carbon of glycine serves as a precursor for the N-methyl carbon of nicotine in the intact tobacco plant. The incorporation of the glycine alpha-carbon into the nicotine methyl group was at least as rapid as the incorporation of the methyl group of methionine and over ten times as rapid as the incorporation of the carbon of formate. Dewey (14) fed calcium glycolate-2-C^{14} to tobacco plants and observed the labeling of the nicotine in the methyl group to be about the same extent as observed in methionine studies.

The rapid incorporation of formaldehyde into the N-methyl group of nicotine and the results obtained in previous studies with methyl precursors in this laboratory led Byerrum et al. (15) to propose that a two-carbon compound possibly in equilibrium with glycine, might be formed in photosynthesis from carbon dioxide which could split to give a one-carbon unit at the oxidation state of formaldehyde. This one-carbon unit would then give rise to the methyl group.

The formation of a 2-carbon intermediate in the photosynthesis of methyl groups, however, is not the only possibility. Vernon and Aronoff (16) suggested that alanine arises from phosphoglycerate and that serine and glycine were

formed from alanine probably by such a pathway as:

pyruvate ⟶ alanine ⟶ serine ⟶ glycine

It was recently demonstrated in this laboratory that the beta-carbon of serine was incorporated to a large extent into the N-methyl group of nicotine in tobacco plant metabolism (15). This finding could indicate that if serine arises directly from pyruvate, the beta-carbon of pyruvate could contribute to the one-carbon pool in metabolism. This possibility seemed of further interest because Arnstein and Keglevic (17) have reported that neither alanine nor pyruvate are efficient serine precursors in the intact rat.

The present study was undertaken in an attempt to prove whether or not the beta-carbon of pyruvate might contribute to the one-carbon pool and then be a precursor of methyl groups in intact photosynthesizing tobacco plants. When pyruvate-3-C^{14} was fed to a group of tobacco plants, the nicotine isolated from them was radioactive. However the radioactivity of the N-methyl group was not greater than would be expected if the carbons of the nicotine molecule were randomly labeled. It would therefore appear that the metabolic pathway suggested by Vernon and Aronoff to occur in plants - the conversion of phosphoglycerate to serine by way of pyruvate and alanine - was operating at best only to a minor extent.

EXPERIMENTAL AND RESULTS

Growth of Plants

The tobacco plants used in this investigation were of a high nicotine strain, Nicotiana rustica L., var. humilis (3). The seeds were planted in the greenhouse in flats containing vermiculite.[1] Within three weeks the seedlings were transplanted allowing about two or three inches between each plant. Occasionally tap water was applied to keep the vermiculite moist and a nutrient solution containing 1 g of $MgSO_4.7H_2O$, 1 g K_2HPO_4 and 5.8 g $Ca(NO_3)_2 \cdot 4H_2O$ in about four liters of tap water was applied twice a week. About two or three months were usually required for the plants to obtain the desired height of about six inches.

To prepare plants for the hydroponic administration of the sodium pyruvate the plants were removed from the flats and the roots were carefully freed from vermiculite first by shaking and then by soaking and washing with tap water. The roots of the plants were then soaked in a 0.01 percent Wyandotte detergent germicide[2] for one hour to reduce the

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[1] A commercial brand of heat expanded mica

[2] Wyandotte detergent germicide, No. 1528, was obtained from the Wyandotte Chemical Corporation, Wyandotte, Michigan.

bacterial population. After rinsing the roots with tap water the plants were placed in 125 ml Erlenmeyer flasks containing 1 ml aureomycin solution (1:1000), 6 drops of a 0.1 percent Wyandotte detergent germicide and 50 ml of an inorganic nutrient medium prepared by diluting with two parts of water, one part of the stock solution. The composition of the stock solution is as follows:

2610 mg	$Ca(NO_3)_2.4H_2O$
500 mg	KCl
756 mg	$MgSO_4.7H_2O$
500 mg	$(NH_4)_2SO_4$
5.6 mg	$FeCl_3.6H_2O$
500 mg	K_2HPO_4
2 liters	Distilled water

Plants were fed the radioactive material in a special fume hood to prevent any health hazard from radioactivity. Artificial light was used in the experimental work. The source of light consisted of two 36-inch, 30-watt fluorescent tubes, placed about 14 inches above the tops of the plants. The lights were left on for twelve hours a day and nutrient solution was added to the flask when required to keep the volume constant. Twice a day, when the lights were turned on and when the lights were turned off, a stream of oxygen was passed through the nutrient solution for two minutes to provide aeration for the roots and to prevent wilting.

