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A Novel Pathway for the Metabolism of Caffeine by a Mixed Culture Consortium

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A new oxidative pathway for the degradation of caffeine(1,3,7-Trimethylxanthine, I) by a mixed culture consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* is presented. The mixed culture does not initiate degradation by N-demethylation either complete or partial, but instead carries out oxidation at the C-8 position resulting in the formation of 1,3,7-trimethyluric acid (TMU, II) which further gets degraded to 3,6,8-trimethylallantoin (TMA, III). Both TMU and TMA are hitherto not shown to be formed in the microbial system. Further degradation of TMA (III) by caffeine grown cells yields dimethylurea (VII) as one of the metabolites. Oxygen uptake studies indicated that caffeine(I) grown cells oxidized TMU(II), TMA (III), glyoxalic acid (VI), dimethylurea(VII), and monomethylurea(V), but not monomethyl and dimethyluric acids. The mixed culture does not accept theophylline(1,3-dimethylxanthine), theobromine(3,7-dimethylxanthine), and paraxanthine(1,7-dimethylxanthine) as the carbon source.

Key Words: **mixed culture; caffeine metabolism; new pathway; absence of N-demethylation; C-8 oxidation; trimethyluric acid; trimethylallantoin.**

The metabolism of caffeine(I, 1,3,7-trimethylxanthine) and related methylated xanthines has been investigated in both microbes and mammals (1,2). Caffeine(I) is generally toxic to bacteria although the concentration required is often relatively high(3). A bacterium capable of utilizing caffeine(I) as the sole source of carbon has been isolated from soil and these studies indicated that the organism carries out partial or complete N-demethylation followed by oxidation at the C-8 position to produce the corresponding uric acids (4,5). Infact caffeine is N-demethylated in two parallel ways via theobromine(3,7-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine), the former appears to be the major pathway for degradation (5). Uric acid is degraded to allantoin and then to allantoic acid. Further degradtion of allantoic acid results in the formation of urea and glyoxalic acid as the end products of caffeine metabolism(5). Infact animals and plants are also known to convert allantoin to glyoxalic acid and urea via allantoic acid(6). Interestingly, the degradation of caffeine(I) in fungi takes places via theophylline(1,3-dimethylxanthine) unlike in bacteria(7).

Mammals metabolize caffeine(I) via two common metabolic reactions, the N-demethylation and the oxidation of the C-8 carbon on the purine ring (8-10). Mammals are also known to metabolize caffeine(I) to 1,3,7-trimethyluric acid (II) which further gets oxidized to 3,6,8-trimethylallantoin (III) (8). Studies carried out *in vitro* have shown the conversion of caffeine (I) to II and substituted diaminouracil derivative(11).

In this communciation we report the isolation of a mixed culture consortium consisting of strains belonging to the genus *Klebsiella* and *Rhodococcus,* capable of complete mineralizatin of caffeine(I). This mixed culture without carrying out N-demethylation degrades caffeine(I) following a pathway hitherto not known in the microbial system.

MATERIALS AND METHODS

The mixed culture was isolated from soil using caffeine(I) as the sole source of carbon. It was shown to be a mixed populations of two strains belonging to the genus *Klebsiella* and *Rhodococcus*. The mixed culture was maintained at 3°C on nutrient agar slants containing 0.3% of I. It was also maintained in a liquid mineral salts medium(12) containing I (0.05%) and glucose(0.1%) and incubated aerobically at 29-30°C. Although the mixed culture accepts I as the sole source of carbon, the growth rate is slow. Hence to increase the growth rate, glucose(0.1%) was added to the medium.

