THE BIOGENESIS OF ALKALOIDS

XX. THE INDUCED BIOGENESIS OF STACHYDRINE*

By GERHARD WIEHLER† and LÉO MARION

(*From the Division of Pure Chemistry, National Research Council, Ottawa, Canada*)

(Received for publication, November 25, 1957)

In a study of the role of ornithine in the biogenesis of stachydrine, Leete, Marion, and Spenser (1) administered ornithine-2-C^{14} to alfalfa seedlings (*Medicago sativa* L. Grimm) but found that the stachydrine isolated from these plants was not radioactive. Morgan and Marion (2) have since confirmed this result and shown that the proline in the plant was not active either, although glutamic acid, aspartic acid, and glycine were. Even in 12 week-old alfalfa, stachydrine and proline were not actively synthesized from ornithine (2). When, however, they administered ornithine-2-C^{14} together with pyridoxal to 19 day-old alfalfa seedlings, and after 12 days harvested the plant and isolated the alkaloid and amino acids, they found that the proline was radioactive although the stachydrine was inactive (2).

It has now been ascertained that glutamic acid-2-C^{14}, when administered together with pyridoxal to 15 day-old alfalfa seedlings, is also converted within 2 days to radioactive proline, although not to stachydrine.

The formation of stachydrine requires the methylation of proline, and this does not seem to take place in the 2 week-old plant. Methionine has been repeatedly shown to be a source of methyl groups in plants (3–5), but the feeding of methionine together with ornithine-2-C^{14} and pyridoxal to alfalfa seedlings failed to bring about the formation of radioactive stachydrine.[1] Sakami and Welch (6) have established that folic acid enhances biological methylation, although it is apparently less effective than tetrahydrofolic acid (7). It is known that rats fed with folic acid and formate-C^{14} incorporate much more C^{14} into body protein than rats deficient in folic acid (8). It was anticipated, therefore, that folic acid might stimulate the methylation of proline by methionine into stachydrine, and indeed alfalfa seedlings, after the administration of methionine-C^{14}, pyridoxal, and folic acid, yielded the radioactive alkaloid.

Both the choline and the alkaloid fraction extracted from these plants were radioactive. In the course of previous work it had been observed that

* Issued as Publication No. 4696 of the National Research Council of Canada.

† Postdoctorate Fellow of the National Research Council of Canada.

[1] Massicot, J., and Marion, L., unpublished results.

stachydrine isolated from alfalfa was not pure, but consisted of a mixture of bases (1, 2). Recently, it has been shown that the mixture consists of stachydrine and its higher homologue, which has been named homostachydrine (9). Homostachydrine which is the *N*-methylbetaine of pipecolic acid always accompanies stachydrine in alfalfa. Its properties are similar to those of stachydrine and, although it has been possible to isolate it in a state of purity, it has not proved possible yet to obtain alfalfa stachydrine entirely free from it (9). After the separation of some pure homostachydrine from the radioactive alkaloid mixture, the remainder contained a greater proportion of stachydrine and showed a higher radioactivity (919 d.p.m. per mg.) than the original mixture isolated from the plant (753 d.p.m. per mg.). Since homostachydrine and stachydrine were clearly separated on a paper chromatogram, it was possible to show that all of the radioactivity of the mixture was contained in the stachydrine by measuring the radioactivity of a paper chromatogram which was cut into strips (Fig. 1).

EXPERIMENTAL

Feeding of Alfalfa with Methionine (Me-C^{14})—Alfalfa seeds (*M. sativa* L. Grimm) (300 gm.) were dusted with the fungicide Arasan[2] and spread evenly on top of a double layer of wetted glass wool in ten Pyrex trays. These were kept in a dark germinating cabinet for 4 days and then brought into the open; 50 ml. of water and 50 ml. of nutrient solution (10) were added on alternate days. On the 8th and 14th days the contents of all trays were washed with distilled water three times. On the 15th day of growth, methionine (Me-C^{14}) (50 mg. with a specific activity of 1.70×10^8 d.p.m. per mmole and a total activity of 5.7×10^7 d.p.m.), pyridoxal (0.5 mg.), and folic acid (0.88 mg. from a stock solution prepared according to Lascelles and Woods (11), and containing 0.044 per cent of folic acid in sodium bicarbonate) were added in water solution to the trays. The same amount of folic acid and pyridoxal was added on the 17th day. The plants received a total of 1.76 mg. of folic acid and 1.0 mg. of pyridoxal and were allowed to grow in contact with the tracer for 6 days. The radioactivity of the fresh leaves was 3×10^3 d.p.m. per mg. on the 18th day and 8.5×10^3 d.p.m. per mg. on the 21st day of growth. After 21 days, each tray was washed with distilled water six times, and the plants were separated from the glass wool as completely as possible and dried on filter paper at room temperature for 3 days.

