THE BIOGENESIS OF ALKALOIDS

XVI. HORDENINE METABOLISM IN BARLEY[1]

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ABSTRACT

Hordenine-α-C^{14} fed to sprouting barley was metabolized in the roots. Exhaustive fractionation of extracts from the dried roots yielded several substances of which only the hordenine, N-methyltyramine, and lignin were radioactive. Phenylalanine and tyrosine were inert. The results show that the methylation of tyramine to hordenine is at least partly reversible, but that the hordenine does not revert to tyrosine or phenylalanine. They also indicate that hordenine or N-methyltyramine become incorporated into the lignin.

It has been established previously that both N-methyltyramine and hordenine, occurring in the roots of germinating barley, originate from the precursor tyrosine (14, 15). The amino acid is first decarboxylated and the resulting tyramine is methylated via methionine to N-methyltyramine and to hordenine (12). According to Raoul (19) and others (6, 27), the alkaloids are present in barley during only approximately 30 days. The concentration of alkaloids starts at zero, since none are present in the seed, reaches a maximum at about 11 days of growth, and gradually decreases to zero after one month. This can be interpreted as meaning that during the first 11 days the rate of synthesis of the alkaloids is greater than the rate at which it is used up, whereas after that period it is degraded at a faster rate than it is formed. It was of interest, therefore, to investigate the transformation of hordenine in the plant.

It has been suggested by Raoul (19) on the basis of *in vitro* experiments performed under supposedly physiological conditions that hordenine is demethylated in the plant to tyramine. The same author also investigated the recarboxylation of the demethylated product, but failed to find proper *in vitro* methods under which this step could take place. It has also been assumed (12) that hordenine could undergo further methylation to the quaternary base candicine, which occurs together with hordenine in *Trichocereus candicans* Gillis (20) and *T. lamprochlorus* (16), but these suggestions have never been verified experimentally in the plant, and no candicine has been detected in barley.

Investigations of the behavior of hordenine toward isolated enzymes have shown that whereas tyrosine is converted by tyrosinase to melanin (4, 9, 19) hordenine is not, because of the substitution on the nitrogen. The product of the oxidation of hordenine by a preparation from Scotch broom containing tyrosinase has been identified as *o*-hydroxyhordenine (3). The presence of tyrosinase in barley has been reported by several investigators (9, 19, 25), but James has recently proved that the enzyme does not occur in the rootlets, at least until the 11th day of germination (11). If, therefore, the conclusion that

[1]*Manuscript received July 27, 1956.*
Contribution from the Division of Pure Chemistry, National Research Council, Ottawa, Canada. Issued as N.R.C. No. 4101.
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the tyrosinase occurs in some parts of the seedling other than in the rootlets is warranted, the presence of N-methyltyramine and hordenine in the rootlets can be explained. In the absence of tyrosinase, decarboxylation becomes the major pathway of tyrosine metabolism, and the product, tyramine, is methylated to form the alkaloids. This would account for the presence of hordenine solely in the roots, although tyrosine as shown by Raoul (19) is plentiful in the grain at the beginning of germination.

Certain microorganisms convert hordenine to *p*-hydroxyphenylethanol (5), while animal livers have been shown to transform hordenine, N-methyltyramine, and tyramine to *p*-hydroxyphenylacetic acid (7) and to *p*-hydroxyphenylethanol (18). These reactions have all been ascribed to oxidative deamination by the enzyme tyramine oxidase (21), but the presence of this enzyme in barley has not been demonstrated.

In the present work we have attempted to trace the path of hordenine metabolism in barley by feeding hordenine-α-C^{14} to the seedlings and following the radioactivity through an exhaustive fractionation.

EXPERIMENTAL

Hordenine-α-C^{14}

Tyramine-α-C^{14} hydrochloride prepared as previously described (13) was dissolved in aqueous ammonia and the solution extracted with ether in a continuous extractor for 72 hr. The ether extract was evaporated and the residual oily base distilled, b.p. 120–130° at 0.01 mm. The base (137 mgm., 1.0 millimole), with a specific activity of 1.15×10^7 disintegrations per min. per millimole,[3] was methylated with formic acid and formaldehyde as described by Raoul (19). The hordenine-α-C^{14} thus obtained was sublimed at 120–130° at 0.01 mm., wt. 70.4 mgm. (44% yield). A paper chromatogram showed that tyramine and N-methyltyramine were absent from the product. It had a total activity of 4.77×10^6 d.p.m., and a specific activity of 1.12×10^7 d.p.m. per millimole.

Administration of Hordenine-α-C^{14} to Barley

Barley (Charlottetown No. 80, 720 gm. dusted with the fungicide Semesan) was grown as previously described (13) except that the seeds were spread on glass wool to minimize the growth of mold. The seeds were about two years old and had lost some of their germinative capacity (35–40% germination). On the sixth day of sprouting the barley was fed (as previously described (13), by addition to the germination trays) a solution of hordenine-α-C^{14} (70.4 mgm.) in water (600 ml.) containing concentrated hydrochloric acid (0.10 ml.). The administration of the alkaloid had no apparent ill effect on the growth of the barley. On the 11th day of sprouting the shoots were clipped off as near as possible to the root mat and dried at 70°, wt. 32 gm., and total activity less than 0.3×10^5 d.p.m. The glass wool mats containing the roots were dried at 110° without separation.

