Biosynthesis of Caffeine in Tea Callus Tissue

By D. B. A. OGUTUGA* AND D. H. NORTHCOTE

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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1. A study of caffeine biosynthesis has been made by following the incorporation of radioactive carbon dioxide and methionine into the methylated purines produced by tea callus tissue. 2. The uptake of the radioactive labels into nucleic acid and caffeine was followed over a period of approximately 9h. 3. The distribution of the radioactive labels in both nucleic acid and caffeine was determined after incorporation and subsequent incubation of the tissue in a non-radioactive medium. 4. The results of the experiments indicated that the caffeine arose from purines released from the breakdown of nucleic acids rather than that it was formed directly from a purine pool. 5. A metabolic scheme to show the production of caffeine from the nucleotides of the nucleic acid is discussed.

Anderson & Gibbs (1962) and Proiser & Serenkov (1963) have demonstrated that the carbon and nitrogen atoms in the purine ring of caffeine are derived from the same precursors as the purine bases in the nucleic acids (Buchanan & Hartman, 1959) and that the methyl groups on N-1, N-3 and N-7 are derived from L-methionine or other C₁ sources. However, it is still not known whether the purine ring in caffeine and other methylated purine bases found in plants is derived directly from the pool of purine precursors in the cell, or whether these arise from the breakdown of some preformed nucleotides.

Camargo (1924), Serenkov (1962) and Warner & Hagemann (1969) have suggested that caffeine is derived from the nucleic acids. Ogutuga & Northcote (1970) have demonstrated that yeast nucleic acids and L-methionine enhance the rate of caffeine formation in a broken-cell autolysate of tea stem-callus tissue. This also suggests that caffeine arises as a result of nucleic acid breakdown.

The mechanism of caffeine formation can be studied on intact plants, on organ culture or on tissue culture of plants that produce caffeine. So far, the organs or the tissues responsible for caffeine formation have not been identified definitely; therefore experiments employing either whole plants or organ cultures will be difficult to interpret because of the unknown effect of transport of substrates, products or both, to and from the appropriate site of synthesis. Tea callus-tissue culture is advantageous for the study of caffeine formation in plants because it is undifferentiated and its environment can be closely controlled. We have

employed such a culture in this work and we have used ¹⁴CO₂ and L-[Me-¹⁴C]methionine to investigate the relationship between the purine pool, nucleic acid purines and caffeine.

MATERIALS AND METHODS

The plant material, growth medium and methods of growth and maintenance of tea callus tissues were as described by Ogutuga & Northcote (1970).

Reagents. NaH¹⁴CO₃ (123 mCi/mmol) and L-[Me-¹⁴C]-methionine (53.6 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; Polyclar AT [polyvinylpyrrolidone (insoluble)] was obtained from GAF (Great Britain) Ltd., Manchester 2, U.K. 7-Methylguanine was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. 7-Methylxanthine and 7-methylxanthosine were prepared as described by Jones & Robins (1963).

Sterilization. The growth medium and instruments were sterilized by autoclaving for 30 min at 15 lb/in².

Addition of radioactive isotope. All operations were carried out under sterile conditions in a box fitted with rubber gloves and u.v. light.

The required quantity of radioactive chemical was added by means of a micropipette to 15 ml of sterile liquid growth medium and thoroughly mixed to ensure uniform distribution. The tea callus was then introduced into the medium by means of long forceps and incubated under diffused light on a rotator (50 cycles/min) in a room maintained at 26°C.

In the 'pulse-chase' experiments, the tissues were washed by shaking with two transfers of fresh sterile growth medium before continuing the chase.

Buffered sucrose medium. This was kept at 0°C and contained (final concns.): sucrose, 0.5 M; KCl, 50 mM; tris, 35 mM; magnesium acetate, 1 mM; EDTA, 2 mM; adjusted to pH 7.8 with 0.1 M-HCl.

Phenol-m-cresol mixture. This contained 70 ml of

* Present address: Cocoa Research Institute of Nigeria, P.M.B. 5244, Ibadan, Nigeria.

redistilled *m*-cresol, 50g of crystalline redistilled phenol, 55ml of water and 0.5g of 8-hydroxyquinoline.