Incubation conditions. Degradation experiments were conducted in 500ml Erlenmeyer flasks containing 100ml sterile salts medium (pH 7.2), glucose (0.1%) and I (0.05%) were inoculated from 36 h-

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grown culture (5.0ml, A_{660}=1.1) and incubated at 29-30°C on a rotary shaker for 36 h. At the end of the incubation period, the contents from all the flasks were pooled, acidified to pH 4-5 and extracted with $CHCl_3$:CH_3OH (2:1)

Metabolism of caffeine(I) by resting cells (i) *without inhibitor and* (ii) *with inhibitor.* Cells grown on I for 36 h were harvested and washed with phosphate buffer (0.03M, pH7.2). The washed cells (2.6g wet weight) were suspended in the same buffer (100ml) and divided into two portions. To this cell suspension (50ml) (i) 100mg of I was added for the experiment without inhibitor and (ii) for the experiment with inhibitor, I (100mg) addition was followed by the addition of N-methylmaleimide (0.01mM). The flasks were incubated at 29-30°C on a rotary shaker for 10 h. Fixed aliquots at definite intervals were withdrawn, centrifuged and the supernatant were subjected to HPLC analysis. To isolate the metabolite in large quantity, the experiment with the inhibitor was repeated. At the end of the incubation period(10 h), the contents were acidified to pH 4-5, extracted with $CHCl_3$:CH_3OH(2:1), the organic layer evaporated and the residue was subjected to column chromatography over silica gel using 3% CH_3OH in $CHCl_3$ as the eluent.

Metabolism of 1,3,7-trimethyluric acid (TMU, II) by resting cells. Resting cells (1.5g, wet weight) suspended in phosphate buffer (0.03M, pH7.2, 50ml) were incubated with TMU(II,100mg) at 29-30°C for 6 h. At regular intervals, aliquots were withdrawn, centrifuged and the supernatant were subjected to HPLC analysis. For isolating the metabolite, the above experiment was repeated and the mixture was incubated only for 2h. At the end of 2h, the incubation mixture was lyophillized and extracted with CH_3OH.

Metabolism of 3,6,8-trimethylallantoin (TMA,III). Resting cells (0.8g, wet weight) suspended in phosphate buffer (0.03M, pH7.2, 25ml) were incubated with TMA(5.0mg) at 29-30°C for 1h. At the end of 1h, the incubation mixture was lyophilized, extracted with CH_3OH and the organic layer was subjected to TLC and mass spectral anyalysis.

Manometric studies. Oxygen consumption was measured with a Gilson 5/6 oxygraph at 30°C using Caffeine(I) grown cells (36 h). Reaction mixture contained freshly washed cells (2mg , dryweight), phosphate buffer (0.03M, pH7.2) and substrate (0.5μ mole in 10 μl of buffer) in a total volume of 2.0ml.

Analytical methods. Thin layer chrmatographic(TLC) analyses were performed on silica gel GF_{254} plates (0.5mm) developed with $CHCl_3$-CH_3OH (85:15, system I) as the solvent system. Compounds were visualized by exposing the plates to UV-light or I_2 vapour. HPLC analysis was carried out on Shimadzu CR &A instrument using ODS reverse phase column with NaOAC(0.1M)-CH_3OH-CH_3CN (70:20:10, v/v) as the solvent system (1ml/min) and eluents were monitored with a UV-detector at 254nm.

RESULTS

Time course studies on the fermentation of caffeine (I) indicated that the growth reached a log phase in 18 h and I was completely metabolized in 36 h (as judged by HPLC analysis). Extraction of the incubation medium at the end of 36 h with $CHCl_3$:CH_3OH (2:1) and subjecting the organic layer to HPLC analysis revealed the presence of a metabolite in extremely low levels (Rt 4.5 min) which was enhanced when mixed with the authentic 1,3,7-trimethyluric acid (TMU, II). Infact the amount of metabolites isolated from a large scale incubation (30 flasks) of I with mixed culture for 36 h was very little which could not be processed further. Therefore all the experiments were carried out using resting cells.