[3]*The activities were determined as thin samples with a Radiation Counters Laboratory "Nucleometer" making the usual corrections for self-absorption, etc.*

Extraction of Roots

The mats consisting of glass wool and the roots were cut into strips and extracted with methanol for 48 hr. The extract after evaporation of the methanol weighed 6.09 gm. and had a total activity of 1.01×10^6 d.p.m. (i.e., 21% of the activity of the hordenine fed to the barley). The roots were now extracted with three portions of 0.5 *N* sodium hydroxide by successive boiling for eight hours and filtering (1). After this treatment the roots were inactive. The three alkaline extracts had the following activities: 3.0×10^6, 6.3×10^5, and less than 0.3×10^5 d.p.m. respectively, totalling 76% of the activity administered. The sum of the activities of all the extracts amounted to 97% of the activity administered as labelled hordenine.

The methanol extract was suspended in water to which was added enough hydrochloric acid to make it acid to Congo red, and the mixture extracted with ether. The aqueous liquor was then treated as described previously (13) to isolate the hordenine (116.3 mgm., specific activity, 8.58×10^5 d.p.m. per millimole) and the N-methyltyramine (34.9 mgm., specific activity, 1.43×10^5 d.p.m. per millimole). Choline was separated from the liquor from which the alkaloids had been removed, via the reineckate by the usual method (12, 15), and isolated as the chloroplatinate. It was inactive. No candicine was present.

Fractionation of the Sodium Hydroxide Extract

The three sodium hydroxide extracts were filtered and acidified separately with 5 *N* sulphuric acid. Precipitates were formed in the first two only (Residue B). The two filtrates were combined with the clear extracts and the combined liquor concentrated to 3 liters, alkalized to litmus with sodium hydroxide, and neutralized with acetic acid. A gelatinous precipitate settled out which was removed by centrifugation (Residue C). The mother liquor was then freed of much of the salt it contained by alternate precipitation with ethanol and concentration. The precipitates were combined with Residue C. After this treatment the aqueous solution D measured 800 ml., was slightly acid to litmus, and had an activity of 8.28×10^5 d.p.m.

One quarter of this solution D was percolated through a cation exchanger (Dowex 50-X4, hydrogen form, chloride-free) and an anion exchanger (Amberlite IR-4B, hydroxyl form, neutral to litmus) contained in columns (40 cm. $\times$3.5 cm.) connected so that the effluate from the cation exchanger passed directly into the anion exchanger. Water was percolated through the assembly until 2 liters of percolate had been collected. The percolate included sugars and other neutral substances which were negligibly radioactive (less than 1.0×10^4 d.p.m.). The columns were disconnected and washed separately with 1 liter of water (washings inactive). The anion exchanger was then eluted with 3 liters of 0.2 *N* hydrochloric acid, giving 1.604 gm. of acids, also negligibly radioactive (less than 2×10^3 d.p.m.). Elution of the cation exchanger with 1 liter of 0.15 *N* ammonia gave a basic fraction (0.990 gm.) with an activity of 6.1×10^4 d.p.m. No further activity was detected on further elution with ammonia or with 0.1 *N* sodium hydroxide.

Fractionation of the Weak Bases

The radioactive eluate from the cation exchanger contained principally the weakly basic amino acids and gave a positive Millon test. It was chromatographed on a charcoal column (2 cm.×9 cm.) (22). The charcoal (Darco G-60, 5 gm.) was pretreated by being heated on the steam bath with 200 ml. of 20% acetic acid, was cooled and washed on a filter with water, and then was transferred to the column in a slurry with 5% acetic acid. The residue left after evaporation of the eluate from the cation exchanger was dissolved in 5% acetic acid (100 ml.) and the solution transferred to the column, which was later eluted with 5% acetic acid until 300 ml. of eluate had been collected. This eluate on evaporation *in vacuo* yielded the non-aromatic amino acids (1.545 gm., activity, 2500 d.p.m.). The charcoal column was further eluted with 300 ml. of a mixture of water, glacial acetic acid, and phenol in the proportion of 80:20:5 (22). This eluate was extracted with ether to remove the phenol and evaporated to dryness under diminished pressure, leaving a residue of aromatic substances (0.198 gm., activity, 5.9×10^4 d.p.m.). A paper chromatogram of the residue developed with 80% phenol and sprayed with ninhydrin showed that it contained phenylalanine and tyrosine, but no tryptophan.