Extraction of nucleic acids. A modification of the method of Loening (1967) was used. All operations were carried out at 4°C. To 1–2g of tea callus tissue was added an equal weight of washed Polyclar AT in a chilled mortar. The tissue was homogenized in the mortar with about 7ml of buffered sucrose medium. The homogenate was centrifuged at 1000g for 10 min and the supernatant was removed. Buffered sucrose medium (7ml) was added to the cell debris and to both the supernatant and the debris was added sodium dodecyl sulphate (0.5%) and sodium 4-aminosalicylate (5%). The mixtures were extracted with an equal volume of phenol-*m*-cresol mixture and the phenol and aqueous phases were separated by centrifugation at 1000g for 10 min. The upper aqueous phases from both extracts were combined and extracted two or three times with phenol-*m*-cresol mixture; the aqueous phase was retained and NaCl was added to it to give a final concentration of 2.5% (w/v). The nucleic acid was precipitated overnight in 2 vol. of 96% (v/v) ethanol at -10°C.

Purification of nucleic acids. The nucleic acid precipitate was separated by centrifugation at 10000g for 2 h and the precipitate was washed with 50, 70 and 96% (v/v) ethanol followed by diethyl ether before being dissolved in 0.15M-sodium acetate, pH 6. The protein that remained in the preparation was removed by shaking the nucleic acid solution vigorously with an equal volume of a mixture of chloroform-octan-1-ol (8:1, v/v), followed by centrifugation at 1000g for 10 min (Sevag, Lackmann & Smollens, 1938). This process was repeated until no white gel was formed on the addition of the chloroform-octan-1-ol mixture. The nucleic acid was precipitated with 96% ethanol overnight at -10°C. The precipitate was centrifuged, washed and dissolved in 0.15M-sodium acetate solution.

Determination of nucleic acid. A comparison was made of the E_{260} of the nucleic acid solution obtained from the tissue culture and solutions of known concentrations of yeast nucleic acid. The spectrophotometric readings were made with a Unicam SP.500 spectrophotometer.

Removal of salt from the nucleic acid solution. The nucleic acid solution was dialysed overnight against water. The nucleic acids were obtained by freeze-drying the residue left in the dialysis tubing.

Acid hydrolysis of nucleic acids. A sample of freeze-dried nucleic acid was introduced into a small glass tube (0.5 cm × 4 cm), and 0.05 ml of 98% (v/v) formic acid was added before the tube was sealed. Hydrolysis was carried out at 175°C for 30 min.

Chromatography of nucleic acid bases. The sealed tubes were opened and the formic acid was evaporated. The hydrolysate was dissolved in 0.05 ml of 1M-HCl and chromatographed on a Whatman no. 1 paper [solvent, propan-2-ol-conc. HCl-water (85:22:18, by vol.) (Wyatt, 1951)].

Caffeine. The methods for extracting the caffeine and the chromatographic method for its determination were as described by Ogutuga & Northcote (1970).

Scintillation fluid. This contained 2,5-diphenyloxazole (3.5g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50mg) dissolved in sulphur-free toluene (1 litre).

Determination of radioactivity of nucleic acid. A sample

(0.1 ml) of the nucleic acid solution (whose nucleic acid content had been determined) was introduced on to a Whatman glass-fibre paper (GF/C) and dried. To this was added 1 ml of scintillation fluid in a 20 ml Packard liquid-scintillation-spectrometer tube. Counting was carried out in a Nuclear-Chicago type L725 liquid-scintillation counter at 4°C. The specific radioactivity was expressed as c.p.m./mg of nucleic acid.

Determination of radioactivity of caffeine. After chromatography of caffeine extracts, strips (4 cm × 1 cm) were cut down the direction of run of each sample, such that the long axis of each strip was at right-angles to the direction of chromatographic movement of the sample. The strips were transferred to glass specimen tubes (5 cm × 1.3 cm), 0.5 ml of scintillation fluid was added to each and their radioactivities were measured on the scintillation counter. The total counts of the area corresponding to caffeine were determined. The caffeine content of an equal volume of sample was determined. The specific radioactivity was expressed as c.p.m./mg of caffeine.

RESULTS

The nucleic acids used in these experiments had a maximum extinction at 260–264 nm, and a minimum at 227–230 nm, at pH 6.0. The $E_{\max.}/E_{\min.}$ ratio in all the experiments was about 1.8.

