

The Effect of Daily Ingestion of Caffeine on the Microsomal Enzymes of Rat Liver*

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Abstract—The effect of repeated ingestion of caffeine on rat-liver microsomal enzymes was studied. In the rat, ingestion of 20–24 mg caffeine/kg/day in the drinking water for more than 2 wk inhibited aminopyrine *N*-demethylase activity. This loss of activity approached 60% at the end of an 8-wk dosing schedule. Acetanilide-hydroxylating activity of rat-liver microsomal enzyme was initially stimulated, with a peak ($145 \pm 11\%$ of control) at 2 wk, and returned to normal levels at the end of 8 wk. Caffeine-treated rats did not respond to the phenobarbitone stimulation of microsomal enzymes to the same extent as did rats treated with phenobarbitone alone.

INTRODUCTION

In addition to the caffeine in coffee, tea and other beverages, caffeine is present in many commercial preparations consumed by the general public (Gleason, Gosselin, Hodge & Smith, 1969), so that substantial amounts of caffeine are ingested by large numbers of people. Caffeine, a xanthine alkaloid, has several pharmacological and biochemical effects, including stimulation of the central nervous system, diuresis (Truitt, 1965), mutagenesis (Ostertag, Duisberg & Stürmann, 1965), inhibition of phosphodiesterase (Beavo, Rogers, Crofford, Baird, Hardman, Sutherland & Newman, 1971) and inhibition of DNA polymerase (Wragg, Carr & Ross, 1967). Psychotropic effects and tolerance to caffeine have also been reported (Colton, Gosselin & Smith, 1968; Goldstein, Kaizer & Whitby, 1969).

Caffeine is readily absorbed following ingestion and is distributed in the body according to the water content of the tissues (Bertoli, Dragoni & Rodari, 1968). It is metabolized in mammals by demethylation to methylxanthines and to methyluric acids by oxidation at the C-8 position (Cornish & Christman, 1957; Khanna, Rao & Cornish, 1972; Parke, 1968).

Acute doses of caffeine have been shown to stimulate hepatic, drug-metabolizing microsomal enzymes in the rat (Mitoma, Sorich & Neubauer, 1968). It has been suggested (Lombrozo & Mitoma, 1970) that the effect of caffeine on cytochrome P₄₅₀ is similar to that of 3-methylcholanthrene. Other than a single reference (Cornish, Wilson & Abar, 1970), reporting that in rats treated with caffeine for 6 wk *N*-dealkylation activity was 66% of that in controls, no information was evident in the literature concerning the effect of repeated ingestion of caffeine on drug-metabolizing microsomal enzymes. Since caffeine is consumed by the public in large quantities, the present study on the effect of prolonged ingestion of caffeine on microsomal enzymes was undertaken.

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EXPERIMENTAL

Animals and maintenance. Male rats of the Sprague-Dawley strain, weighing 250–300 g, were used for these studies. They were fed Rockland Rat Chow *ad lib.* and housed in stainless-steel cages.

Conduct of experiments. Caffeine was administered in the drinking-water (0.2 mg/ml), the daily dose being calculated on the basis of a daily consumption of 25–30 ml water/rat. In the first experiment, groups of rats (six treated animals and two controls) were killed at intervals between 4 and 56 days from the start of caffeine administration for hepatic microsomal-enzyme determinations. In the second study, caffeine was administered for 6 wk, on the last 3 days of which sodium phenobarbitone (25 mg/kg/day) was injected ip into some of the treated and control animals. Enzyme induction of the liver microsomal enzymes was determined by measurement of acetanilide-hydroxylation and aminopyrine-*N*-demethylation activities. Values in the caffeine/phenobarbitone-treated rats were compared with those in rats treated only with caffeine or only with phenobarbitone and with those in untreated rats of the same age. In the third study, caffeine was administered for 14 wk, and phenobarbitone (25 mg/kg) was injected ip on the last 3 days of treatment. Animals were killed 24 hr after the last dose for the determination of hepatic microsomal levels of cytochrome P₄₅₀ and cytochrome b₅.

Fractionation of liver samples. The rats were anaesthetized with ether and killed by open-chest surgery. They were bled by heart puncture with the aid of vacutainers. Liver samples (5 g) were immediately frozen on dry ice and stored at –20°C before use. Each 5 g sample was homogenized in 10 ml 0.05 M-tris buffer (pH 7.4) containing 1.15% KCl by means of glass homogenizers and a teflon pestle fitted to a mechanical drill press. The homogenate was centrifuged at 13,000 g in a Sorvall refrigerated high-speed centrifuge. This supernatant was used for microsomal-enzyme analysis.

