Increases in plasma concentrations of steroids in the rat after the administration of caffeine: comparison with plasma disposition of caffeine

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ABSTRACT

It was demonstrated that the altered endocrine environment caused by caffeine consumption could be equated with a stress-like pattern of response. A single acute treatment with caffeine (30 or 60 mg/kg) to male rats approximately 85 days old caused plasma concentrations of corticosterone, progesterone, testosterone and Na^+ to rise significantly above control values. These changes were evident 3 min after caffeine administration and were maintained for 1–4 h before returning to normal. In animals exposed to daily chronic caffeine treatment for 10 days or more the levels of progesterone fell and Na^+ rose significantly compared with control values at 24 h after administration. Following a single treatment of 30 mg/kg, caffeine was detected in blood plasma after 3 min, and reached peak levels by 1 h. After 24 h, less than 2% of the peak levels of caffeine remained. Metabolites of caffeine were detectable within 6 min and reached their peak levels 4 and 12 h later for theophylline and theobromine respectively.

It is suggested that high steroid levels may in the long-term cause an altered hepatic clearance pattern affecting both steroid metabolism and caffeine elimination. A preliminary study of the morphology of livers from males chronically exposed to caffeine revealed that the hepatic cells lost cytoplasmic matrix, and that the sinusoids did not show up as clear spaces, compared with those in the controls.

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INTRODUCTION

Concern over the effects of caffeine, a universally consumed methylxanthine alkaloid, on reproductive capacity has been reviewed by James & Paull (1985). Reproductive disfunctions may be induced by either maternal or paternal use of caffeine. Maternal consumption of caffeine during pregnancy causes significant retardation of fetal growth and increased post-natal mortality of both sexes. The subsequent growth rates are also affected. The pups grow more slowly (with growth plateauing at the same age) resulting in smaller adults. A significant decrease in testosterone biosynthesis in the fetal testes at critical times for testicular differentiation has also been observed, and the offspring of a second generation were also affected (Pollard, Jabbour & Mehrabani, 1987). Similarly, exposure of a male rat to caffeine before and during mating also affects his progeny and the progeny of a second generation. The testes of Fo sires exposed to caffeine show a marked degeneration characterized by significant overall size reduction, breakdown of germinal epithelium and the accumulation of cellular debris in the lumen of the seminiferous tubules (Pollard & Smallshaw, 1988). In both male and female studies the caffeine-induced effects show close similarities to stress effects of psychological origin, which bring about non-adaptive reproductive changes (Pollard, 1984, 1986; Pollard & Dyer, 1985). It has been suggested that the action of caffeine may be the result of (i) a direct toxic effect on cellular function thus affecting the sperm (or germ cell) or the growing fetus and/or (ii) an indirect effect through changes in maternal or paternal physiological function mediated by an altered endocrine environment. Endocrinologically, caffeine is known to increase plasma levels of catecholamines (Levi, 1967), and affect pituitary function (Spindel, Arnold, Cusack & Wurtman, 1980). It may also be relevant that caffeine is a purine analogue and that the testis and placenta do not act as a barrier to its passage.

The present study tests the hypothesis that caffeine causes an altered endocrine environment which can be equated with a form of anxiety stress. Steroid profiles

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of plasma corticosterone, progesterone and testosterone, as well as Na^+, were obtained and compared with the plasma levels of caffeine plus its two major metabolites (theophylline and theobromine) after exposure to caffeine by gavage. Concentrations of the above steroids were also monitored in a long-term (chronic) study in which caffeine was administered daily for 5, 10 and 20 days. The livers of male rats which had received caffeine for 38 consecutive days were also studied by light microscopy.

