Br. J. Pharmacol. (1990), **100**, 435–440

Caffeine-induced behavioural stimulation is dose- and concentration-dependent

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1 The relationship between plasma and brain caffeine and metabolite concentrations and behavioural stimulation was investigated over a 4 h time course.

2 CD-1 mice receiving single intraperitoneal doses of caffeine-sodium benzoate solution (caffeine doses: 0, 20, and 40 mg kg^{-1}) were evaluated in an activity monitor, and their plasma and brain caffeine and metabolite concentrations were determined by high performance liquid chromatography (h.p.l.c.).

3 Kinetic variables for caffeine at low and high caffeine doses were: volume of distribution (Vd), 1.16 and 0.88 l kg^{-1}; plasma elimination half-life ($t_{1/2}$), 1.25 and 1.62 h; brain $t_{1/2}$, 0.93 and 1.30 h; clearance, 0.64 and 0.38 l h^{-1} kg^{-1}, respectively, with Vd and brain $t_{1/2}$ differing significantly between the two caffeine doses.

4 Low-dose caffeine stimulated vertical behaviours significantly more than high-dose, during the first 150 min post-dosage; both doses stimulated vertical behaviours significantly more than vehicle treatment.

5 Low-dose and high-dose caffeine stimulated horizontal and stereotypic behaviours equivalently, during the first 150 min post-dosage; both doses stimulated these behaviours significantly more than vehicle.

6 Only later, 150 min post-dosage, did high-dose caffeine stimulate all behaviours significantly more than both low-dose and vehicle treatment; this occurred when caffeine concentrations had fallen to approximately 10 $\mu g\,g^{-1}$ in the high-dose group.

7 The maximal stimulant effects of caffeine occurred in an intermediate concentration range, between 10–20 $\mu g\,g^{-1}$, while lower and higher concentrations produced either no additional stimulation or decrements in activity.

Introduction

Caffeine appears to be a model compound to study pharmacologically-induced anxiety. The clinical syndrome of caffeinism has been described as being indistinguishable from anxiety states (Greden, 1974). A positive correlation between daily caffeine consumption and scores of anxiety was found in a group of patients with anxiety disorders (Boulenger & Uhde, 1982). Caffeine, at doses of 10 mg kg^{-1}, precipitated panic attacks in patients with pre-existing panic disorder (Charney *et al.*, 1984; 1985). Although these studies demonstrated the anxiogenic effects of caffeine, they did not define relationships between caffeine doses, plasma concentrations and anxiety.

Animal studies have begun to correlate better caffeine dosage and concentration with behavioural stimulation. Caffeine has anxiogenic effects in two animal models of anxiety, the social interaction test and the elevated plus maze test (Baldwin *et al.*, 1989). Preliminary studies in rats have examined dose-response relationships for the stimulant effect of caffeine on motor activity (Thithapandha *et al.*, 1972). More recent studies have evaluated caffeine pharmacokinetics in greater detail and have shown a relationship between brain caffeine concentrations and locomotor stimulation (Kaplan *et al.*, 1989).

Several authors have demonstrated that at higher doses (30 mg kg^{-1} caffeine or greater) there is a decline in the stimulant effects on motor and exploratory activity (Thithapandha *et al.*, 1972; Buckholtz & Middaugh, 1987; File *et al.*, 1988). In addition, at higher brain caffeine concentrations (> 20 $\mu g\,g^{-1}$) there is a decrement in motor stimulation compared to that seen at low and intermediate concentrations (Kaplan *et al.*, 1989). From these previous studies, it appears that a nonlinear relationship exists between caffeine dose and concentrations and behavioural stimulation. The purpose of this study is to delineate more carefully relationships between caffeine dose, plasma and brain concentration and behavioural stimulation.