Enzyme and other analyses. The incubation mixture (total volume 5 ml) contained supernatant (1 ml), substrates (17.8 μM acetanilide for hydroxylation and 3.5 μM aminopyrine for demethylation) and the following co-factors: glucose 6-phosphate (25 μM), NADP (2.25 μM), nicotinamide (20 μM), NAD (2.25 μM) and magnesium sulphate (12 μM) in 4 ml 0.1 M-phosphate buffer (pH 7.4). This mixture was incubated under oxygen for 20 min in 25 ml Erlenmeyer flasks using a Dubnoff shaking incubator at 37°C. For *N*-demethylation studies, aminopyrine was used as a substrate and 4-aminoantipyrine was measured as the reaction product by the method outlined by Brodie & Axelrod (1950). Hydroxylation activity was determined essentially by the method of Brodie & Axelrod (1948), using acetanilide as a substrate. The enzyme activity was expressed as μg product formed/g liver/20 min. Cytochrome P₄₅₀ and cytochrome b₅ values were determined by the method of Omura & Sato (1964) and expressed as nmoles/mg protein. A Beckman DK-2A Spectrophotometer was used for absorbancy measurements. Protein concentrations were measured as described by Lowry, Rosebrough, Farr & Randall (1951). The Student's *t* test was used for statistical evaluation of the results.

RESULTS

It was established that the average increase (233 ± 24 g) in the body weight of rats given caffeine (20–24 mg/kg/day) orally for a period of 12 wk was essentially the same as that of controls (202 ± 49 g). The liver weight/100 g body weight (3.58 ± 0.11 g) of rats fed caffeine was also comparable with that of untreated rats (3.67 ± 0.12 g).

Table 1 shows the effect of daily caffeine ingestion (20–24 mg/kg) on the mixed-function drug-metabolizing microsomal enzymes of the livers of rats killed at intervals of 4–56 days

Table 1. *Hepatic microsomal-enzyme activity in rats given 20–24 mg caffeine/kg/day in the drinking-water*

Duration of dosing (days)	Acetanilide hydroxylation (% of control)*	Aminopyrine <i>N</i> -demethylation (% of control)*
4	123 ± 4	115 ± 7
8	127 ± 12	104 ± 11
14	145 ± 11	90 ± 6
28	139 ± 7	73 ± 3
56	93 ± 9	38 ± 5

*Values are means for six treated rats at each sacrifice period ±SEM. Two control rats were killed at each time.

from the start of treatment. Acetanilide hydroxylation and aminopyrine *N*-demethylation activities of these enzymes appeared to be minimally stimulated after a period of 4 days to approximately the same level (hydroxylation 123 ± 4% and *N*-demethylation 115 ± 7% of controls). The hydroxylation activity of these enzymes increased gradually with time, having a peak (145 ± 11% of control) at 2 wk and returning essentially to control level at 8 wk (93 ± 9%). In contrast, the mean activity of the *N*-demethylating enzyme showed only a slight initial elevation, with values declining over the subsequent study period. A marked decrease in *N*-demethylation activity was observed after 4 wk (73 ± 3% of control, $P < 0.05$). This loss of activity progressed as treatment continued, the *N*-demethylation enzyme levels of caffeine-treated animals being only 38 ± 5% of that of controls at the end of the 8-wk period.

In the second study, the phenobarbitone-treated animals showed the highest levels of acetanilide-hydroxylating activity (200 ± 13%) and aminopyrine *N*-demethylating activity (186 ± 19%), whereas animals treated with caffeine plus phenobarbitone had enzyme activities of 157 ± 12 and 140 ± 9% respectively (Table 2). This suggests that caffeine-treated animals were not able to respond to the phenobarbitone treatment to the same extent as did animals treated only with phenobarbitone.

Table 2. *Effect of phenobarbitone on the hepatic microsomal enzymes of rats given 20–24 mg caffeine/kg/day in the drinking-water for 6 wk*

Treatment	No. of animals/group	Acetanilide hydroxylation (% of control)*	Aminopyrine <i>N</i> -demethylation (% of control)*
None	3	100 ± 8	100 ± 5
Caffeine	6	110 ± 12	80 ± 11
Phenobarbitone†	5	200 ± 13	186 ± 19
Phenobarbitone† + caffeine	6	157 ± 12	140 ± 9

*Values are means for the numbers of animals shown ±SEM.