MATERIALS AND METHODS

Animals and experimental design

Experimentally naive male Wistar rats, 80–90 days old and 400–450 g in weight were housed in pairs under identical conditions of constant temperature and humidity (21 ± 0·5 °C; 46% relative humidity) with food and water available *ad libitum* and subjected to a natural light:darkness regimen for a minimum of 1 week before experimentation. During this time the animals were accustomed to daily handling. Thus, normal unstressed males were chosen at random to represent control and caffeine-treated groups, with six animals per experimental and three animals per control group at each time-interval. For the endocrine study two dose levels of caffeine (30 and 60 mg/kg) were administered by gavage. These levels are, in terms of human consumption, approximately equivalent to caffeine intakes of 10 and 20 cups of brewed coffee daily respectively. The controls were given vehicle (water) only. For the acute study, groups of rats were gavaged and killed 3, 6, 12 and 24 min and 1, 4, 12 and 24 h after caffeine or water exposure. For the chronic study, treatments were continued daily for 5, 10 and 20 days. These animals were then killed on the next day (i.e. 24 h after their last exposure to either caffeine or vehicle). For the disposition study, only one level of caffeine (30 mg/kg) was used and the animals were killed on the same-time schedule as that used in the acute study.

A light microscope study of the livers from four caffeine-exposed rats and four control rats from another experiment was undertaken. The experimental groups had received a daily dose of caffeine (30 mg/kg) for 38 consecutive days and were killed 24 h later.

Assay for corticosterone

A description of the steroid assay procedures has been given by Pollard (1986). Corticosterone (an 11-hydroxycorticoid) is the glucocorticoid secreted in quantity by the rat. Corticosterone levels in plasma were determined by a competitive protein-binding assay modified from the cortisol assay of Murphy (1967). For corticosterone estimation, 100 µl aliquots of plasma were vortexed with 1 ml redistilled dichloromethane. The aqueous phase was aspirated and a 0·5 ml aliquot of the dichloromethane extract transferred to fresh assay tubes and evaporated to dryness.

A corticosterone-binding globulin-isotope solution (16% Hyland control serum I; Travenol Laboratories Pty Ltd., Old Toongabbie, NSW, Australia (containing sufficient [^{3}H]corticosterone to produce maximum binding of available receptor sites)) was added to the tubes and mixed by vortexing. The tubes were then incubated at 45 °C for 15 min, followed by immersion in an ice bath for 45 min. The unbound steroid was then removed by the addition of 80 mg of 60–100 mesh Florisil (Selby's Scientific, North Ryde, NSW, Australia). A sample (0·5 ml) of supernatant fraction containing the bound hormone was then transferred to scintillation fluid and counted in a Packard 3375 scintillation counter. Standard curves were obtained using known quantities of unlabelled corticosterone and plotted as per cent bound against corticosterone concentration. Cross-reactivity at 50% displacement only occurred, to any appreciable extent, with progesterone (20%).

Assay for testosterone and progesterone

Concentrations of testosterone and progesterone were measured by radioimmunoassay using the kit supplied by New England Nuclear (Division of Serle Nucleonics Aust., Sydney, NSW, Australia). For testosterone estimations, 200 µl aliquots of plasma were vortexed in 5 ml freshly redistilled dichloromethane and centrifuged. The aqueous phase was aspirated and the dichloromethane serially washed with 0·5 ml NaOH (0·1 mol/l), acetic acid (0·1 mol/l) and distilled water to remove potentially interfering substances. Before aspiration of the final water wash the mixture was again centrifuged. The dichloromethane extract was then evaporated and the residue reconstituted with 2 ml absolute ethanol. For progesterone estimation, 200 µl aliquots of plasma were extracted in 6 ml freshly redistilled hexane. The hexane was separated from the aqueous phase by freezing. The solvent was evaporated and reconstituted in 2 ml absolute ethanol. Recoveries (in the order of 85%) were routinely checked in each assay using [^{3}H]testosterone or [^{3}H]progesterone (3000 d.p.m.).

For the assay, 100 µl (testosterone) or 200 µl (progesterone) aliquots of the ethanol extract were transferred and evaporated to dryness in assay tubes. Samples and standards were incubated overnight at 4 °C together with 0·1 ml antiserum and 0·1 ml of the corresponding ^{3}H-labelled steroid (12 000 d.p.m.).

Separation of free and antibody-bound steroid was by the addition of dextran-coated charcoal to the incubation mixture.

Antisera were raised in rabbits against testosterone-3-oxime–bovine serum albumin or progesterone-11α-hemisuccinate–bovine serum albumin. Cross-reactivity with testosterone at 50% displacement only occurred to any appreciable extent with dihydrotestosterone (56%) and was negligible for progesterone.

Determination of sodium ion concentration

Plasma Na^+ concentrations were determined by a standard flame photometry procedure. Sample concentrations were determined against a standard calibration curve over the range of 100 to 150 mmol/l using a EEL 100 flame photometer.

