UDC 582.823 : 581.134

200. **Takao Inoue and Fumiko Adachi** : Studies on Biogenesis of Tea Components. III.[1] The Origin of the Methyl Groups in Caffeine.[*1]

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Recently it has been proved, for various alkaloids,[2~5] that their N- and O-methyl groups take place by transmethylation from methionine as well as utilization of some active 1-carbon compounds, while Byerrum, *et al.*[6,7] demonstrated that methyl groups were transferred to nicotine and methoxy groups of lignin, as a whole, by feeding with doubly labelled methionine-(methyl-$^{14}CD_3$). These findings show that the transmethylation reaction generally arises from methionine in higher plants.

As to the caffeine biosynthesis in plant, Bonner[8] presumed that xanthine was methylated step by step through the way of heteroxanthine and theobromine. It seems that this suggestion is probably based on natural occurrence of heteroxanthine and observation of Weevers that theobromine changed to caffeine in *Theobroma* germinating in the dark, but there is no precise proof of such a metabolic pathway.

It is, therefore, of interest to confirm the utilization of methionine for three methyl groups of caffeine. As it was shown that caffeine formed in tea leaves,[1] in the present work methionine-(methyl-^{14}C) was administered with the nutrient solution to excised tea shoots and after feeding for a week the radioactivities of the degradation products of isolated caffeine and the derivatives of the methyl groups obtained from them were compared.

Experimental

Administration of Methionine-(methyl-^{14}C)——*Thea sinensis* L. grown in Tea Experimental Station, Saitama Prefecture, was used as a plant material. The culture solution, recommended by Yoshie,[9] which is most suitable to the hydroponic cultivation of tea plant, was prepared and methionin-(methyl-^{14}C) was added to the solution (Table I). The 120 tea shoots, 20 cm. long, were excised on May 16, 1960 and immediately dipped in the culture solution. The cultivation lasted for a week under lighting in a draught chamber. At the end of cultivation, the material was removed from the solution, washed sufficiently with H_2O and dried. The amount of the solution absorbed was 480 ml. Dried wt. : 51 g.

Caffeine was isolated by the method described in the previous paper.[10] Yield, 0.65 g. (1.27 % of

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[*1] Reported at 81th Annual Meeting of Pharmaceutical Society of Japan (July, 1961). After the completion of manuscript of this report, the authors found that L. Anderson reported on the incorporation of methionine-(CH_3-^{14}C) to methyl groups of caffeine in "the biosynthesis of caffeine" using Coffea leaves, in 41th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic city (April, 1961); cf. Fed. Proc., **20**, 374 (1961).

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Table I. Components of Culture Solution

	(mg.)		(mg.)
$Ca(NO_3)_2$	29.9	$MgSO_4$	43.9
$(NH_4)_2SO_4$	24.1	$C_6H_5O_7\cdot Fe$	15.3
KH_2PO_4	24.8	DL-Methionine-(methyl-^{14}C)*3 (total activity 0.3 mc.)	
K_2SO_4	111.3		44.0

in 1 L. distilled H_2O; pH 4.5

dried wt.). Caffeine gave a single radioactive spot at Rf 0.75 on paper chromatogram (BuOH-AcOH-H_2O=5:1:4).

Degradation Procedure——Degradation of caffeine was carried out as shown in Chart 1.

(1) **Decomposition to Amalic Acid and Methylurea**——After radioactive caffeine (0.5 g.) from the plant material was diluted to 10 times by adding inactive caffeine (4.5 g.) as carrier, caffeine was decomposed to amalic acid and methylurea according to the method described by Fischer.[11]

To a mixture of caffeine (5 g.), conc. HCl (7 g.) and H_2O (15 ml.), $KClO_3$ (2.3 g.) was added in small portions at 50° with continuous stirring during 2.5 hr. when chlorocaffeine first separated out from the solution disappeared to complete solution. After cooling, the mixture was diluted with the same volume of H_2O and was saturated with H_2S. Amalic acid deposited along with S was filtered, extracted with large amount of hot H_2O and allowed to crystallize. Amalic acid was recrystallized from hot H_2O. Yield, 0.85 g. (19.3 %). *Anal.* Calcd. for $C_{12}H_{14}O_8N_4$: N, 16.37. Found : 16.03.

The mother liquor left after separation of amalic acid and S was neutralized with basic lead carbonate and concentrated in a reduced pressure to give a syrupy residue which on addition of conc. HNO_3 afforded crystals of methylurea nitrate. These were filtered, washed with small amount of cold EtOH and recrystallized from EtOH. The aqueous solution of nitrate was neutralized with $Ba(OH)_2$, the excess of Ba was removed with CO_2 gas and evaporated to dryness in a reduced pressure. From the residue methylurea was extracted with EtOH, crystallized by the addition of Et_2O to the concentrated solution and recrystallized from EtOH, m.p. 101°. Yield, 0.47 g. (24.7 %).

