The biosynthesis of caffeine in the coffee plant

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The carbon precursors utilized in the biosynthesis of the purine ring have been identified for a variety of heterotrophic systems, including birds, mammals, bacteria, and yeast. With the use of isotopically labeled substrates, carbon atoms 2 and 8 of the purine ring have been shown to derive from formate (2) or from materials which can give rise to active 1-carbon compounds (3), carbon atom 6 from CO₂ (3-5) and carbon atoms 4 and 5 from glycine (3, 4, 6, 7). Thus far, no studies of this kind have been reported on the biosynthesis of purines in higher plants. Another aspect of this problem pertinent primarily to the higher plant is the biosynthesis of the methylated purines. Although there are reports on the formation of the methyl group in higher plants (8, 9), no attention has been given to the relationship between methyl group and purine ring formation. In addition, the effect of light on these processes has not been reported. These considerations prompted the present study of the biosynthesis of caffeine (1,3,7-trimethylxanthine) in Coffea arabica. Caffeine is found in relatively high concentrations in the leaves and seeds of this plant, thus facilitating an investigation of the metabolic pathways involved.

The results presented here indicate (a) that caffeine is synthesized from the same carbon precursors utilized for purine and methyl group synthesis in other systems and (b) that light enhances the formation of the purine ring of caffeine.

Experimental Procedure

Administration of Labeled Compounds—The leaves used in these experiments were selected at random from stock coffee plants grown in a greenhouse. They were deep green in color and approximately 4 cm in length. The leaves were washed with a dilute detergent solution, rinsed with distilled water, and chopped into squares ranging in size from 0.25 to 0.75 cm². Of the leaf squares, 5 g were floated in a medium consisting of 80 ml of water, 2 ml of 0.5 M potassium phosphate buffer, pH 7, and the isotopically labeled compound in 250-ml Erlenmeyer flasks. In the dark experiments, the flasks were wrapped in aluminum foil. In the light experiments, the light source consisted of two 150-watt, 120-volt floodlights. This provided a light intensity of approximately 1000 foot-candles at the surface. The temperature was maintained inside the desiccators at 20-25°C. Carbon dioxide was generated within the closed desiccators by the addition of 4 N lactic acid to a solution of NaHCO₃ through a separatory funnel.

Corresponding light and dark experiments were performed on the same or consecutive dates.

Isolation and Purification of Caffeine—Experiments were terminated by grinding the leaves or leaf squares in a blender at full speed with approximately 50 ml of water until they were pulverized and then adding boiling water to make a total volume of approximately 200 ml. The mixture was heated for 40 minutes on the steam bath. The hot water extract was filtered with suction through dry Whatman No. 1 filter paper which had previously been washed with hot CHCl₃. The pulverized leaves were re-extracted twice with 50-ml portions of boiling water, the mixtures being heated for 30 minutes on the steam bath before filtration. A saturated solution of basic lead acetate was added to the combined filtrate to precipitate proteins, followed by 8 N NH₄OH to precipitate tannins; the solution was filtered with hot water-washed Whatman No. 1 filter paper. H₂SO₄ (22 N) was added to bring the pH to 7, and lead sulfate was filtered onto whatman No. 1 filter paper. The filtrate was extracted three times with equal volumes of CHCl₃. After the CHCl₃ was removed by a stream of air, crude caffeine remained. This preparation was chromatographed on a column (diameter, 21 mm; final height, 250 mm) consisting of 25 g of dry, ether-washed Celite analytical filter aid moistened with 15 ml of 0.1 N H₂SO₄. The eluent was 0.1 N H₂SO₄-washed CHCl₃, 200 ml of which were passed through the column before addition of the sample. Elution of caffeine was followed by measuring optical density at 273 mp. Caffeine was found to emerge from the column in the 70- to 100-ml fraction. The solution of the alkaloid was taken to dryness with a stream of air. The dry material was taken up in 30 ml of CHCl₃ and this solution was extracted three times with 60-ml portions of 1.8 N H₂SO₄. The resulting solution was neutralized (pH 7) with NaOH, and the caffeine was brought back into CHCl₃ by extracting the aqueous solution three times with equal volumes of CHCl₃. The solvent was removed with a stream of air. The resultant caffeine was sublimed from a battery of Sylvania Daylight fluorescent tubes under the flasks. The flasks were shaken gently for 10 hours at room temperature.