Extraction and Isolation of Stachydrine—The dried alfalfa plants (220 gm.) were ground and extracted with three 2 liter portions of boiling water

[2] Arasan, a product of du Pont de Nemours, Inc., has for its active principle tetramethylthiuramdisulfide.

as previously described (1, 2). The total activity of the 6 liter water extract was 7.4×10^6 d.p.m., representing 13 per cent of the activity present in methionine fed to the plant. The extract was treated with lead acetate in the usual way, and the lead-free solution was concentrated *in vacuo* to a volume of 250 ml. Part (20 per cent) of this solution was set aside and the remaining 200 ml. were further concentrated to half volume. The resulting dark brown viscous syrup was boiled with 95 per cent alcohol (350 ml.) and the mixture filtered after cooling. The insoluble residue was again boiled with two 150 ml. portions of 95 per cent alcohol. The combined alcoholic filtrates were evaporated to dryness, the brown residue was dissolved in water and made alkaline (pH 8) with aqueous ammonia, and the choline was precipitated as the reineckate. The crude choline reineckate was purified via the chloride and the mercuric salt complex by the standard procedure. The choline chloride (80 mg.) thus obtained was found by a paper chromatogram to be pure. Its activity was 501 d.p.m. per mg. or 6.9×10^4 d.p.m. per mmole.

The reddish brown choline-free aqueous filtrate was acidified with concentrated hydrochloric acid (50 ml.), a 5 per cent solution of reinecke salt in methanol (50 ml.) was added, and the precipitated mixture of stachydrine and homostachydrine reineckates was filtered with suction and washed with *n*-propanol. The mixture was dissolved in acetone (100 ml.), the solution filtered to remove a brown insoluble impurity, and the filtrate evaporated to dryness. The residue of mixed reineckates (1.41 gm.) was dissolved in acetone, converted to the chlorides (12), and purified by being treated twice with mercuric chloride as already described (9). After recrystallization from alcohol and acetone, the colorless crystalline mixture of stachydrine and homostachydrine hydrochlorides (133 mg.) melted at 197–199° (decomposed).[3] Chromatography on Whatman No. 1 paper with Munier's Bc-20 solvent (13) showed the two red-purple spots characteristic of stachydrine and homostachydrine after the paper was sprayed with Dragendorff's reagent (9). The activity of this choline-free mixture was 753 d.p.m. per mg.

Separation of Stachydrine and Homostachydrine—The separation of homostachydrine from stachydrine was carried out by chromatography on cellulose powder (50 mg.), Whatman standard grade, as described previously (9). A solution of the stachydrine and homostachydrine hydrochlorides was applied to three disks of filter paper (diameter, 24 mm.) and the paper was dried. The paper disks were placed on top of a cellulose powder column (25 × 280 mm.) and eluted with Munier's Bc-20 solvent. A total of 55 fractions was collected. Fractions 11 to 19 contained homostachydrine, Fractions 20 to 24 a mixture of homostachydrine and stachy-

[3] All melting points are corrected.

drine, and Fractions 25 to 27 yielded a small quantity of stachydrine. Fractions 20 to 29 were pooled and evaporated *in vacuo*. The residual hydrochlorides were purified via the mercuric salt complex (9) and recrystallized from alcohol-ether. The colorless crystalline salt (8 mg.) melted at 208–211° (decomposed). A paper chromatogram of this salt showed a larger spot of stachydrine and a smaller spot of homostachydrine. The activity of this stachydrine-enriched mixture was 919 d.p.m. per mg.