†Phenobarbitone (25 mg/kg) was injected ip on each of the last 3 days of the caffeine treatment.

Cytochrome P₄₅₀ and cytochrome b₅ values of rats fed caffeine for 14 wk are given in Table 3. Rats dosed orally with caffeine (20–24 mg/kg) daily for 14 wk showed no change

Table 3. *Effect of phenobarbitone administration on cytochrome P₄₅₀ and b₅ levels of hepatic microsomes of rats given 20–24 mg caffeine/kg/day for 14 wk*

Treatment	No. of animals/group	Cytochrome P ₄₅₀ (nmoles/mg protein)*	Cytochrome b ₅ (nmoles/mg protein)*
None	4	1.10 ± 0.09	1.09 ± 0.04
Caffeine	6	1.13 ± 0.05	0.98 ± 0.06
Caffeine + phenobarbitone†	6	1.60 ± 0.11	0.74 ± 0.05

*Values are means for the numbers of animals shown ± SEM.

†Phenobarbitone (25 mg/kg) was injected ip on each of the last 3 days of caffeine treatment. The animals were sacrificed 24 hr after the last dose.

in their mean cytochrome P₄₅₀ or cytochrome b₅ values when compared with the controls. However, caffeine-treated rats subsequently administered sodium phenobarbitone (25 mg/kg, ip) for the last 3 days of the treatment showed an increase in P₄₅₀ level in comparison with both controls and caffeine-dosed animals. Cytochrome b₅ levels were essentially the same in caffeine-treated animals and in controls. In contrast to cytochrome P₄₅₀ values, however, cytochrome b₅ levels were decreased in rats given both caffeine and phenobarbitone. The decrease was statistically significant ($P < 0.01$) when compared with caffeine-treated rats.

DISCUSSION

The prolonged ingestion of 20–24 mg caffeine/kg/day by normal, healthy rats does not seem to affect their growth rate. However, alterations in environmental temperature and the amount and quality of the diet have been reported to affect the toxicity of caffeine. Muller & Vernikos-Danellis (1970) showed that a slight increase or decrease in the normal environmental temperature ($22 \pm 7^\circ\text{C}$) of mice enhanced the toxicity of caffeine, and when food intake was less than half the voluntary intake, an increase in the toxicity of caffeine was noted (Peters, 1966).

The effect of short-term exposure to caffeine on the drug-metabolizing liver microsomal enzymes has been previously reported (Mitoma *et al.* 1968; Mitoma, Lombrozo, Le Valley & Dehn, 1969). Mitoma *et al.* (1968) reported that caffeine, tea and coffee in acute doses induced the *O*-demethylation of *O*-nitroanisole and the hydroxylation of acetanilide by rat-liver microsomes. Similarly, the present study shows some initial elevations of the microsomal-enzyme activity. However, our results (Table 1) show that the prolonged ingestion of caffeine by the rat markedly reduced the aminopyrine *N*-demethylation activity of rat liver. In contrast to *N*-demethylation, acetanilide-hydroxylating activity of the liver, although initially stimulated, was nearly normal after 8 wk of caffeine ingestion.

The mechanism of the stimulation of the drug-metabolizing enzymes of rat-liver microsomes by acute dosing with caffeine has not been satisfactorily elucidated. Stimulation of these microsomal enzymes by caffeine was shown to be partially inhibited by actinomycin D (Mitoma *et al.* 1969). Proof of enzyme induction, as measured by the increase in the incorporation of labelled amino acids into the microsomal proteins, was inconclusive. This

suggests that caffeine may stimulate microsomal-enzyme activity without causing an increase in enzyme synthesis. Caffeine and phenobarbitone in acute doses were shown by Mitoma *et al.* (1968) to have an additive effect with respect to the stimulation of the hydroxylation of acetanilide by the microsomal enzymes. On the other hand, this effect was not additive when *O*-nitroanisole or aminopyrine was used as the substrate. In the present study, phenobarbitone stimulated hepatic microsomal enzymes in rats that had been fed caffeine for 6 wk. This stimulatory effect of phenobarbitone on both the hydroxylation of acetanilide and the *N*-demethylation of aminopyrine was not additive (Table 2). In fact, it appears that, on prolonged ingestion, caffeine hinders the induction of microsomal enzymes by phenobarbitone.