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Materials

Anhydrous caffeine and paraxanthine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Theobromine and the high-performance liquid chromatography (h.p.l.c.) internal standard beta-hydroxyethyltheophylline were obtained from Sigma Chemical Co. (St. Louis, MO). Theophylline was obtained from Chemical Dynamics Co. (So. Plainfield, NJ). Other reagents were obtained from standard commercial sources. Caffeine-sodium benzoate solution for parenteral administration (250 mg each of caffeine and sodium benzoate in 2 ml) was obtained from Quad Pharmaceuticals Inc. (Indianapolis, IN). The parenteral preparation of caffeine was initially diluted with distilled water to yield a working solution containing 6 mg ml^{-1} each of caffeine and sodium benzoate for high-dose administration. This in turn was diluted with sodium benzoate solution (6 mg ml^{-1}) for low-dose administration. The final volume of injectate was always 0.2 ml and the concentration of sodium benzoate was always 6 mg ml^{-1}.

A stock solution of caffeine (100 $\mu g\,ml^{-1}$), theophylline (50 $\mu g\,ml^{-1}$), paraxanthine (50 $\mu g\,ml^{-1}$) and theobromine (50 $\mu g\,ml^{-1}$) was prepared by weighing appropriate amounts of each standard individually and then dissolving them together in 100 ml of methanol. Another working solution of the mixed standards was prepared by a 1:10 dilution with methanol.

Methods

High-performance liquid chromatography

The h.p.l.c. instrumentation has been described previously (Kennedy *et al.*, 1987). The column (Supelco, Bellefonte, PA)

was stainless-steel, reverse-phase, 15 cm in length × 4.6 mm internal diameter packed with LC-18DB (particle size 5 μm). The mobile phase consisted of 1.75 μM aqueous orthophosphoric acid:acetonitrile:tetrahydrofuran (97:02:01). The organic and aqueous components were filtered and degassed before use. Operating conditions were: detector, 273 nm and 0.02 AUFS; mobile phase flow rate, 1.2–1.4 ml min^{-1}; injection volume varied from 5 to 30 μl.

Extractions of methylxanthines from plasma and brain

The extraction of methylxanthines (caffeine and metabolites) from plasma and brain was performed as previously described by Kaplan and coworkers (1989). Briefly, calibration curves were prepared by using varying amounts of mixed standard solution and a fixed amount of internal standard. Diluted plasma samples were extracted in 4 ml of ethyl acetate:isoamyl alcohol (98:2) by agitation in a vortex mixer. The solution was centrifuged at room temperature and the upper organic layer was transferred to a conical centrifuge tube and evaporated to dryness. The residue for h.p.l.c. analysis was reconstituted with 300 μl of mobile phase.

Mouse brain samples were weighed and homogenized with a Polytron power control unit (Brinkmann Co., Lucerne, Switzerland). The homogenate was extracted in 5 ml of chloroform, agitated in a vortex mixer and then centrifuged. The lower organic phase was transferred by pipette to conical centrifuge tubes. The same extraction procedure was performed an additional two times and the contents of the three organic phases were combined and evaporated in the conical tubes. The residue for h.p.l.c. analysis was reconstituted in the mobile phase.

Behavioural testing procedures

Mouse locomotor behaviour was measured by the Digiscan Model RXYZCM (Columbus, OH). This infrared sensor system monitors the following animal activities: horizontal activity, total distance, number of horizontal movements, horizontal movement time, vertical activity, number of vertical movements, vertical movement time, stereotypy count, number of stereotypies and stereotypy movement time. The animal monitor determines the number of times that the mouse interrupts an infrared beam in the measured direction, during a variable time interval. Stereotypic behaviour is identified if the animal repeatedly breaks the same beam or set of beams. This typically happens during grooming and head bobbing. The data for each animal are printed in separate columns representing the given monitored behaviours.

Mice were individually tested in a dimly lit sound-controlled area. They were removed from their colony and placed in the middle of the activity monitor. They were allowed to settle for at least one hour before the testing procedure began. Mice were injected intraperitoneally (i.p.) with caffeine-sodium benzoate solution to deliver either 0, 20, or 40 mg kg^{-1} of caffeine. Activity counts were cumulated and printed out at 5 min intervals. At 240 min the testing ended, and mice were decapitated for trunk blood and brain specimens. Plasma and brain specimens were prepared and extracted for methylxanthines as described above.