Uptake of Sodium Pyruvate

Before the administration of radioactive sodium pyruvate it was necessary to ascertain the extent of uptake of the pyruvate by the plant and to determine whether the pyruvate was destroyed by microorganisms on the roots.

The most sensitive method for the determination of pyruvic acid depends upon its precipitation with nitrophenylhydrazines (18). Whereas 4-nitrophenylhydrazine may be used, 2,4-dinitrophenylhydrazine was preferred because its solution is stable and it reacts more rapidly with the pyruvate (18). The 2,4-dinitrophenylhydrazine reagent was prepared by grinding 100 mg of the phenylhydrazine with 100 ml of hydrochloric acid, prepared by diluting one part concentrated HCl with 5 parts of water. The solution was filtered and kept in the refrigerator while not in use. The determinations were carried out as follows: a three ml aliquot of sample solution was placed in a water bath for ten minutes at 25°C. One ml of 2,4-dinitrophenylhydrazine reagent was added and the solution was mixed. After five minutes, eight ml of ethyl acetate were added and a stream of nitrogen was bubbled through the mixture for two minutes. The lower aqueous layer was removed and discarded. Exactly 6 ml of a 4 percent solution of sodium carbonate were added to the solvent mixture, and nitrogen was rapidly passed through the solution again for two minutes. After the two phases had separated, 5 ml were withdrawn from the lower layer. To this solution five ml

of a six percent sodium hydroxide solution were added, mixed and allowed to stand for five minutes. The optical density of the solution was determined in a Beckman Spectrophotometer Model B, at 420 mμ and 520 mμ, 5 to 10 minutes after the addition of the alkali.

A standard curve was made by using various standard sodium pyruvate solutions containing from 5 to 20 micrograms per milliliter. Determinations were carried out as explained above. Optical density was plotted against concentration and a straight line curve was obtained.

To determine the uptake of sodium pyruvate by the tobacco plants, four plants were prepared as described previously. Then 2 ml of sodium pyruvate solution (1 mg/ml) were added to each flask. Four other flasks were prepared with the same solutions but, instead of plants, contained six root pieces of about one-centimeter each. Four other flasks containing the 50 ml nutrient solution, one ml aureomycin and six drops of the 0.1 percent Wyandotte detergent germicide were used as controls. All twelve flasks were put in the hood and the lights were left on for twelve hours a day. After 48 hours, the plants were removed from the nutrient solution. The roots were washed with a stream of distilled water and the washings were collected in the respective flasks. The content of each flask was filtered through Whatman number one paper. The filtrate was collected in 100 ml volumetric flasks and

made up to the mark with distilled water. Three ml of each flask were analyzed for the presence of pyruvic acid as explained before. It was found that 90 percent of the pyruvate had been absorbed by the intact plants during the 48 hours. No significant decrease in the original amount of pyruvate was indicated in the analysis of the solution which had contained root fragments or in the control solutions. Due to the rapid absorption of pyruvate by the plants a growing period of seven days was used for the administration of the radioactive pyruvate.

Administration of the Radioactive Pyruvate

1.5 mg of sodium pyruvate containing 10^6 counts per minute as C^{14} were fed to each plant.[3] The molar quantity of pyruvate was calculated to be equivalent to the amounts of methionine and other methyl precursors used in previous studies in this laboratory. However, the amount of radioactivity was ten times more than the amount used by previous workers.

Isolation and Purification of Nicotine

Seven days after the administration of the radioactive material the plants were removed from the flasks. The roots

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[3]Obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Ill.

were washed under the tap and blotted with a cheese cloth. The plants were then cut with scissors into very small pieces that were dried as soon as possible under two infra-red lamps. The dried material was ground in a mortar with 20 percent of its weight of calcium hydroxide. The resulting powder was transferred to a Kjeldahl flask and steam distilled into a flask containing 6 ml of 6N HCl, until the distillate was clear upon the addition of two drops of silicotungstic acid (12 g per 100 ml water). Usually from one to two liters of distillate were collected. The distillate was concentrated in vacuo to a volume of about ten milliliters. Following the procedure of Smith (19), the concentrate was made alkaline and azeotropically distilled twice to purify the nicotine. Every time the distillate was collected in hydrochloric acid as described above. The final distillate was concentrated in vacuo to dryness. One ml of distilled water was added to the nicotine hydrochloride and this solution was again concentrated in vacuo to dryness. The salt was dissolved in one milliliter of water, and then the flask was washed out with small amounts of methanol until the total volume was about 20 ml. An equal amount of methanol saturated with picric acid was then added to precipitate the nicotine as the dipicrate. After the mixture had stood for about 30 minutes, the nicotine dipicrate was filtered off, washed with methanol and recrystallized from hot water. The