Phenylalanine and Tyrosine

The aromatic fraction described above was dissolved in a small volume of 4 *N* hydrochloric acid, transferred to a column (1 cm.×50 cm.) of Dowex 50-X12 prepared according to Wall (28), and eluted with 4 *N* hydrochloric acid. The eluate was collected in 5 ml. fractions, and aliquots were chromatographed on paper to spot the amino acids, which were detected with ninhydrin. Fractions 20–48 and 54–84 contained tyrosine and phenylalanine respectively. Each of these two acids was dissolved in 5% acetic acid (5 ml.) and the solution percolated through columns of Dowex 1-X8 in the acetate form to convert the hydrochloride to the free bases (10). The two eluates (each 50 ml.) were evaporated to dryness. Neither the tyrosine (43.9 mgm.) nor the phenylalanine (53.4 mgm.) after recrystallization was radioactive.

Hordenine and N-Methyltyramine

The mother liquor from the first phenylalanine crystallization and fractions 85–154 from the Dowex 50–X12 column gave a strong Millon test. A paper chromatogram under conditions previously described (13) showed the presence of hordenine and N-methyltyramine, but no tyramine nor tyrosine. The combined fractions 85–154 (activity, 4.3×10^4 d.p.m.) were alkalized with ammonia and extracted with ether for 48 hr. The extract (activity, 3.3×10^4 d.p.m.) contained hordenine and N-methyltyramine. The alkaloids were isolated directly from the remaining three-quarters of solution D by alkalizing the solution with ammonia and extracting with ether in the usual manner. To this extract were added the bases obtained above and the solution chromatographed on alumina to separate the two alkaloids. After sublimation the hordenine weighed 22.8 mgm. and had the specific activity 6.28×10^5 d.p.m. per millimole, and the N-methyltyramine weighed 32.2 mgm. and had the specific activity 1.5×10^5 d.p.m. per millimole.

Lignin

Residue B (activity, 1.95×10^5 d.p.m.) was treated by the procedure of Bondi and Meyer (1) in order to isolate the lignin. The product, a brown amorphous powder weighing 0.704 gm., had a total activity of 0.96×10^5 d.p.m. or 136 d.p.m. per mgm. Found: ash, 1.47%; N (ash-free), 5.81%; OCH_3 (ash-free), 4.53%.

A sample of this lignin was oxidized with nitrobenzene and alkali as described by Stone and Blundell (26). A product was obtained which gave a brick-red derivative with 2,4-dinitrophenylhydrazine. This derivative was completely inactive, but could not be crystallized and was not further identified.

A further quantity of lignin was isolated from Residue C. This residue consisted of dark brown gelatinous material and salts. It was taken up in water (1 liter) and dialyzed for one week to remove the salts and any remaining substances of low molecular weight. The amorphous, insoluble material was collected by centrifugation, and stirred in 1.25 *N* sodium hydroxide (500 ml.). The alkali-soluble material was treated as before (1) and yielded 1.670 gm. of brown amorphous powder with an activity of 2.1×10^5 d.p.m. or 126 d.p.m. per mgm. No proof of the identity of the two lignin preparations was possible, but the activities per mgm. are in good agreement.

DISCUSSION

Since the methanol and the sodium hydroxide extracts together accounted for 97% of the activity fed to the plant as labelled hordenine, any loss to the air as carbon dioxide was negligible, and it appeared probable that hordenine was not degraded to one-carbon fragments, except for the methyl group. The only compounds isolated that were radioactive were hordenine, N-methyltyramine, and lignin. Hordenine was not broken down to fragments capable of taking part in sugar synthesis, for the sugars were inert. Phenylalanine and tyrosine were also inert, and no tyramine was present. Consequently hordenine seems to undergo a partial demethylation only, giving rise to N-methyltyramine. No recarboxylation of the bases back to amino acids takes place since the amino acids isolated were not radioactive.

It has been shown by Freudenberg and Bittner (8) and by Siegel (23) that simple C_6, C_3 compounds can be lignin precursors. More recently Siegel (24) has demonstrated that in the presence of cellulose, eugenol is converted by peroxidase – hydrogen peroxide to a polymeric substance giving positive color tests for lignin. Also, Brown and Neish (2) have shown that C_6, C_3 amino acids such as phenylalanine and tyrosine do act as lignin precursors.

It is known that the lignin of very young plants unlike that of older plants contains nitrogen which appears to be an integral part of it. The lignin of two-week-old barley seedlings has been stated to contain as much as 8% nitrogen by Phillips (17), who further showed that the nitrogen content fell steadily as the plants matured.

The isolation of radioactive lignin in the present investigation, where the

only source of C^{14} was the hordenine, indicates that a C_6, C_2 fragment can also take part in lignin synthesis. The results also lead to the inference that hordenine is the source of at least part of the nitrogen in barley lignin. This inference, however, can only be drawn with caution and will require more definite proof.

ACKNOWLEDGMENT

The authors are indebted to Mr. R. B. MacLaren of the Experimental Station, P.E.I., for supplying the barley used in these experiments.

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