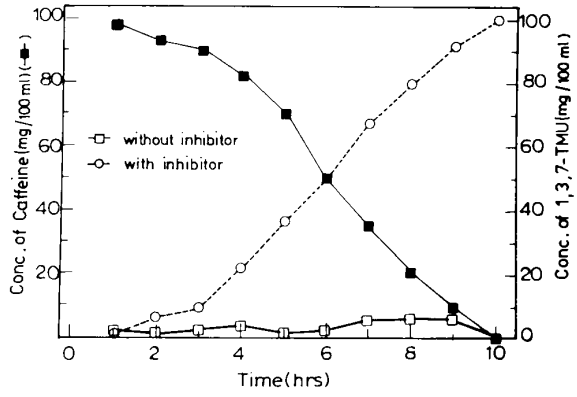

FIG. 1. Time course experiment: metabolism of caffeine (**I**) by resting cells (i) in the presence of inhibitor (N-methylmaleimide, 0.01mM), — ○ — ○ — represents concentration of TMU (**II**), and (ii) in the absence of inhibitor [— □ — □ —, level of TMU (**II**)]. — ■ — ■ — represents the concentration of caffeine. Experimental details are mentioned in text.

Transformation of I carried out with resting cells in the presence of N-methylmaleimide showed that the level of I dropped gradually with the concomitant increase in the level of TMU (II, Fig.1). At the end of 10 h, all the I was converted into II and no other metabolite was seen during this period. In the absence of inhibitor, accumulation of II in the medium was not noticed (Fig.1). Formation of II was confirmed by performing a large scale incubation of I with resting cells in the presence of inhibitor for 10 h and from the incubation medium a metabolite (Rf 0.69, system I) was isolated in almost quantitative yield (≈98% isolated yield). This metabolite (II) was identified as 1,3,7-trimethyluric acid(II) by comparison of its NMR and mass spectral data with the corresponding authentic sample. The spectral data is also in agreement with the earlier report for this compound (13).

Transformation of TMU (II) by resting cells: The ability of caffeine grown cells to metabolize TMU(II) is shown in Fig. 2. HPLC analysis of the incubation medium indicated the formation of metabolite (Rt 1.2 min) in low levels (Fig. 2). This polar metabolite (Rf. 0.22 system I) isolated (10mg) by preparative TLC gave positive reaction to the Young-Conway test (14) which suggested that it is a derivative of allantoin. The metabolite had the following spectral characteristics: IR spectrum (Nujol)ν_{max}: 3400-3300 cm^{-1} (Secondary amide), 1675cm^{-1} (carbonyl), 1622 and 1526 cm^{-1} (amide group); ^{1}H NMR (CF_3COOD, 400 MHz, Fig.3) δ: 3.92 [3H, S, $NHCH_3$ (8)], 3.85 [3H, s, NCH_3 (6)], 3.79 [3H, s, NCH_3 (3)] and 5.76 (accounts less than one H, s, C-5); ^{13}C NMR (100 MHz, CF_3COOD): 157.357 (C-4), 153.68 (C-2), 139.435 (C-7), 103 (C-5), 32.93[$NHCH_3$ (8)], 30.76 [NCH_3 (6)], 30.58[NCH_3 (3)]; mass spectra

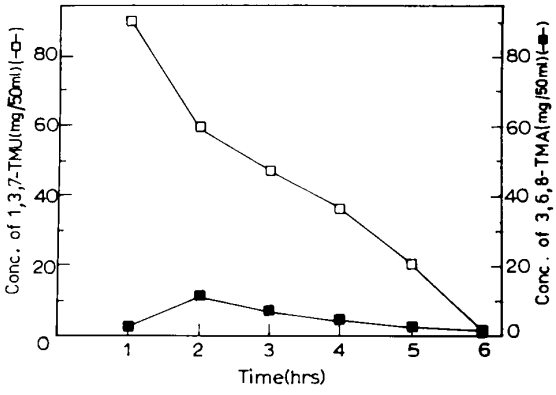

FIG. 2. Time course experiment: metabolism of TMU (**II**) — □ — □ — by resting cells. — ■ — ■ — represents concentration of TMA (**III**). Details are as mentioned in the text.