(2) **Demethylation of Amalic Acid and Methylurea**——Procedure adopted for demethylation of nicotine by Brown and Byerrum[2] was employed to obtain the solid derivatives of methyl groups of amalic acid and methylurea. Apparatus for estimation of N-methyl or N-ethyl groups by Furter[12] was prepared, amalic acid or methylurea were treated with HI and MeI obtained was absorbed into a solution of Et_3N in EtOH cooled in a Me_2CO-dry ice bath. Then, methyl groups of amalic acid and methylurea gave triethylmethylammonium iodide. Yield, 43.5 mg. (A) from amalic acid 35 mg. (43.8 %); 75.5 mg. (B) from methylurea 31 mg. (74.2 %). *Anal.**4 Calcd. for $C_7H_{18}NI$: I, 52.20. Found : 52.50 (A), 52.71 (B).

Determination of Radioactivity——The radioactivity of all products was determined with a "Q"-gas flow counter (Windowless Flow Counter SC-16, Tracerlab Inc.) attached to the scaler (Superscaler SC-18A, Tracerlab Inc.). Methylurea was estimated as its nitrate (m.p. 127°) after varying to nitrate by the addition of HNO_3, because of the deliquescent property.

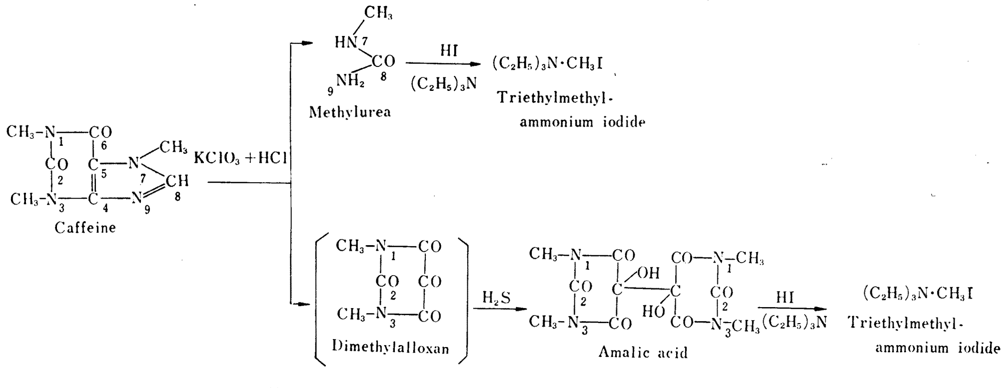

Chart 1. Degradation Procedure of Caffeine

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*3 Supplied from Daiichi Pure Chemicals Co. Ltd.

*4 Determined by method of Uyeo and Sakamoto (Yakugaku Zasshi, 58, 711 (1938)).

11) E. Fischer : Ann., **215**, 257 (1882).

12) M. Furter : Helv. Chim. Acta, **21**, 1144 (1938).

Results and Consideration

The radioactivity of caffeine isolated from the plant material was 1.13×10^6 c.p.m./m mole and it showed considerably rapid incorporation of methyl group of methionine to caffeine formed in excised tea shoots.

Results obtained by present work are shown in Table II. As there was no suitable method to separate the methyl groups in N_1 and N_3, they were taken out together, but in view of the approximate activity of two quaternary iodides from amalic acid and methylurea it is probable that the incorporation to three methyl groups occurred at the same rate in a week's cultivation. As shown in Table II, the radioactivity of caffeine was almost served from its three methyl groups, indicating that methyl group of methionine was utilized as a precursor of the methyl groups of caffeine through the transmethylation reaction between xanthine and methionine. However, very little amount of radioactivity may incorporate to other carbon besides the methyl groups of caffeine.

Table II. Radioactivity of Degradation Products

Position of CH_3		c.p.s./mole	Quaternary iodide c.p.m./mole	Recovery[a] of counts (%)
$\frac{1}{2}(N_1+N_3)$	Amalic acid	13.00×10^4	2.73×10^4	84.0
N_7	Methylurea nitrate	2.88×10^4	2.43×10^4	84.4

a) Amalic acid was noted to have 4 CH_3-groups.

Mackenzie,[13] and Weinhouse and Friedmann[14] demonstrated that labile methyl groups of methionine and sarcosine was oxidized to formate and finally to carbon dioxide in animal tissues. Buchanan, *et al.*[15] has etablished that both of C_2 and C_8 of uric acid originated from formate in studies on purine biosynthesis, which seems to arise in higher plants similarly to animal bodies. By these results, the possibility that methyl carbon of methionine might partly participate in C_2 and C_8 of caffeine was considered, though it was not ascertained in this case.

The authors wish to thank Prof. S. Shibata, University of Tokyo, for his kind help and valuable advice. The authors are indebted to Mr. H. Yoshida, Tea Experimental Station, Saitama Prefecture, for supplying tea plants used in this work and to the members of Radioisotope Laboratory, National Institute of Hygienic Sciences, for their kind help in determination of radioactivity. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, to which the authors are grateful.

Summary

By feeding tea shoots with methionine-(methyl-^{14}C) it was proved that the methyl group of methionine served as a precursor of the methyl groups of caffeine in tea plants.

(Received December 22, 1961)

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[13] C.G. Mackenzie: J. Biol. Chem., **186**, 351 (1950).

[14] S. Weinhouse, B. Friedmann: *Ibid.*, **197**, 733 (1952).

[15] J.C. Sonne, J.M. Buchanan, A.M. Delluva: *Ibid.*, **173**, 69 (1948); J.M. Buchanan, J.C. Sonne, A.M. Delluva: *ibid.*, **173**, 81 (1948).