Kinetic study

A separate group of mice were used in the kinetic study. They were injected i.p. with caffeine, 20 mg kg^{-1} or 40 mg kg^{-1}, as described previously. At each of the following post-dosage time points, groups of three mice were decapitated, and plasma and brains were prepared for methylxanthine extraction: 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3 and 4 h.

Kinetic and behavioural statistical analyses

Mean plasma and brain caffeine concentrations at each dose were fitted to the following equation, consistent with a one-compartment open pharmacokinetic model:

$$C = C_0 e^{-kt}$$

where C is the plasma or brain concentration at time t after dosage. The fitting procedure yields parameter estimates for C_0, the extrapolated initial plasma concentration at time zero, and for k, the apparent first-order elimination or disappearance rate constant, as well as estimates of the standard error or standard deviation of each. By use of standard pharmacokinetic methods (Harmatz & Greenblatt, 1987), the parameter estimates were used to calculate the following derived kinetic variables:

$$\text{Volume of distribution (Vd)} = \frac{\text{Dose}}{C_0}$$

$$\text{Elimination half life } (t_{1/2}) = \frac{\ln 2}{k}$$

$$\text{Clearance (Cl)} = \frac{\text{Dose} \times k}{C_0}$$

By use of the standard deviations of the original parameter estimates C_0 and of k, standard deviations of the derived variables can be calculated based on error propagation methods. With their respective mean values and standard deviations, Student's *t* test was used to evaluate the effect of dose on Vd, $t_{1/2}$, and Cl for plasma caffeine, and $t_{1/2}$ for disappearance of caffeine from brain.

To distinguish early and late behavioural effects of caffeine, each behavioural score was expressed as the sum of individual scores from the time of dosage through to 150 min ('early') and from 155 to 240 min ('late'). For each summed 'early' and 'late' score, analysis of variance (ANOVA) was used to evaluate the overall significance of the effect of caffeine (0, 20, 40 mg kg^{-1}). When ANOVA was significant, the significance of individual differences was tested by Student-Newman-Keuls test.

Results

Kinetic studies

As in a previous study (Kaplan *et al.*, 1989), there were moderate variations in plasma and brain concentrations at each caffeine dose. For example, the mean plasma caffeine concentration at a dose of 40 mg kg^{-1}, at 180 min post-injection, was 10.7 μg g^{-1}. The range was 5.51 to 16.50, and the coefficient of variation was 51%. In other cases, the coefficient of variation for a given dose at a given time was as low as 3%. There was a linear relationship between plasma and brain concentrations of caffeine. The slope of the regression line passing through the origin was 1.03 ± 0.05 (mean ± s.e.). Similar linear relationships were found between plasma and brain concentrations of theophylline, paraxanthine, theobromine, with regression slopes of 0.37 ± 0.02, 0.30 ± 0.02, and 0.53 ± 0.02, respectively. These values were similar to those derived from a one-hour time course study (Kaplan *et al.*, 1989). These linear relationships show that plasma concentrations of caffeine and metabolites consistently reflected brain concentrations up to 4 h post-caffeine administration.

Plasma and brain caffeine concentrations at both doses (20 and 40 mg kg^{-1}), over the 4 h time course, are shown in Figure 1. This figure demonstrates that absolute brain concentrations consistently reflect plasma concentrations at each time point. Figure 2 shows brain concentrations of caffeine and metabolites at each dose over the 4 h study. Caffeine concentrations were consistently greater than metabolite concentrations throughout the study.