Cytochrome P₄₅₀ and the cytochrome b₅ contents of rat hepatic microsomes were evaluated in order to study the effects of caffeine on the electron transport system of mixed-function oxygenases. Cytochrome P₄₅₀ levels in the liver microsomes of rats dosed with caffeine were essentially the same as those in the untreated rats. Similar results on cytochrome P₄₅₀ contents of hepatic microsomes from rats given caffeine in acute doses had been reported by Lombrozo & Mitoma (1970). However, these workers suggested a possible alteration in the nature of the cytochrome P₄₅₀ of liver microsomes from caffeine-treated rats. Recently, cytochrome b₅ of hepatic microsomes has been shown to be involved in the hepatic drug-metabolizing microsomal-enzyme system (Estabrook, Franklin, Cohen, Shigamatzu & Hildebrandt, 1971). Cytochrome b₅ is postulated as being responsible for the transfer of an electron to cytochrome P₄₅₀ during the metabolism of substrates. It is rate-limiting only when the NADPH levels are low in the reaction mixture. Interestingly enough, cytochrome b₅ values are shown (Table 3) to be significantly decreased in the rats given caffeine and phenobarbitone. Studies in our laboratories (K. L. Khanna & H. H. Cornish, unpublished data, 1971) have shown that rats treated with phenobarbitone alone have increased levels of cytochrome P₄₅₀ but not of cytochrome b₅. The mechanism by which caffeine and phenobarbitone-treated rats inhibit the synthesis or estimation of cytochrome b₅ is not clear.

In conclusion, rats fed caffeine for more than 2 wk begin to show a diminution of aminopyrine-*N*-demethylating activity in the liver. This loss of activity approaches 60% at the end of an 8-wk dosing schedule. Acetanilide-hydroxylating activity was not similarly affected. Although phenobarbitone stimulated microsomal-enzyme activity in caffeine-treated animals, the extent of induction was considerably below that found in control animals receiving only phenobarbitone. In addition, phenobarbitone stimulated production of cytochrome P₄₅₀ in caffeine-treated animals, but cytochrome b₅ levels in the same animals were considerably below normal.

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Effet de l'ingestion quotidienne de caféine sur les enzymes microsomiques du foie chez le rat

Résumé—On a étudié chez le rat l'effet de l'ingestion répétée de caféine sur les enzymes microsomiques du foie. L'ingestion quotidienne, pendant plus de 2 semaines, de 20–24 mg/kg de caféine ajoutée à l'eau de boisson a inhibé l'activité de l'aminopyrine-*N*-déméthylase. Cette diminution d'activité s'est élevée à près de 60% à la fin d'un traitement de 8 semaines. L'activité d'hydroxylation de l'acétanilide de l'enzyme microsomique du foie du rat était d'abord stimulée, atteignait un maximum ($145 \pm 11\%$ par rapport aux animaux témoins) à 2 semaines et revenait à des niveaux normaux à la fin des 8 semaines. Les rats traités à la caféine ne réagissaient pas au même degré à la stimulation par le phénobarbital des enzymes microsomiques que ceux traités uniquement au phénobarbital.

Der Einfluss täglicher Ingestion von Koffein auf die mikrosomalen Enzyme der Rattenleber

Zusammenfassung—Der Einfluss wiederholter Ingestion von Koffein auf mikrosomale Enzyme der Rattenleber wurde untersucht. Bei der Ratte hemmte die Aufnahme von 20–24 mg Koffein/kg/Tag im Trinkwasser für die Dauer von über 2 Wochen die Aktivität von Aminopyrin-*N*-demethylase. Dieser Aktivitätsverlust kam gegen das Ende einer 8-Wochen-Verabreichungsperiode 60% nahe. Die Acetanilidhydroxylierungsaktivität des mikrosomalen Rattenleberenzym wurde anfänglich angeregt, wobei nach 2 Wochen ein Maximum ($145 \pm 11\%$ der Kontrolle) erreicht wurde, und kehrte nach 8 Wochen zu normalen Werten zurück. Koffeinbehandelte Ratten reagierten nicht auf die Phenobarbitone-Stimulierung von mikrosomalen Enzymen im gleichen Ausmass wie Ratten, die nur mit Phenobarbitone allein behandelt worden waren.