High-performance liquid chromatography (HPLC) separation of caffeine, theophylline and theobromine

All reagents were HPLC grade. Plasma concentrations of caffeine (1,3,7-trimethylxanthine) and the two major metabolites theophylline (1,3-dimenthylxanthine) and theobromine (3,7-dimethylxanthine) were measured by a method described by Klassen & Stavric (1983). Standard stock solution of caffeine (10 μg/ml) and the internal standard 8-chlorotheophylline (20 μg/ml) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were prepared in 0·9% (w/v) NaCl. The extraction procedure was as follows. Standard solution or sample of plasma (typically 100 μl) was added to 1·2 g NH_4HCO_3 and 100 μl internal standard. Extracting solvent (6 ml chloroform; isopropanol, 85:15, v/v) was then added and the mixture vortexed for 60 s and centrifuged. The aqueous phase was aspirated and the organic phase decanted into clean tubes and evaporated to dryness. The residue was reconstituted with 200 μl eluting solvent (freshly prepared mobile phase consisting of 10 ml acetic acid, 40 ml acetonitrile and 40 ml isopropanol made up to 1000 ml with milli-Q water, filtered and degassed), vortexed for 40 s, and the tubes inserted for 15–20 s into a warm (50 °C) water bath to ensure complete dissolution. Samples were frozen overnight before assay.

The operating procedure was as follows. A Walters Associates (Model 450) high-performance liquid chromatograph was used with a Regis Hi-Chrom Reversible HPLC column (5 μm spherical packing, 25 cm × 4·6 mm internal diameter; Regis Chemical Co., Morton Grove, IL, U.S.A). The column was at ambient temperature and the flow at 1·5 ml/min (15 MPa), with the u.v. wavelength set at 276 μm. Sample volumes between 50 and 100 μl were injected per analysis and run times were approximately 20 min. An LCD/Milton Roy integrator was used for the recording and processing of the data. The method separates paraxanthine (1,7-dimethylxanthine) from theophylline, which have identical retention times under most solvent conditions published.

Statistical analyses

Data for blood steroid and Na^+ concentrations were analysed using one-way analysis of variance (ANOVA).

RESULTS

Plasma steroid and Na^+ profile

Table 1 summarises the effect of a single and Table 2 repeated exposure of 30 or 60 mg caffeine/kg on plasma concentrations of corticosterone, progesterone, testosterone and Na^+. One-way ANOVA showed no significant difference between the controls over the duration of the experiment so each timed experimental group was analysed against the same pooled control value. It may be seen from Table 1 that in the acute phase following both caffeine doses there was an initial significant rise of corticosterone, progesterone and testosterone which was maintained for the first 1–4 h after administration of caffeine before returning to normal. Concentrations of Na^+ increased significantly only in response to the higher (60 mg/kg) dose and returned to normal by 1 h. At 6 min after exposure to caffeine the concentration of progesterone and Na^+ were not different from the controls. Table 2 summarises the chronic study with animals killed 24 h after the last exposure to caffeine. The progesterone level was significantly lower whilst Na^+ (60 mg/kg only) was significantly higher than the controls from day 10 onwards.

Plasma disposition of caffeine

Text-figure 1 depicts the plasma caffeine, theophylline and theobromine profiles for the first 24 h after exposure to 30 mg caffeine/kg. Caffeine was in the circulation within 3 min and reached a peak level at 1 h. It had mostly disappeared after 24 h, with less than 2% of peak levels remaining. The intermediate metabolites were detectable in the blood after 6 min and reached their peak levels 4 and 12 h later for theophylline and theobromine respectively.