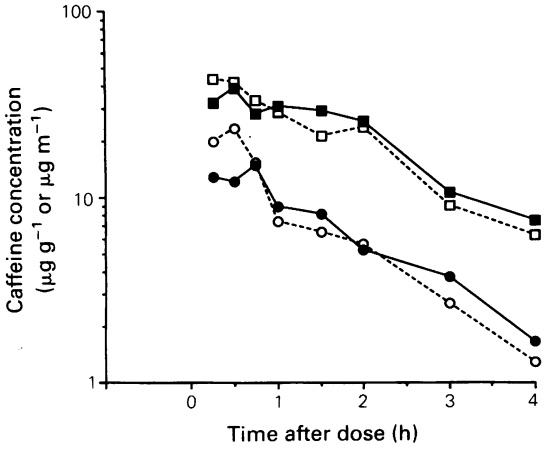

Figure 1 Plasma and brain concentrations of caffeine at both caffeine doses of 20 and 40 mg kg^{-1} over a 4 h time course. Each point represents the mean concentration for 3 animals at the corresponding time. Symbols are: after the 20 mg kg^{-1} dose, plasma (●——●); brain (○---○). After the 40 mg kg^{-1} dose, plasma (■——■); brain (□---□).

Table 1 shows values of Vd, $t_{1/2}$ and Cl determined from plasma and brain concentrations of both caffeine doses. Although differences in plasma $t_{1/2}$ and Cl between the two doses was not significant, there was a trend toward longer $t_{1/2}$ and lower Cl at the higher dose. However, there is a significant difference between the values for Vd ($P < 0.001$) and brain $t_{1/2}$ ($P < 0.01$). No differences were found between methylxanthine concentrations in animals run in the activity monitor (at 240 min) versus those used in the kinetic study, indicating that behavioural testing *per se* is unlikely to alter the kinetics of caffeine.

Table 1 Effect of dose on caffeine pharmacokinetics

	Caffeine dose	
	20 mg kg^{-1}	40 mg kg^{-1}
Volume of distribution (Vd) (l kg^{-1})	1.16* (±0.02)	0.88* (±0.01)
Plasma half-life ($t_{1/2}$) (h)	1.25 (±0.27)	1.62 (±0.54)
Clearance (l h^{-1} kg^{-1})	0.64 (±0.48)	0.38 (±0.13)
Brain half-life ($t_{1/2}$) (h)	0.93* (±0.21)	1.30* (±0.25)

* Significant differences between doses are seen with V_d ($t = 46.72$, $P < 0.001$) and brain $t_{1/2}$ ($t = 3.16$, $P < 0.01$).

Dose-behaviour relationships

Figure 3 shows horizontal activity (HA) versus time at the three caffeine doses (0, 20, 40 mg kg^{-1}). Soon after administration, low-dose and high-dose caffeine appear to stimulate activity equivalently, and both stimulate it greater than vehicle treatment. Later, this stimulant effect is sustained in the high-dose group but not in the low-dose group. As caffeine