Detection of Radioactive Stachydrine on Paper Chromatogram—The determination was made by using the ascending method of paper chromatography (14). Two spots of 0.2 mg. of the mixture of stachydrine and homostachydrine hydrochlorides (m.p. 197–199° decomposed) were applied each on a sheet of Whatman No. 3 paper and the chromatograms developed with Munier's Bc-20 solvent. One of the papers was sprayed with Dragendorff's reagent, revealing two spots which corresponded to stachydrine and homostachydrine, respectively. The other paper was cut into strips 1 cm. in width. Each strip was threaded onto a platinum wire, suspended inside a reflux condenser, and extracted with boiling methanol (30 ml., 30 minutes). The methanol extracts were concentrated and then evaporated on separate aluminum disks. The activity of these extracts (the actual counts observed above the background without any corrections) plotted against the distance of the strips from the starting point is shown in Fig. 1. This shows that the position of maximal activity corresponds to the position of stachydrine (11 to 13 cm. from the starting line), while the position of homostachydrine (13.5 to 15.5 cm. from the starting line) reveals no activity.

Administration of Glutamic Acid-2-C^{14}—Alfalfa seeds (300 gm.) dusted with Arasan were germinated as described above. On the 15th day, a total of 0.5 mg. of pyridoxal hydrochloride dissolved in water was added to the trays. On the 16th day, 0.5 mg. of pyridoxal hydrochloride and 9.96 mg. of glutamic acid-2-C^{14} were added (specific activity 7.48×10^8 d.p.m. per mmole, total activity, 4.9×10^7 d.p.m.). On the 18th day the trays were washed carefully with distilled water and the plants, together with the glass wool, extracted with three portions of 6 liters of boiling water. The combined extracts had an activity of 4.0×10^6 d.p.m., representing 8.2 per cent of the total activity of the glutamic acid fed to the plant. Part of the extract (600 ml.) was set aside, and the rest used for the isolation of the alkaloids. The mixture of stachydrine and homostachydrine hydrochlorides (0.163 gm.) was isolated as described above. The activity of the crude mixture was 37.2 d.p.m. per mg. but it decreased upon further crystallization and became negligible after several crystallizations from alcohol.

Isolation of Proline—The 600 ml. of the aqueous extract that had been

set aside were evaporated to dryness and the residue was dissolved in 0.5 M acetic acid (5 ml.). The amino acids were separated as described

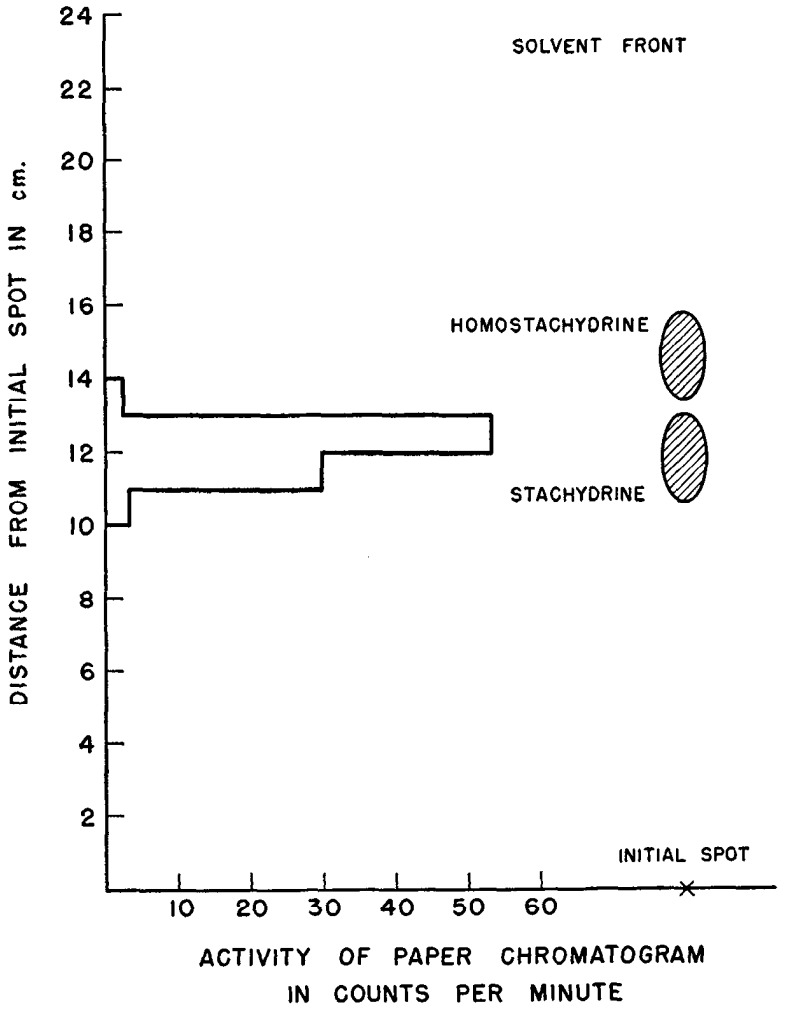

FIG. 1. Paper chromatogram showing the separation of the bases and their radioactivity.