Liver morphology

A preliminary investigation of the liver of male rats which had received a daily dose of 30 mg caffeine/kg for 38 consecutive days showed overall morphological differences. The liver cells making up the hepatic plates in the experimental group showed a loss of cytoplasmic matrix. The most obvious difference,

TABLE 1. Effect of a single acute dose of 30 or 60 mg caffeine/kg body weight on plasma concentrations of corticosterone, testosterone, progesterone and Na^+ at various times after administration. Values are the means ± S.E.M. for 33 rats per pooled control and six animals per treatment group

	Corticosterone (μmol/l)	**Progesterone** (nmol/l)	**Testosterone** (nmol/l)	**Na^+** (mmol/l)
Pooled control	0·17 ± 0·02	7·0 ± 0·6	10·6 ± 1·4	134·1 ± 1·2
30 mg/kg				
3 min	0·31 ± 0·07*	12·1 ± 2·2*	23·9 ± 6·6**	139·7 ± 2·3
6 min	0·38 ± 0·05***	9·9 ± 1·6	17·7 ± 0·7*	135·5 ± 7·2
12 min	0·79 ± 0·12***	13·7 ± 1·3**	51·3 ± 2·8***	133·0 ± 1·8
24 min	0·34 ± 0·08**	8·3 ± 1·6	37·8 ± 14·9***	131·2 ± 1·4
1 h	0·44 ± 0·13***	12·4 ± 2·2*	29·1 ± 5·2***	135·9 ± 2·0
4 h	0·44 ± 0·14***	4·1 ± 0·6	15·9 ± 2·4	139·5 ± 1·5
12 h	0·18 ± 0·01	9·2 ± 1·9	14·9 ± 4·5	137·8 ± 1·6
24 h	0·11 ± 0·02	4·1 ± 1·3	12·5 ± 2·4	140·3 ± 3·3
60 mg/kg				
3 min	0·34 ± 0·09**	12·2 ± 1·6**	20·5 ± 7·6**	149·0 ± 7·5**
6 min	0·38 ± 0·10**	9·5 ± 1·9	25·7 ± 1·1*	132·0 ± 1·8
12 min	0·37 ± 0·06**	12·1 ± 2·2*	26·4 ± 8·7**	151·3 ± 10·8**
26·1 ± 13·9***	142·3 ± 3·6*			
1 h	0·45 ± 0·03***	23·5 ± 2·9*	23·9 ± 7·3**	132·5 ± 2·5
4 h	0·27 ± 0·05	7·3 ± 1·6	10·7 ± 1·4	137·4 ± 2·9
12 h	0·28 ± 0·03	9·2 ± 0·3	17·0 ± 3·8	136·5 ± 1·4
24 h	0·21 ± 0·03	5·1 ± 0·3	13·5 ± 5·5	134·3 ± 1·5

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with the pooled control value (one-way analysis of variance).

TABLE 2. Effect of chronic daily administration of 30 or 60 mg caffeine/kg body weight on plasma concentrations of corticosterone, testosterone, progesterone and Na^+ in rats killed 24 h after the last administration. Values are means ± S.E.M. for 33 animals per pooled control and six animals per treatment group

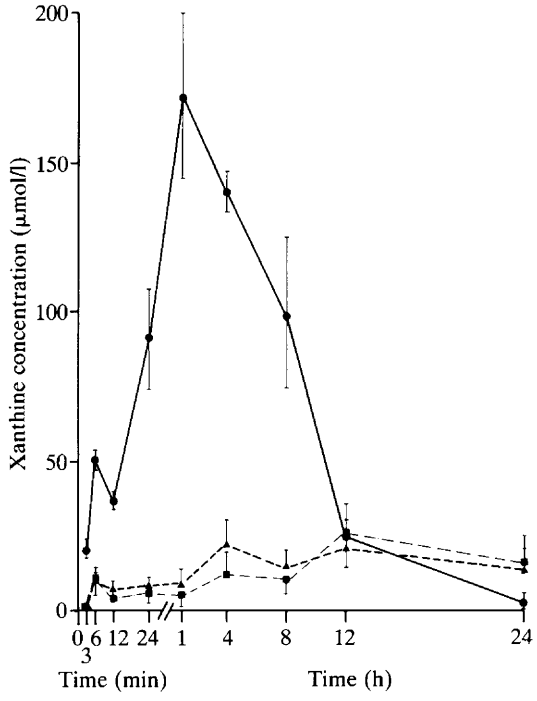
	Corticosterone (μmol/l)	**Progesterone** (nmol/l)	**Testosterone** (nmol/l)	**Na^+** (mmol/l)
Pooled control	0·17 ± 0·02	7·0 ± 0·6	10·6 ± 1·4	134·1 ± 1·2
30 mg/kg				
5 days	0·20 ± 0·06	4·5 ± 1·0	16·3 ± 2·4	136·7 ± 1·3
10 days	0·22 ± 0·02	4·8 ± 0·3	14·2 ± 4·2	139·8 ± 3·3
20 days	0·24 ± 0·04	2·5 ± 0·3*	8·0 ± 0·7	139·2 ± 3·5
60 mg/kg				
5 days	0·20 ± 0·04	4·1 ± 0·6	13·2 ± 3·8	134·1 ± 1·0
10 days	0·18 ± 0·01	2·9 ± 0·3*	9·7 ± 1·4	140·8 ± 2·0*
20 days	0·18 ± 0·01	1·9 ± 0·3**	9·4 ± 3·1	143·5 ± 2·7**