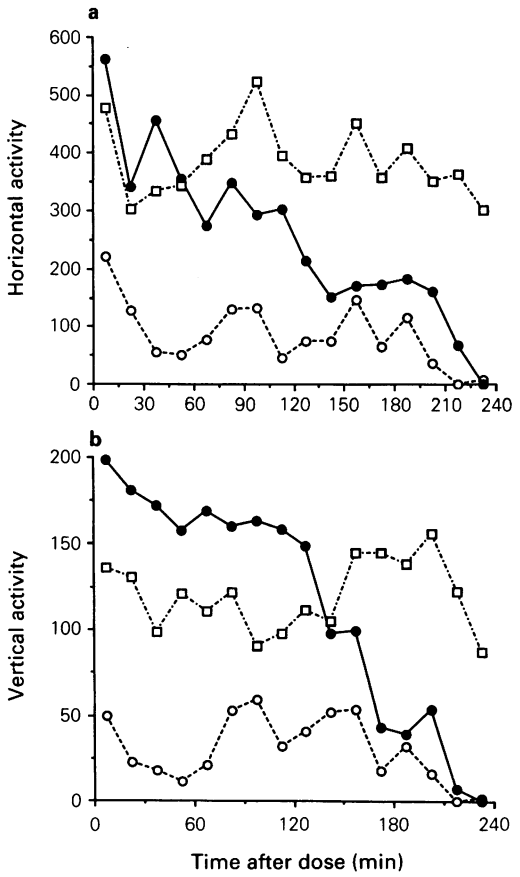

Figure 3 Horizontal activity (a) and vertical activity (b) over a 4 h time course, in animals given caffeine doses of 0, 20, and 40 mg kg^{-1}. Each point represents the mean of the behavioural parameters for 4 animals, averaged over a 15 min time period. Symbols are: caffeine dose = 0 (vehicle control) (○---○); 20 mg kg^{-1} (●——●); 40 mg kg^{-1} (□——□).

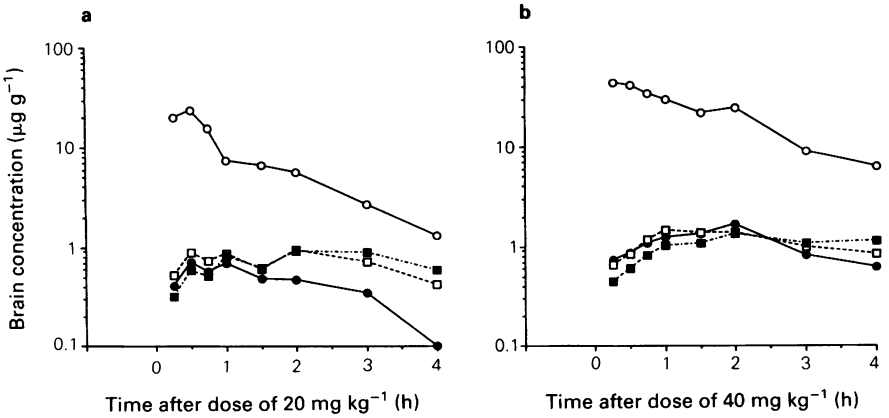

Figure 2 Brain concentrations of caffeine and metabolites of caffeine after doses of 20 mg kg^{-1} (a) and 40 mg kg^{-1} (b) caffeine over a 4 h time course. Each point is the mean concentration for 3 animals at the corresponding time. Symbols are: caffeine (○——○); theophylline (●——●); paraxanthine (□---□); theobromine (■---■).

Table 2 Statistical comparison of behaviour scores in different dosage groups

		Mean value at caffeine dose			Value of F from ANOVA	Significance of individual comparisons		
		0	20	40		20 vs 40	0 vs 20	0 vs 40
Horizontal								
Horizontal activity	Early	2947	9877	11747	5.99 ($P < 0.03$)		*	*
	Late	1112	2263	6686	15.34 ($P < 0.002$)	*		*
Horizontal movements	Early	152	596	742	6.14 ($P < 0.03$)		*	*
	Late	37	116	241	10.28 ($P < 0.005$)	*		*
Total distance	Early	341	2058	2832	5.57 ($P < 0.03$)			*
	Late	69	295	643	7.99 ($P < 0.02$)	*		*
Movement time	Early	45	289	382	5.97 ($P < 0.03$)		*	*
	Late	9	43	95	10.65 ($P < 0.005$)	*		*
Vertical								
Vertical activity	Early	1072	4817	3365	4.14 ($P = 0.053$)		*	
	Late	363	728	2379	44.67 ($P < 0.0002$)	*		*
Vertical movements	Early	261	1115	530	6.04 ($P < 0.03$)	*	*	
	Late	75	192	304	12.99 ($P < 0.003$)	*	*	*
Vertical time	Early	737	1920	2161	2.44 ($P = 0.14$)			
	Late	279	370	1080	21.65 ($P < 0.0005$)	*		*
Stereotypic								
Stereotypy count	Early	1464	5998	6897	6.51 ($P < 0.02$)		*	*
	Late	589	1320	4575	16.12 ($P < 0.002$)	*		*
Stereotypy number	Early	325	902	997	15.01 ($P < 0.002$)		*	*
	Late	120	232	554	24.06 ($P < 0.0003$)	*		*
Stereotypy time	Early	297	1112	1213	9.47 ($P < 0.007$)		*	*
	Late	129	270	874	22.20 ($P < 0.0004$)	*		*

Asterisk (*) indicates significant difference ($P < 0.05$) between behavioural scores of given dosage groups.

and metabolite concentrations decline below $4\,\mu g\,g^{-1}$ in the low dose group (Figures 1 and 2), the behavioural effect of caffeine diminishes and appears similar to vehicle treatment.