precipitate was left overnight in a vacuum desiccator and the next day a determination of melting point was run for purity. The final product melted at 223°-224°C as compared with the recorded value of 224°C (20).

The dipicrate was ground in a mortar and then plated in a tared aluminum dish for counting. The samples were weighed, counted in a windowless flow counter and corrected for self absorption. (For calculations see Appendix I, page 21). The results are presented in Table I. It may be seen that after feeding pyruvate-3-C^{14} to tobacco plants, the nicotine isolated from them was radioactive.

Demethylation of the Nicotine

As the nicotine was found to be radioactive, it was of interest to determine how much of the radioactivity was in the methyl group. The method used was that suggested by Pregl (21) for the determination of methylimino groups. Brown's modification (3) of the procedure was used in order to recover the methyl carbon in a suitable form for counting. The procedure consisted of the conversion of the alkylimino substance in the presence of hydriodic acid into the quaternary ammonium salt, which on heating was decomposed with separation of the alkyl iodide. The liberated methyl iodide was made to react with triethylamine to yield triethylmethyl ammonium iodide. The demethylation apparatus was similar to that described by Pregl.

In order to obtain the nicotine in a more soluble form about 200 mg of the nicotine dipicrate were dissolved in sodium hydroxide and the nicotine recovered by azeotropic distillation through a Widmer column. The distillate was collected in 2 ml of 6N hydrochloric acid and was concentrated to dryness in vacuo. The final concentration of the solution was done in the demethylating flask. To the nicotine hydrochloride in the demethylating flask were added 45 mg NH_4I, 2 drops of 5 percent gold chloride solution, and 3 ml of hydriodic acid, (specific gravity 1.5). The gas washing vessel contained 0.75 ml of a 5 percent cadmium sulfate solution and 0.75 ml of a 5 percent sodium thiosulfate solution as described by Pregl to remove iodine and hydrogen iodide. The receiver contained a 5 percent ethanolic solution of triethylamine which was cooled in a solid carbon dioxide methyl cellosolve bath. After the apparatus was assembled a slow stream of nitrogen was passed through the side arm and the reaction flask was heated in a cupric oxide bath. The temperature was raised to 200°C in 20 to 25 minutes, then slowly to 350-360°C and held there for 45 minutes. The heat was removed and the apparatus was flushed with nitrogen until the reaction flask had cooled. The receiving tube was then taken off, corked tightly, shaken and allowed to stand overnight at room temperature. The next day the excess alcohol and triethylamine were evaporated in a steam bath and then the last traces were removed in a vacuum desiccator. The white triethylmethyl ammonium iodide was dissolved

in less than one ml of absolute ethanol and was plated in tared aluminum dishes for counting. The samples were dried in a vacuum desiccator and counted in the flow counter. The results of these counts are also presented in Table I.

TABLE I

LOCATION OF RADIOACTIVITY IN THE NICOTINE MOLECULE AFTER THE ADMINISTRATION OF PYRUVATE-3-C^{14}

Trial	Number of Plants	Maximum Specific Activity (Counts per minute per millimole)		
		Nicotine Dipicrate	Triethylmethyl Ammonium Iodide	Percent Recovered
1	16	1.43×10^3	81	6
2	6	5.93×10^2	37	6
3	23	1.22×10^3	50	4

The results (Table I) show that less than 10 percent of the radioactivity of nicotine from pyruvate-3-C^{14} fed plants was located in the N-methyl group.

DISCUSSION

From the results obtained in the present study it appears that the beta-carbon of pyruvate does not contribute to any appreciable extent to the one-carbon pool in metabolism.