*$P < 0·05$, **$P < 0·01$ compared with the pooled control value (one-way analysis of variance).

however, was in the hepatic sinusoids between the hepatic plates. In the control group the sinuosoids (which eventually open into a vein) were apparent as clear spaces, whilst in the experimental group they were filled with a dense cloudy material (Plate). Further studies as to the effect of caffeine on liver metabolism are now being undertaken.

DISCUSSION

The present study has demonstrated that ingestion of caffeine (30 and 60 mg/kg) produced a profound endocrine response characterized initially by significant increases in plasma levels of corticosterone, progesterone and testosterone, and, at the higher caffeine dose, Na^+. Animals exposed daily to chronic caffeine treatment from 10 days onwards had plasma levels of progesterone significantly below and Na^+ significantly above control values 24 h after administration. Changes in Na^+ concentration are a likely consequence of caffeine-induced changes in mineralocorticoid secretion and/or a result of changed antidiuretic hormone secretion. It has long been known that caffeine acts on the kidney to produce diuresis (Greden, 1974). Body water redistribution following



TEXT-FIGURE 1. Concentrations of caffeine (●), theophylline (▲) and theobromine (■) in the plasma of rats after a single administration of 30 mg caffeine/kg. Values are means ± S.D. for three animals per treatment group.

changes in blood pressure due to the sudden impact of caffeine on the gastric mucosa may also be explained by the observation that 6 min after administration Na^+ and progesterone concentrations seemed normal. The present study also demonstrates a dual response pattern to caffeine consumption: an initial physiological 'shock' response immediately following acute caffeine exposure followed by a long-term readjustment of steroid secretion (progesterone is a precursor for both corticosterone and testosterone synthesis) in response to prolonged caffeine exposure. The caffeine disposition (i.e. biotransformation and elimination) profile over the first 24 h after a single exposure demonstrated that the drug reached the circulation within 3 min and rose to peak levels by 1 h with the bulk having disappeared by 24 h. The metabolite concentration is low because of rapid excretion by the kidney and further hepatic degradation to a variety of methyluric acids. This disposition pattern is in good agreement with the results of other investigators (Latini, Bonati, Castelli & Garattini, 1978; Bonati, Latini, Galetti *et al.* 1982).

The effect of caffeine consumption on steroid metabolism has not been extensively studied. However, the pattern of the endocrine changes described resembles that seen in stress and is in accord with the hypothesis that medium to high caffeine doses produce a stress-like pattern of endocrine response. Spindel, Griffith & Wurtman (1983) have also reported that caffeine, as well as its major metabolites such as theophylline and theobromine, increases serum corticosterone levels. There are also strong parallels with a previous study dealing with a form of anxiety stress when caffeine was given daily for 60 days. In the short-term, stress caused significantly increased metabolic activity of all anterior pituitary trophic cell types, whilst between 5 and 10 days a degree of adaptation occurred. In the long-term a shift toward a decreased secretory pattern was observed (Pollard, Bassett & Cairncross, 1976). There are, however, important differences in the caffeine response when compared with the stress model. Under anxiety stress testosterone secretion was always less than 25% above control values (Pollard, Bassett & Joss, 1980) whilst both doses of caffeine used caused a minimum doubling of testosterone over control values. Conversely, stress- induced corticosterone levels were approximately eight times higher than normal, a much greater response than that found for caffeine. Pollard & Smallshaw (1988) have already reported a marked effect of caffeine on reproductive capacity coupled with visible degeneration of the testes of caffeine-fed male rats.