The findings were borne out statistically. Scores for early HA, up to 150 min, were not significantly different for the two caffeine doses (Table 2); both are significantly greater than scores of the vehicle-treated group. In the late period (150–480 min), the high-dose HA score was significantly greater than both the low-dose and vehicle HA scores, which were not statistically different from each other. Similar results occurred with horizontal movement, total distance, and movement time. Nearly identical trends were found with all stereotypic behaviours (Tables 2 and 3). In summary, for horizontal and stereotypic behaviours, high-dose and low-dose caffeine initially stimulated behaviour equivalently. The group receiving high-dose caffeine was more stimulated than the low-dose and vehicle groups, only after 150 min, when caffeine concentrations had fallen to a concentration of $10\,\mu g\,g^{-1}$ for this group (Figures 1 and 2).

The effects of caffeine were somewhat different when the various vertical behavioural measures were studied. For vertical movement and activity (Figure 3), early effects of low-dose treatment were greater than either vehicle or high-dose treatment. This difference was statistically significant in the case of vertical movements (Table 2). However, this trend was not seen when the parameter of vertical movement time was looked at. For all vertical measures, effects of the high-dose were significantly greater than either the low-dose or the vehicle, during the late period of treatment. These late-stage results are similar to those seen with all stereotypic and horizontal behaviours. A summary of these findings are presented in Table 3.

Table 3 Summary of caffeine dose-behavioural response relationships

	Comparison of behavioural scores in different dosage groups† Time period:	
Type of behaviour	*Early*	*Late*
Horizontal	20 = 40 Both > 0*	0 = 20 Both < 40*
Stereotypic	20 = 40 Both > 0*	0 = 20 Both < 40*
Vertical	20 > 40 > 0*	0 = 20 Both < 40*

Asterisk (*) indicates significant ($P < 0.05$) difference between behavioural scores in different dosage groups.

Kinetic-behaviour relationships

Figure 4 demonstrates the relationship between mean brain concentrations and activity scores at corresponding times. It indicates that the stimulant effects of caffeine were maximal at concentrations of approximately $10–20\,\mu g\,g^{-1}$ and that concentrations exceeding this range did not cause additional stimulation. In fact, stereotypic counts declined at the highest brain concentrations.

Discussion

Our study provides information about caffeine pharmacokinetics, and relationships between caffeine dose, concentration and behavioural stimulation. The pharmacokinetic results indicate that plasma concentrations consistently reflect brain concentrations of caffeine and metabolites over a 4 h course. This is consistent with previous results (Kaplan *et al.*, 1989) and suggests that plasma caffeine and metabolite concentrations may be accurate indicators of brain concentrations in man as well. The diminished clearance and prolonged plasma and brain half-life associated with higher doses, suggest dose-dependent caffeine kinetics, at least in mice.

Dose-response and concentration-response relationships are delineated in our study. High-dose caffeine does not stimulate activity to a greater degree than low-dose, early after treatment. In fact, low-dose caffeine stimulates certain behaviours to a greater degree than high dose. High-dose caffeine only stimulates activity more than low-dose, 150 min or more after treatment, when concentrations in the high-dose group have fallen to more intermediate levels. The graph of mean brain concentrations versus motor activity (Figure 4) plateaus at intermediate brain concentrations of caffeine, between $10–20\,\mu g\,g^{-1}$; in the case of stereotypic counts, this graph resem-