This finding is in contrast with Vernon and Aronoff's suggestion that serine and glycine arise from phosphoglycerate by the following pathway:

phosphoglycerate ⟶ pyruvate ⟶ alanine ⟶ serine ⟶ glycine

If this pathway were operating appreciably in the intact tobacco plant, the beta-carbon of pyruvate would correspond to the beta-carbon of serine and a larger amount of radioactivity would have been found in the N-methyl group of nicotine than was observed experimentally. This assumption is based on the findings of Byerrum *et al.* (15) who have showed that the beta-carbon of serine contributes to the one-carbon pool in metabolism and that it is incorporated to a large extent in the N-methyl group of nicotine. Thus it appears probable that serine does not arise directly from pyruvate in the intact photosynthesizing tobacco plant. The results are also in disagreement with those of Newburg and Burris (22) who suggested that alanine arises from pyruvate without rupture of C-C bonds and that carbon-2 of pyruvate

becomes carbon-1 of glycine. If this were the case, the beta-carbon of pyruvate would correspond to the alpha-carbon of glycine, and it has been shown by Byerrum et al. (13) that the alpha carbon of glycine is a good methyl precursor in the tobacco plant. There is, however, a significant difference in the method of study followed in each case that would possibly explain the difference in results. Vernon and Aronoff used soybean leaves in the light, Newburg and Burris employed tobacco leaves in the dark, and in the present study, intact tobacco plants were used in the light. It is possible that different metabolic pathways exist in each case.

One suggestion for the metabolic pathway involved in the incorporation of pyruvate-3-C^{14} into nicotine in the intact tobacco plant is that pyruvate is completely oxidized to CO_2 and H_2O by way of the Krebs cycle and that then this CO_2 is metabolized in photosynthesis. Under these conditions the radioactivity would be randomly distributed within the nicotine molecule. The results show that about 5 percent of the radioactivity of nicotine from pyruvate-3-C^{14} fed plants was in the methyl group. Since nicotine contains 10 carbons, one would expect 10 percent of the total radioactivity to be in the methyl group if random labeling had resulted. The counts of triethylmethylammonium iodide were so low that great counting accuracy was not possible.

The present results may therefore actually indicate a random distribution of the beta-carbon of pyruvate in the nicotine molecule.

The idea of random distribution does not discard the possibility of finding most of the radioactivity in some definite location in any one of the rings of the nicotine molecule. The fact that less than the theoretical 10 percent labeling expected for random distribution was found in the N-methyl group gives a point for speculation. Another possible metabolic pathway may be the conversion of pyruvate to glutamate. Calvin et al. (23) fed labeled pyruvate to algae and found that glutamate had over two times as much radioactivity as any other single product. Pyruvate-3-C^{14} may be metabolized to alpha ketoglutarate by way of the Krebs cycle, and then by transamination be converted to glutamic acid labeled in the gamma position. The glutamic acid may cyclize and form the pyrrolidine ring labeled in the three or four position. This proposition is speculation and needs further study. Nothing can be concluded definitely since the nicotine in the present study was not degraded beyond demethylation. It is interesting to point out that Dewey (24) fed labeled ornithine-2-C^{14} to tobacco plants and obtained labeling in the two and five positions of the pyrrolidine ring of nicotine. The interconversion of ornithine and glutamic acid has not been shown to occur in plants but it has been demonstrated in animal metabolism (25).

SUMMARY

Pyruvate labeled with carbon-14 in the beta-carbon was administered to tobacco plants and was further metabolized. The nicotine isolated from the tobacco plants was found to possess radioactivity. Demethylation experiments showed that about 5 percent of the radioactivity of the nicotine was in the N-methyl group.

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APPENDIX I

The formula used in correcting the observed count for self-absorption was

$$Am = \frac{Co \cdot M}{W \cdot b}$$

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

Co = observed count (counts/minute)

M = molecular weight of compound

W = weight of sample counted (milligrams)

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

Sample calculation:

Nicotine dipicrate...Co = 33 c.p.m., W = 36 mg, M = 620, T = 12.7 mg/cm^2

$$Am = \frac{33 \times 620}{36 \times 0.39} = 1.45 \times 10^3 \text{ c.p.m./mM}$$

VITA

The writer was born in Puerto Rico and received her secondary education at Mayaguez High School in Mayaguez, Puerto Rico. In August, 1947, she entered the College of Agriculture and Mechanic Arts of the University of Puerto Rico, and was graduated in May, 1951 with a Bachelor of Science Degree. Since graduation she has been working as a chemistry instructor for the same institution. On June, 1955 she was granted a leave of absence for advanced studies at Michigan State University. After graduation the writer will return to her teaching position in Puerto Rico.

Date Due