The caffeine counted for radioactivity had the properties described by Ogutuga & Northcote (1970). Small amounts of 7-methylguanine, 7-methylxanthine and 3,7-dimethylxanthine (theobromine) were detected on the chromatograms on which the caffeine extract was run.

Incorporation of $^{14}\text{CO}_2$ into the nucleic acid and caffeine of the tissue culture. Four masses of callus tissue (0.9g fresh wt. each), harvested late in the

Fig. 1. Incorporation of radioactivity from $^{14}\text{CO}_2$ into nucleic acids (○) and caffeine (●) by tea callus tissue. The callus was incubated in a growth medium containing $\text{NaH}^{14}\text{CO}_3$ (5.0 $\mu\text{Ci/ml}$). Callus tissue (1g) contained 45 μg of nucleic acid and approx. 60 μg of caffeine.

Table 1. Incorporation of ^{14}C from $^{14}\text{CO}_2$ into the nucleic acids and caffeine of tea callus tissue

Tea callus tissue was incubated in a growth medium to which $\text{NaH}^{14}\text{CO}_3$ (5.0 Ci/ml) was added, for 5.5 h, followed by incubation for a further 3 h in a non-radioactive growth medium.

Time of incubation (h)	Incorporation of ^{14}C from $^{14}\text{CO}_2$	
	Into nucleic acids (c.p.m./g dry wt. of tissue)	Into caffeine (c.p.m./g dry wt. of tissue)
5.5	11500	2100
8.5	7200	5600

Fig. 2. Incorporation of radioactivity from L-[Me- ^{14}C]methionine into the nucleic acids (■), caffeine (○) and theobromine (●). The callus was incubated in a growth medium containing L-[Me- ^{14}C]methionine (6.6 $\mu\text{Ci/ml}$).

active growth phase (14 days from subculture), were transferred into each of six tubes containing 15 ml of sterile liquid growth medium and $\text{NaH}^{14}\text{CO}_3$ at a concentration of 5 $\mu\text{Ci/ml}$. At 1, 2.5, 5.5, 7.0 and 8.5 h after the beginning of the experiment, a tube was withdrawn and two of the callus tissues were extracted for nucleic acids, and the remaining two, plus the medium, were analysed for caffeine. The pattern of incorporation of the radioactive label into the nucleic acids and caffeine is shown in Fig. 1. After formic acid hydrolysis of the nucleic acids and chromatography, radioactivity was located in the purine rings.

Incorporation of $^{14}\text{CO}_2$ into the nucleic acid and caffeine followed by incubation of the tissue in non-

radioactive media ('pulse-chase' experiment). The experiment was similar to that described above, except that two tubes were used, each tube containing four masses of callus tissue. A tube was withdrawn for extraction of nucleic acids and caffeine from the callus tissue after 5.5 h. Callus tissue in the other tube was washed by transferring it into sterile liquid growth medium twice, before incubating in fresh non-radioactive medium for a further 3 h. Two callus tissue masses from each tube were extracted for nucleic acids and the remaining two, plus the medium used for incubation and washing, were extracted for caffeine. The pattern of incorporation into nucleic acids and caffeine is shown in Table 1.

Incorporation of L-[Me- ^{14}C]methionine into the nucleic acid and caffeine of the tissue culture. The procedure used in this experiment was the same as that described above. L-[Me- ^{14}C]methionine was added to the growth medium (6.6 $\mu\text{Ci/ml}$) and samples were taken after 1, 3, 5, 7 and 9 h. The pattern of incorporation of radioactivity into the nucleic acids, caffeine and theobromine is shown in Fig. 2.

Chromatograms of nucleic acid hydrolysates of tissues incubated with L-[Me- ^{14}C]methionine, when scanned for radioactivity, showed radioactive peaks (up to 80 c.p.m.) at the spot corresponding to authentic 7-methylguanine (R_F 0.35). Radioactive material was also detected at spots with R_F values of 0.7 and 0.8.

Incorporation of L-[Me- ^{14}C]methionine into the nucleic acid and caffeine followed by incubation of the tissue in non-radioactive media ('pulse-chase' experiment). The procedure used in this experiment was similar to that described above. Four tubes were used and the tissue was incubated with radioactive L-methionine for 3 h. After 3 h, the contents of one tube were extracted for determination of nucleic acids and of caffeine. The callus tissues in the other tubes were separately washed by shaking twice in fresh non-radioactive medium before incubation in non-radioactive medium. Samples were taken for nucleic acid and caffeine extraction after further incubation for 3, 5 and 20 h. Fig. 3 shows the pattern of incorporation of radioactivity into caffeine and nucleic acids.