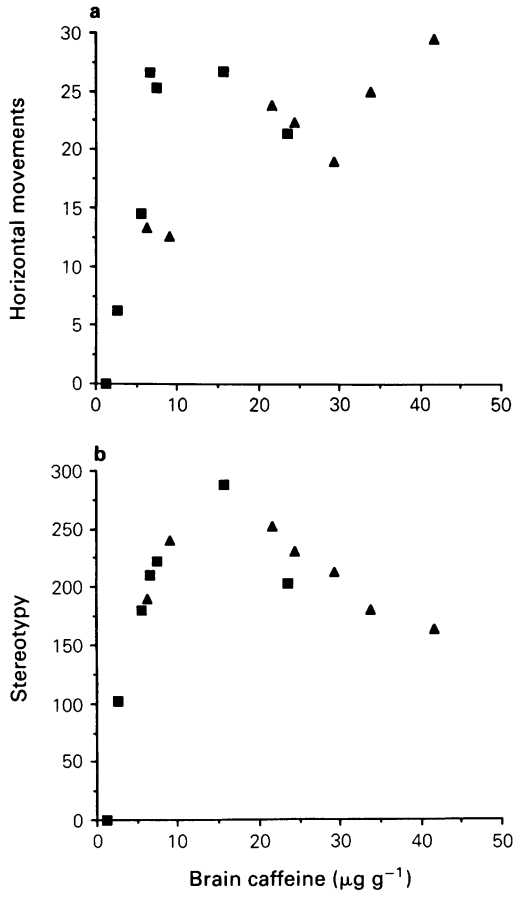

Figure 4 Mean brain caffeine concentrations (x-axis) versus horizontal movements (a) and stereotypy (b) among animals that received 20 or 40 mg kg^{-1} of caffeine. Each point is the mean value for all animals at corresponding times (30 min to 4 h). Symbols are: caffeine dose = 20 mg kg^{-1} (■); 40 mg kg^{-1} (▲).

bles an inverted U. This suggest that the maximal stimulant effects of caffeine occur in a concentration range of 10–20 μg g^{-1}. Concentrations below or above this range are associated with submaximal behavioural stimulation.

Caffeine-induced behavioural stimulation may relate to the complex effects of caffeine on central benzodiazepine, adenosine and monoamine systems (Daly *et al.*, 1981). Snyder and coworkers (1981) were the first to correlate the stimulant effects of caffeine with its antagonism of central adenosine receptors. They also demonstrated that caffeine had a weak inhibitory effect on benzodiazepine receptor binding. Several authors have confirmed that caffeine significantly alters central adenosine receptor binding (Boulenger *et al.*, 1983; Hawkins *et al.*, 1988; Daval *et al.*, 1989) and benzodiazepine receptor binding (Marangos *et al.*, 1979; Weir & Hruska, 1983; Boulenger *et al.*, 1983; Wu & Coffine, 1984; Kaplan *et al.*, 1989). Future studies can examine how various caffeine doses and concentrations affect adenosine and benzodiazepine receptor binding differently, ultimately resulting in different behavioural responses to the drug.

Our results may be useful in their application to man. As in our animal study, Bruce and coworkers (1986) showed that in man, the effects of caffeine on the central nervous system, as measured by electrophysiological changes, were maximal at intermediate plasma concentrations (between 4–6 μg ml^{-1}). High doses and plasma concentrations (10–12 μg ml^{-1}) did not produce these central effects. Therefore, a specific caffeine concentration range for maximal stimulatory effects may be found in man as well as animals. Future studies in man can be done to evaluate more carefully the relationship between caffeine plasma concentration and pharmacodynamics (e.g., mood, motor performance and autonomic effects). In this way, we can better understand pharmacologically-induced anxiety.

We are grateful for the assistance of Jerold S. Harmatz, Soong T. Chiang, and Lawrence G. Miller, M.D. This work was supported in part by Grants MH-34223, AG-00106 and DA-05258 from the Department of Health and Human Services.

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(Received August 28, 1989
Revised February 21, 1990
Accepted February 26, 1990)