In the CO₂ experiments, approximately 20 to 25 g of intact leaves were used, and the petioles were placed in water in vials inside either a 1-liter black desiccator or a 2-liter glass desiccator. In the light experiment, the light source consisted of two 150-watt, 120-volt floodlights. This provided a light intensity of approximately 1000 foot-candles at the surface. The temperature was maintained inside the desiccators at 20-25°C. Carbon dioxide was generated within the closed desiccators by the addition of 4 N lactic acid to a solution of NaHCO₃ through a separatory funnel.
at atmospheric pressure in an apparatus which consisted of a "cold finger" condenser (a large ice-filled test tube) suspended within and approximately 5 mm from the bottom of a 40-ml round bottomed centrifuge tube by means of a glass wool collar. Caffeine which had been dried in the centrifuge tube was heated with a micro burner until it sublimed onto the condenser. The sublimate was taken up in a known volume of glass-distilled water, an aliquot was removed, and the optical density was determined at 273 μμ. When necessary, carrier caffeine was added at this point. The solution was divided, placed in the appropriate oxidation or degradation flasks, and taken to dryness on the steam bath.

Degradation of Caffeine—Caffeine decomposes to give CO₂ (C-2 + C-6), CO (C-8), CH₃NH₃ (N-1 + N-3 methyl carbon atoms), NH₃ (N-9), and sarcosine (C-4, C-5, and N-7 methyl carbon atoms) when heated with 25 ml H₂SO₄ (10, 11). After 20 hours, the degradation is approximately 50% complete, as shown by recovery of CO₂ from the fractions derived from carbon atoms 2, 6, and 8 and the N-1 + N-3 methyl carbon atoms. A degradation train similar to that described by Goldthwait and Greenberg was used (12). Approximately 250 μmoles of caffeine were dried in a 100-ml 3-necked flask. The latter was attached to a reflux condenser and dry ice trap, 10 ml of 25% H₂SO₄ were added to the reaction flask, and this portion of the degradation train was flushed with N₂ for 40 minutes. At the end of this period, the first NaOH trap (containing 2 ml of 2 N NaOH, a tube containing anhydrous magnesium perchlorate, a tube containing Schütze reagent (13), and a tube containing granulated zinc were attached, and the N₂ flushing was continued for another 10 hours. The second NaOH trap, also containing 2 ml of 2 N NaOH, and another tube containing anhydrous magnesium perchlorate were then attached. The reaction flask was heated for 20 hours in a 185-189° oil bath while IS² was bubbled through the solution. The dry ice trap, a slush of dry ice and methyl Cellosolve, served to freeze out water which escaped the condenser and to trap SO₂. CO₂ from C-2 and C-6 of caffeine was trapped in the first NaOH bubbler, the anhydrous magnesium perchlorate tube following this trap removed traces of water from the stream of N₂. The CO from C-8 was converted to CO₂ by the Schütze reagent, and the resulting CO₂ was taken up in the second NaOH bubbler.

At the end of 20 hours, the reaction train was dismantled. The CO₂ trapped in the NaOH bubblers was converted to BaCO₃ by the method of Steele and Sfortunato (14). The acidic reaction mixture was cooled in ice and diluted with 30 ml of water. NaOH (10 N) was added to the phenolphthalein end point, and the alkaline mixture was heated between 90° and 100° in a water bath for 3 hours while N₂ was bubbled through the solution. The liberated CH₃NH₃ and NH₃ were trapped in 0.1 N H₂SO₄. The acidic methyleneamine solution was concentrated to a syrupy residue on the steam bath and oxidized by the wet oxidation method of Van Slyke, Plazin, and Weisiger (15).

After the removal of CH₃NH₃, it was possible to isolate sarcosine from the reaction mixture. The sarcosine fraction was not degraded further or oxidized.

Total oxidation of the isolated caffeine was performed by the wet oxidation method of Van Slyke, Plazin, and Weisiger (15). All samples were converted to BaCO₃ before isotopic assay. Isotopic activity is expressed as counts per minute at infinite thickness. Where carrier caffeine was added, specific activity values have been corrected.

**Reagents**—Glycine-1-C⁴ and glycine-2-C⁴ were obtained from New England Nuclear Corporation; HC¹⁴ΟNa, HC¹⁴ΟH, L-methionine-methyl-C⁴, l-histidine 2 C⁴, glycolic acid 2 C⁴, and acetone-1, 3-C⁴, from Volk Radio-Chemical Company; HC¹⁴Ο, OONa and L-serine-3-C⁴, from Nuclear-Chicago Corporation. Carrier formaldehyde was prepared by distillation of a solution of p-formaldehyde in water. The concentration of formaldehyde in the distillate was determined colorimetrically after reaction with chromotropic acid by the method of Koiusso (16). Carrier caffeine was a product of Eastman Organic Chemicals.