(Fig.3, inset): m/z 200 (M^+, 50%), 142 (M^+-$CONHCH_3$, basepeak), 127 [(M^+-$CONHCH_3$)-CH_3, 30%], 111[(M^+-$CONHCH_3$)-NCH_3, 20%] and 58($CONHCH_3$, 95%). From the spectral characteristics, the compound was identified as 3,6,8-trimethylallantoin (TMA, III).

Resting cells experiment carried out with TMA (III) as described under Methods and analysis of the organic extract of the incubation medium by GC-MS revealed the presence of a metabolite with the molecular mass peak at 88 and the fragmentation pattern identical with that of authentic 1,3-dimethylurea (VII). To obtain corrobarative evidence for the pathway of degradation of caffeine by the mixed culture, oxygen uptake studies were carried out with metabolites isolated from the culture media as well as synthetic probable intermediates (Table I). These studies indicated that compounds II, III,V,VI and VII showed comparable oxygen uptake whereas monomethyluric acids and 1,3-, 3,7- and 1,7-dimethyluric acids were not oxidized by the caffeine (I) grown cells (Table I).

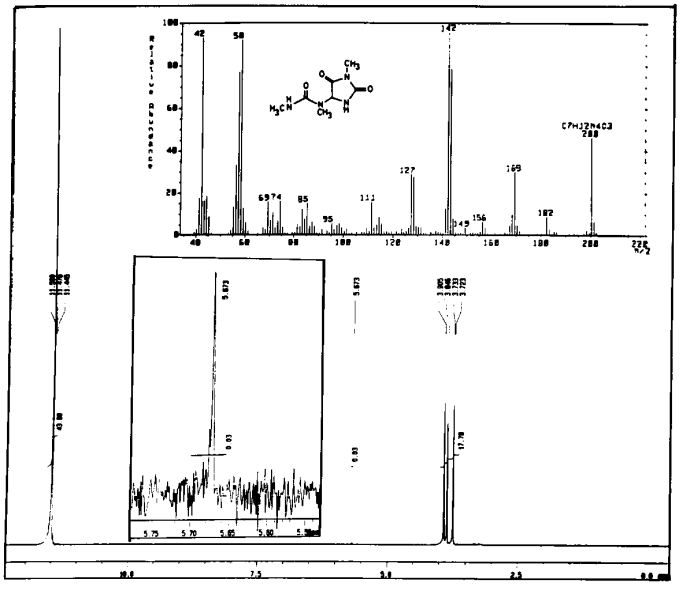

FIG. 3. ^{1}HNMR spectra and electron impact mass spectra (inset) of 3,6,8-trimethylallantoin TMA (**III**).

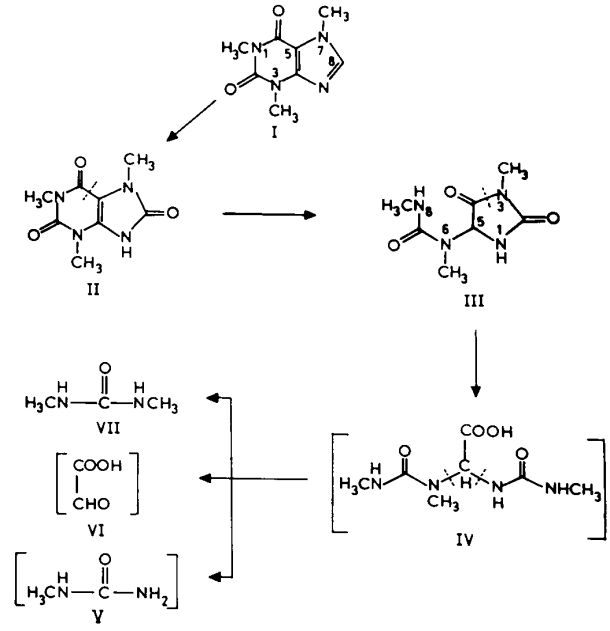

FIG. 4. Proposed pathway for the metabolism of caffeine (**I**) by a mixed culture.