DISCUSSION

The pathway of biosynthesis of caffeine is still not definitely known. Scheme 1 is a representation of two possible pathways. The pattern of incorporation of radioactivity into caffeine has indicated that pathway II is the more likely.

If caffeine synthesis results directly from a metabolic pool of purines, then when a radioactive precursor of the purine ring such as $^{14}\text{CO}_2$ is fed

Fig. 3. Incorporation of radioactivity from L-[Me-¹⁴C]-methionine into nucleic acid (○) and caffeine (●). The callus was incubated in a growth medium containing L-[Me-¹⁴C]methionine (6.6 μCi/ml) for 3 h (arrow) and was then transferred to a non-radioactive medium.

Scheme 1. Possible pathways of caffeine biosynthesis. The methylation steps of the particular N atom of the purine ring are indicated by 7Me-N, 1,3Me-N and 1,3,7Me-N.

continuously to the tissue, total radioactivity should continue to rise steadily in the caffeine; and in a 'chase' experiment there should be a decrease in the rate of incorporation during the 'chase' since the metabolic pool will be diluted with unlabelled purine precursors. A similar result should be obtained if the caffeine is derived from the nucleic acid as indicated in pathway II, since the radioactive precursor is steadily released and methylated to form caffeine.

When ¹⁴CO₂ was fed to the tissues, the pattern of incorporation into caffeine was similar to that predicted for either pathway I or II (Fig. 1 and Table 1). The appearance of the radioactivity in the caffeine was slower than that in the nucleic acid.

The fall in the radioactivity of nucleic acids after 5.5 h was probably due to the exhaustion of ¹⁴CO₂ in the incubation medium; the similar pattern of incorporation observed in the pulse experiment gave support to this view.

If a radioactive methyl-group donor is fed to the tissue, the pattern of incorporation will allow a distinction between the two pathways. If pathway I is operative, then in an experiment in which L-[Me-¹⁴C]methionine is fed continuously, incorporation of radioactivity should be fast and linear with time, whereas in a 'chase' experiment the amount of radioactivity should remain constant during the 'chase'. On the other hand, if pathway II is operative the pattern of incorporation will be biphasic. Incorporation should be initially slow while radioactivity is building up in the nucleic acid, and then it should rise when the nucleic acid has become saturated, whereas in a 'chase' experiment the radioactivity in the nucleic acids should fall during the 'chase' whereas that of caffeine should rise, since the methylated base that eventually gives rise to caffeine will still be available from the degradation of nucleic acid.

When L-[Me-¹⁴C]methionine was used as a precursor, the result with the 'chase' experiment strongly supported pathway II (Scheme 1) as the more likely route of caffeine formation (Fig. 3). During incubation in the non-radioactive medium, the radioactivity of the caffeine rose, but that in the nucleic acid fell. The additional radioactivity found in the caffeine was probably derived from the specific methylated purine precursor of caffeine that was released during nucleic acid turnover. The radioactivity of caffeine remained fairly constant when that of the nucleic acid did not show any further decrease (Fig. 3).

During the continuous feeding experiment with L-[Me-¹⁴C]methionine, there was an initial low rate of incorporation of radioactivity, which increased when the nucleic acid was saturated; theobromine, a suspected precursor of caffeine, gave a similar pattern. It has been shown that theobromine is only very slightly retained in the tissues (Ogutuga & Northcote, 1970). The incorporation of more radioactivity into theobromine than into caffeine may be because the amount of theobromine excreted into the medium exceeded the amount of theobromine that was methylated to caffeine in these experiments. Before the nucleic acid was fully saturated with the label, the methylated base released for caffeine formation should be a mixture of radioactive and non-radioactive material, and the proportion of the former will steadily increase as saturation is approached. This will lead to a slow but steady increase in the amount of label in the caffeine; but at saturation all the three methyl sites on the caffeine will carry radioactive labels, and thus

Scheme 2. Suggested routes of caffeine biosynthesis.

the rate of incorporation of radioactivity will increase (Fig. 2).

The results presented in this paper have confirmed that CO_2 is a precursor of the purine ring, and that L-methionine is a methyl-group donor in caffeine biosynthesis (Anderson & Gibbs, 1962).