An altered endocrine balance in the long term may be due to a caffeine- and/or steroid-induced changed pattern of hepatic clearance. Such a hypothesis is not inconsistent with the morphological changes in the livers of rats chronically exposed to caffeine reported here. Caffeine metabolism is possibly catalysed by the hepatic microsomal mixed-function oxidases, in particular, forms of cytochrome P-450 (Bonati, Latini, Marzi, *et al.* 1980). Increased plasma concentrations of steroid may alter microsomal function. An analogous situation may be pregnancy where steroid concentrations are also raised. Gilbert, So, Klassen *et al.* (1986) reported that pregnancy decreased caffeine elimination in the monkey (*Macaca fascicularis*) resulting in a significant increase in serum caffeine levels when compared with those in the non-pregnant animal. Preliminary results from our laboratory indicate a similar slower elimination of caffeine in pregnant rats. Such findings give cause for concern about the habitual use of caffeine as well as more specific concern for the next generation.

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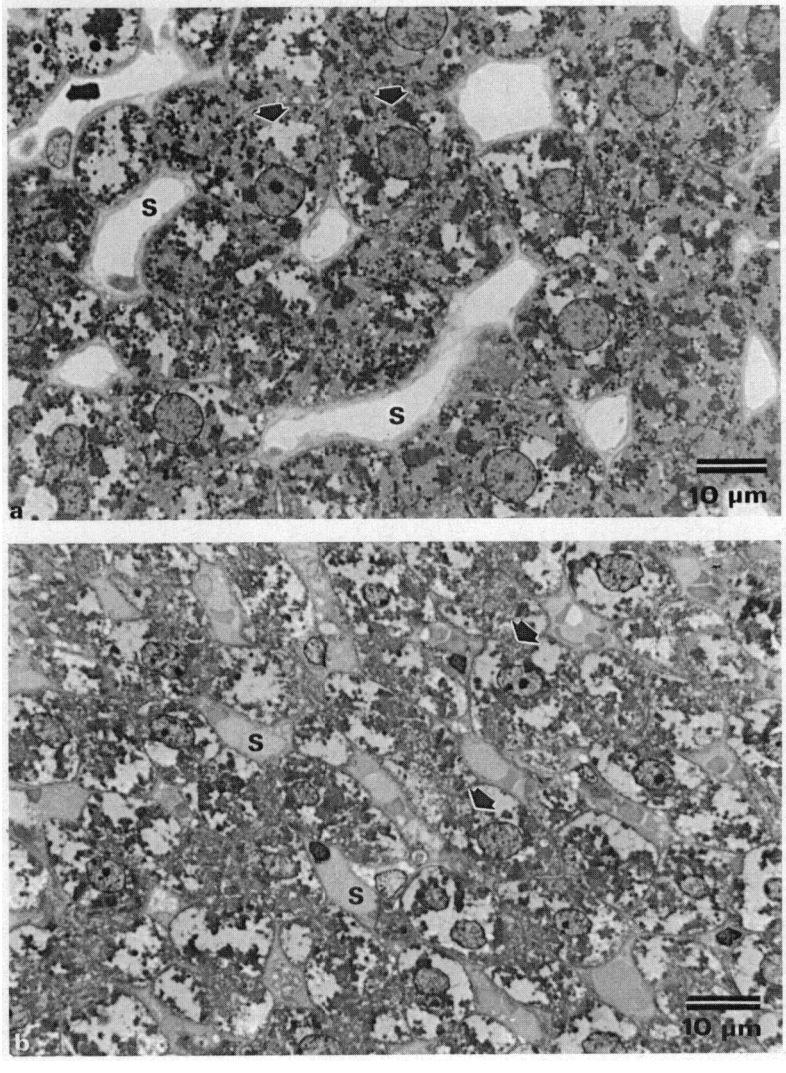

DESCRIPTION OF PLATE

Light microscope sections of a typical view of the liver from control and caffeine-treated rats.
(a) The control liver showing healthy hepatic cells (arrows) and sinusoids (S) seen as clear spaces.

(b) The liver of an experimental rat which had received a daily caffeine dose of 30 mg/kg for 38 consecutive days. Hepatic cells exhibited a loss of cytoplasmic matrix (arrows) and sinusoids (S) were filled with a cloudy material.