by Morgan and Marion (2) first by means of a column (35 × 1.8 cm.) of Dowex 1-X8 ion exchange resin. Besides glutamic acid, the aspartic acid (146 mg.) was also radioactive (2.4×10^4 d.p.m. per mmole). The mixture of neutral and basic amino acids (1.394 gm.) was dissolved in N hydrochloric acid and separated on Dowex 50-X4. The proline isolated

(36 mg.) had an activity of 75.3 d.p.m. per mg. or a specific activity of 0.86×10^4 d.p.m. per mmole.

DISCUSSION

2 week-old alfalfa seedlings can convert ornithine into glutamic acid, but only after being fed pyridoxal can they convert ornithine and glutamic acid into proline. The failure to produce proline from these precursors by the normal plant at this stage of growth must be attributed to the absence of the proper enzyme system. Pyridoxal does not induce the methylation of proline, which does take place, however, in the presence of folic acid.

Experiments of Dinning, Keith, and Day (15) with chick livers suggest that folic acid is involved in transmethylation from betaine to methionine. The mature alfalfa plant contains folic acid (16), but since the methylation of proline to stachydrine in 2 week-old seedlings does not take place even after feeding methionine (6) unless folic acid is also fed, it can be concluded that the young plant is deficient in this acid, and also that folic acid is involved in the transmethylation from methionine to stachydrine.

What determines the biogenesis of an alkaloid, therefore, is not only the presence of the precursor amino acids but also the presence of the coenzymes or vitamins necessary to induce the proper reactions. In alfalfa, these inducing factors are formed only at a later stage of growth, probably in the late flowering period, since the alkaloid is stored in the seeds.

SUMMARY

The 2 week-old alfalfa plant can convert ornithine into glutamic acid, but not to proline. In the presence of pyridoxal, both ornithine and glutamic acid are converted to proline.

The feeding of methionine to 2 week-old alfalfa plants does not bring about the methylation of proline to yield stachydrine.

When the plant is fed folic acid together with methionine and pyridoxal, both the formation of proline and the methylation reaction are induced, and the stachydrine isolated from the plant is radioactive.

BIBLIOGRAPHY

1. Leete, E., Marion, L., and Spenser, I. D., *J. Biol. Chem.*, **214**, 71 (1955).
2. Morgan, A., and Marion, L., *Canad. J. Chem.*, **34**, 1704 (1956).
3. Brown, S. A., and Byerrum, R. U., *J. Am. Chem. Soc.*, **74**, 1523 (1952).
4. Dubeck, M., and Kirkwood, S., *J. Biol. Chem.*, **199**, 307 (1952).
5. Matchett, T. J., Marion, L., and Kirkwood, S., *Canad. J. Chem.*, **31**, 488 (1953).
6. Sakami, W., and Welch, A. D., *J. Biol. Chem.*, **187**, 379 (1950).
7. Kisliuk, R. L., and Sakami, W., *J. Am. Chem. Soc.*, **76**, 1456 (1954).
8. Plaut, G. W. E., Betheil, J. J., and Lardy, H. A., *J. Biol. Chem.*, **184**, 795 (1950).

9. Wiehler, G., and Marion, L., *Canad. J. Chem.*, **36,** 339 (1958).
10. Leete, E., *J. Am. Chem. Soc.*, **78,** 3520 (1956).
11. Lascelles, J., and Woods, D. D., *Biochem. J.*, **58,** 486 (1954).
12. Kampfhammer, J., and Bischoff, C., *Z. physiol. Chem.*, **191,** 179 (1930).
13. Munier, R., *Bull. Soc. chim. biol.*, **33,** 857 (1951). Munier, R., and Macheboeuf, M., *Bull. Soc. chim. biol.*, **32,** 192 (1950).
14. Leete, E., Kirkwood, S., and Marion, L., *Canad. J. Chem.*, **30,** 749 (1952).
15. Dinning, J. S., Keith, C. K., and Day, P. L., *J. Biol. Chem.*, **189,** 515 (1951).
16. Polizzi, C. V., Thesis, University of Chile (1953); *Chem. Abstr.*, **49,** 14228 (1955).