Scheme 2 summarizes a hypothesis that has been developed from the experimental results available from this work and that of other investigations concerning the mechanism of caffeine biosynthesis.

Apart from the work reported in this paper, there are other results that support the route suggested for caffeine biosynthesis outlined in pathway II (Scheme 1) and in Scheme 2. (1) The withering of tea leaves in tea manufacture results in an increase in caffeine content (Wood & Chanda, 1955) and a net loss of RNA (Bhattacharyya & Ghosh, 1968). (2) Tea leaves infiltrated with yeast RNA produce more caffeine (Serenkov, 1962). (3) The rate of caffeine formation in an autolysing tea callus can

be increased by the addition of RNA (Ogutuga & Northcote, 1970).

It is now well established that the methylation of purine bases in nucleic acids takes place after the formation of the polynucleotide (Fleissner & Borek, 1963), so that the methylated bases are released during the turnover. By feeding 7-[Me- ^{14}C]-methylguanine to rats, Craddock, Mattocks & Magee (1968) have shown that it is not incorporated into nucleic acids, but excreted unchanged. We believe that N-7 of guanine is the first site of methylation. When the tea callus was incubated with L-[Me- ^{14}C]methionine and the nucleic acid was hydrolysed by formic acid, a radioactive peak corresponding in R_f to authentic 7-methylguanine was detected in the hydrolysate; both 7-methylguanine and 7-methylxanthine co-accumulate with theobromine and caffeine in the growth medium of tea callus. The 7-methylxanthine might have arisen after deamination of 7-methylguanine.

Another result suggesting that this conclusion is correct is the presence of 7-methylxanthosine in the supernatant of homogenized tea leaves centrifuged at 105 000g (Ogutuga, 1969).

Jones & Robins (1963) carried out the chemical methylation of purine nucleosides under physiological conditions (pH 5–6; 20°C). They showed that N-7 of guanosine and xanthosine and N-1 of adenosine and inosine were preferentially methylated. Anderson & Gibbs (1962) and Proiser & Serenkov (1963) degraded the radioactive caffeine obtained from feeding labelled methyl-group donors to coffee and tea leaves respectively. They showed that there was less methylation of N-7 than of N-1 and N-3, presumably because part of the caffeine resulted from the methylation of non-radioactive 7-methylguanine already released from the nucleic acids at the time of exposure to the radioactive precursors.

The formation of theobromine and caffeine occurs at the same time during the growth of the callus tissue. Both purines became labelled in a similar manner when radioactive precursors were fed to the tea callus. Theobromine and caffeine have a similar chemical structure, and they usually occur together in tissues such as tea callus, tea leaves, coffee beans and cocoa beans. All these facts suggest that they are formed through the same synthetic pathway.

The presence of theophylline (1,3-dimethylxanthine) in tea leaves (Michl & Haberler, 1954) cannot, however, be explained as the result of the methylation on a 7-N-methyl base released from the nucleic acid. Xanthine is known to be a catabolic product of purine metabolism (Scheme 2). Pullman & Pullman (1958) studied the most chemically reactive nitrogen atom of xanthine and other purine bases. They found that in xanthine the order of reactivity of the three nitrogen atoms of the ring was N-3 > N-7 > N-1. Presumably therefore the formation of theophylline could occur from xanthine if N-3 is methylated first, then N-1 (Scheme 2). Caffeine could then be formed from the dimethyl purine by methylation at N-7. However, if this pathway is operative it must only be a minor route since only very little theophylline is found in the tea leaf (Michl & Haberler, 1954).

The usual fate of purines derived from nucleic acid catabolism is further breakdown into uric acid, allantoin, allantoic acid, urea and CO₂. The form in which it is finally excreted depends on the presence or absence of the enzymes involved. The young

tea leaves in which caffeine normally accumulates have been shown by Kalberer (1964) to be incapable of breaking down radioactive caffeine into these excretory intermediates, whereas they were found in the older leaves in which the caffeine content is appreciably lower. It is possible that the methylating systems in plants that produce caffeine have been developed to cope with the catabolic purines. The methylation of the purines makes them more hydrophilic and therefore makes their excretion easier.

In conclusion, these experiments demonstrate that the turnover rate of caffeine and theobromine is very low.

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