**RESULTS**

The radioactivity and distribution of isotope in caffeine with CO₂⁴, L-serine-3-C⁴, glycine-2-C⁴, HC¹⁴ΟNa, HC¹⁴ΟH, and L-methionine-methyl-C⁴ as substrates in the light and in the dark are recorded in Table I. In addition, the table contains data from experiments with glycine 1 C⁴ and CH₃OH as substrates in the dark.

**DISCUSSION**

The labeling patterns in caffeine (Table I) obtained when glycine-1-C⁴, glycine-2-C⁴, CO₂⁴, serine-3-C⁴, HC¹⁴ΟNa, HC¹⁴ΟH, OONa, and CH₃OH were administered indicate that the overall scheme of purine formation in C. arabica is analogous to that in animals, bacteria, and yeast.

The β-carbon atom of serine, the α-carbon atom of glycine, and formaldehyde, formate, and methanol carbon atoms were incorporated into C-8 and the C-2 + C-6 fraction of caffeine (Table I). The indicated carbon atoms of these compounds, with the exception of methanol, have been shown to be incorporated in carbon atoms 2 and 8 of the purine ring in animals (2, 3) and, in the case of glycine and formate, in yeast (6). Presumably, isotope in the C-2 + C-6 fraction is located in C-2 by analogy to other systems (3-5).

In the present investigation, CO₂⁴, when administered in the dark, was incorporated primarily into the carbon atoms obtained as the C-2 + C-6 fraction of degradation (Table I). If it is assumed that carbon atoms 2 and 8 are approximately equivalent with regard to isotopic content, almost all of the CO₂ incorporated into caffeine in the dark can be accounted for in C-6. A similar finding was observed in animal and bacterial preparations (3-5).

When glycine-1-C⁴ was the substrate, approximately 15% of the isotope incorporated into caffeine was located in the C-2, C-6, C-8, N-1, and N-3 methyl carbon atoms. An indirect value for incorporation into either C-4, C-5, or N-7 methyl carbon atoms of caffeine can be obtained by subtracting the counts in the remainder of the molecule from the counts incorporated into caffeine: (C-4, 5 + N-7 methyl) = (caffeine × 8) - 2(C-2 + C-6) - (C-8) - 2(N-1 + N-3 methyl). In the experiment recorded in Table I, this value is 408, or 65% of the total isotope incorporated into caffeine. Since the carboxyl carbon of glycine has not been reported to give rise to methyl carbon atoms, this indicates that the isotope is located in C-4 or C-5 of caffeine. This is similar to the incorporation pattern reported for other systems (3, 4, 6, 7). Analogous to the other organisms, isotope is presumably located in caffeine carbon atom 4.

When serine-3-C⁴, glycine-2-C⁴, HC¹⁴ΟNa, HC¹⁴ΟH, or C⁴-H₂O were administered, incorporation was higher into C-2 than into C-8, as is shown by the C-2 to C-8 ratio in Table I. This indicates that in these preparations there is a relatively large quantity of a purine precursor in the plant which contains C-8...
but not C-2 of caffeine. This compound may be 5-aminomidazo[4,5-b]pyridine, an intermediate of purine metabolism which has been found to accumulate in bacterial and animal systems (17, 18). In addition, it will be noted that for serine-3-\(^{14}C\), glycine-2-\(^{14}C\), and light-dependent \(^{14}CO_2\) uptake, incorporation into C-2 is only slightly higher than incorporation into C-8, whereas for formate, formaldehyde, and methanol, the incorporation into C-2 is approximately 3-fold higher than the incorporation into C-8. This suggests that there is more synthesis of the purine ring from the indicated carbon atoms of the two amino acids and photosynthetically reduced \(^{14}CO_2\) than from formate, formaldehyde, and methanol.

Whereas the methyl carbon of methionine has been reported to be incorporated into the uric acid carbon atoms of uric acid in an avian system (19), the methyl carbon of methionine did not give rise to carbon atoms 2 and 8 of caffeine (Table I). Thus, in this respect, plant and animal appear to differ either in the utilization of 1-carbon precursors or in purine ring synthesis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total given</th>
<th>Activity per carbon atom</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Isolated</td>
</tr>
<tr>
<td>(^{14}CO_2)</td>
<td>80</td>
<td>352</td>
</tr>
<tr>
<td>(^{14}CH_3)</td>
<td>80</td>
<td>352</td>
</tr>
<tr>
<td>Glycine-1-(^{14}C)</td>
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<td>175</td>
</tr>
<tr>
<td>Glycine-2-(^{14}C)</td>
<td>72</td>
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<tr>
<td>Glycine-2-(^{14}C)</td>
<td>72</td>
<td>25</td>
</tr>
<tr>
<td>L-Serine-3-(^{14}C)</td>
<td>68</td>
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<tr>
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</tr>
<tr>
<td>(^{14}C)</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

* C-2 = 2(C-2 + C-6); C-6 is assumed to derive exclusively from CO_2 and is considered to be inactive with those substrates for which a ratio is given.