DISCUSSION

We report for the first time the metabolism of caffeine (I) using a mixed culture. The present study has clearly established that the bacterial system metabolizes I fol-

TABLE I

Oxygen Uptake by Mixed Culture in the Presence of Various Substrates[a]

Substrates	Oxygen consumed[b] (nmol/min/mg dry weight)
Caffeine (I)	15.68
1,3,7-TMU (II)	13.44
3,6,8-TMA (III)	16.80
Mono methyl urea (V)	10.08
Dimethyl Urea (VII)	6.72
Glyoxalic acid (VI)	12.32
1,3-dimethyluric acid	0
3,7-dimethyluric acid	0
1,7-dimethyluric acid	0
1-methyluric acid	0
3-methyluric acid	0

[a] Experimental details are described in text.
[b] All values were corrected for endogenous respiration.

lowing a pathway hitherto not known. Caffeine (I) degrading microorganisms are known to posses both N-demethylation and C-8 oxidation activities (4-7). Caffeine (I) is also metabolized extensively by mammals and N-demethylation is the predominant pathway (8-11). However, present studies have demonstrated that the mixed culture does not carry out N-demethylation, but instead initiates the degradation by C-8 oxidation to yield 1,3,7-trimethyluric acid (TMU, II). Infact TMU (II) accumulates in the medium (Fig. 1) in the presence of N-methylmaleimide since it is known that thio-group blocking reagents inhibit urate oxidase (15). It is interesting to note that monomethyluric acids and dimethyluric acids are not oxidized by caffeine grown cells (Table I) indicating that the urate oxidase has a rigid substrate specificity. This also suggests that N-demethylation does not take place after the formation of TMU (II). TMU (II) has never been shown as one of the metabolites of I in the microbial system although its formation in the mammalian system has been established (8).

It appears that the mixed culture degrades TMU(II) through oxidative cleavage of the pyrimidine ring between N(1) and C(6) yielding 3,6,8-trimethylallantoin (TMA, III), a compound never been shown to be formed in the microbial system. However, one of the minor metabolites isolated and tentatively identified from the urine of rats treated with caffeine(I) was TMA(III) (8). In fact this minor urinary metabolite was characterized based only on mass spectral fragmentation pattern (8). In the present study, we have isolated TMA (III) in quantities sufficient for detailed spectral analysis (^{1}H NMR, IR and MS). In the ^{1}H NMR spectrum, the proton at C-5 does not account fully for one proton mainly because of the enolisation of carbonyl at C-4 at the cost of proton at C-5. Mammals also metabolize TMA(III) by cleaving the imidiazole ring between C(8) and N(9) which leads to the formation of substituted diaminouracil derivative (11).

CONCLUSIONS

The mixed culture which utilizes caffeine (I) as the sole source of carbon initiates degradation by C-8 oxidation to yield TMU (II) which further gets oxidized to TMA (III). It is reasonable to assume that the mixed culture oxidizes TMA (III) to 1,6,8-trimethylallantoic acid(IV) which upon hydrolysis yield polar and water soluble metabolites such as glyoxalic acid(VI), mono-(V) and dimethyl urea (VII). This assumption stems from the fact that VII has been shown as one of the metabolites of TMA (III) and compounds V, VI and VII are oxidized by caffeine grown cells (Table I). Based on these evidences, a new pathway for the metabolism of caffeine (I) in the mixed culture has been proposed (Fig.4). Our results clearly suggest that in certain respects, the metabolism of caffeine (I) in the mixed culture resembles the way it gets degraded in the mammalian system. Unlike all the earlier reports on the microbial degradation of caffeine (I), the mixed culture used in the present study does not carry out N-demethylation during the mineralization of I.

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