† Light experiments; all other experiments in the table were run in darkness.

Methionine appears to be an important source of methyl groups in the coffee plant. The transfer of methyl groups from methionine to the unmethylated or partially methylated purine is apparently direct, since of all substrates used in this study, isotopic dilution (specific activity of precursor divided by specific activity of caffeine) was lowest with methionine-methyl-\(^{14}C\).

Whether the methyl groups of caffeine are derived through "active" 1-carbon compounds or by transmethylation from methionine, incorporation of isotope into the N-7 methyl carbon atom is apparently less than incorporation into one or both of these carbon atoms in the fraction isolated as methylamine. This is concluded, since, if the counts incorporated into C-2, C-6, C-8 and N-1 + N-3 methyl carbon atoms are subtracted from the total activity incorporated into caffeine when serine-3-\(^{14}C\), glycine-2-\(^{14}C\), formate, formaldehyde, methanol, and methionine were administered, little isotope is located in the C-4, C-5, and N-7 methyl fraction. The highest value for these three carbon atoms was 10% of the total activity when the substrate was serine-3-\(^{14}C\). With other substrates, this value was essentially nil. The apparent low incorporation into the N-7 methyl carbon atom would suggest an incorporation of a methyl group into this position relatively early in the formation of caffeine. Our degradation method does not permit us to distinguish whether isotopic activity is equally divided between the N-1 and N-3 methyl carbon atoms or whether it is located only in one.

The data presented here, together with the classic studies of Greenberg and Jaenicke (17) and Buchanan et al. (18) and the observation of Weevers (21) that seedlings of Theobroma can convert theobromine to caffeine, suggest the following as the possible steps in the biosynthesis of methylated purines: (a) synthesis of the carbon portion of the xanthine ring from glycine, CO_2 and formate catalyzed by a series of enzymes similar to those elucidated for pigeon liver and (b) methylation of the xanthine to produce heteroxanthine or 3-methylxanthine and, in turn, theobromine and caffeine.

**Effect of Light on Caffeine Biosynthesis**—The primary effect of light on caffeine biosynthesis in C. arabica is an apparent enhancement of purine ring formation rather than an increase in methyl group synthesis. This is supported by the observation that when glycine-2-\(^{14}C\), serine-3-\(^{14}C\), HC\(^{14}OONa\), or HC\(^{14}OH\) was administered, incorporation into C-5 relative to incorporation into the N-1 and N-3 methyl carbon atoms was approxi-
inhibition of methyl group formation in the light rather than an effect by light (Table I).

methylated purines, does not appear to be affected significantly by light (Table I).

Further confirmation that light enhances the formation of the purine ring is an approximate 2.5-fold increase in incorporation of C\textsubscript{14}O\textsubscript{2} into carbon atom 6 in the presence of light (Table I).

Incorporation of Other Compounds into Caffeine—It has been shown that the \(\alpha\)-carbon atom of glycolate (22, 23), the methyl carbon atoms of acetone (24), and ring carbon 2 of histidine (25) can give rise to metabolically active 1-carbon units and could function as precursors of caffeine. However, coffee leaf squares did not incorporate isotope from acetone-1,3-C\textsubscript{14} or L-histidine-2-C\textsubscript{14} into caffeine under the conditions described under “Experimental Procedure.” With leaf squares and intact seedlings and in the presence or absence of light, there was only limited incorporation of the \(\alpha\)-carbon atom of glycolate into caffeine. The glycolate was taken up by the preparations as indicated by assay (26). This contrasts with the tobacco plant, in which the \(\alpha\)-carbon atom of glycolate is incorporated into the methyl group of nicotine (22).

SUMMARY

1. It was concluded that the xanthine base of caffeine is synthesized from the same precursors as are purines in all of the other systems which have been studied. The xanthine is converted to 7 (or 3)-methylxanthine and, in turn, to theobromine and caffeine.

2. The \(\beta\)-carbon atom of serine, the \(\alpha\)-carbon atom of glycine, formaldehyde, formate, methanol, and the methyl carbon atom of methionine are active precursors of the N-1, N-3, or both methyl carbon atoms of caffeine.

3. The primary effect of light is an enhancement of purine ring formation.

4. The methyl carbon atom of methionine is not a precursor of the ureido carbons of caffeine.

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REFERENCES

21. WERNER, T., Arch. urol. urologe, 111 (1930).