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Genotoxicity of cocoa examined by microbial and mammalian systems

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Summary

Unroasted or roasted cocoa powder dispersed in water and applied to Chinese hamsters by stomach tube caused elevated numbers of SCEs in the sister-chromatid exchange test (bone-marrow cells). Roasted cocoa freed from rat produced distinctly higher SCE values with a linear dose–response relationship, whereas cocoa butter had no influence on SCE levels. Positive results in the SCE test (1.5-fold values of the controls) were obtained after application of about 5 g cocoa/kg b.w. Presumably, because of the smaller quantities that could be administered in this way, positive test results were not found when cocoa was given in the diet instead of being administered by stomach tube. Cocoa from which theobromine was extracted by chloroform did not affect SCE levels. Pure theobromine increased SCE levels in a dose-dependent way. Theobromine was also positive in the micronucleus test at 2×40 mg/animal and negative in the chromosome aberration test at 1×40 mg/animal. Cocoa and the theobromine were negative in the Salmonella/mammalian microsome mutagenicity test both with and without metabolic activation.

The occurrence of mutagenic substances in heated foods has been reported by several groups of authors using systems in vitro, especially in the Salmonella/mammalian microsome mutagenicity test (Nagao et al., 1977; Commoner et al., 1978; Matsumoto et al., 1978; Sugimura and Nagao, 1979; Spingarn and Weisburger, 1979). However, published results have not permitted a final and reliable interpretation of the relevance of such data (Sugimura, 1978).

Cocoa is roasted during processing. As a constituent of chocolate it is mainly consumed by children, whose exposure to potentially mutagenic substances should command special attention. Fermented cocoa beans are imported from tropical countries. After the grinding and roasting, chocolate liquor is obtained, from which

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cocoa powder is produced. Separation from cocoa butter yields cocoa powder containing 20 or 8% fat. Commercial cocoa brands contain various amounts of theobromine (1.4–2.7%) and caffeine (0.1–0.3%) (Zoumas et al., 1980).

Experimental

The animals used were Chinese hamsters from our own breeding colony (outbred stock), kept under standardized conventional conditions, 10–14 weeks old, weighing 30 ± 2 g. The controls and test groups consisted of equal numbers of males and females. In all mammalian tests, bone-marrow cells were used. Animals had undergone 22 h of starvation at the beginning of the tests.

The *sister-chromatid exchange (SCE) test* was performed with 5-bromodeoxyuridine tablets (Boehringer, Mannheim) which were implanted subcutaneously into the experimental animals, as recommended by Allen et al. (1977). The test procedure described by Renner (1979) was used: 50 mg tablets/animal and 26 h BrdU-treatment time; 4 animals/dose; 50 metaphases/animal.

Theobromine suspended in corn oil was administered by stomach tube as a single dose 2 h after BrdU implantation, as indicated in the test procedure. To make cocoa suitable for this procedure, the cocoa powder dispersed in water (1 : 1) was given at a dose of 0.1 or 0.2 g/animal by stomach tube, at the same time as the BrdU implantations. This was followed by 2 further administrations of 0.2 g each at 90-min intervals. It was not possible to give the full dose at once because of the limited stomach volume of the animals.

The *micronucleus test* was performed according to the standard procedure (Schmid, 1973). Administration of cocoa (0.2 g) 30, 28.5 and 27 h before the animals were killed. In theobromine testing, 2 equal doses were applied (suspended in corn oil) by stomach tube 30 and 6 h before the animals were killed (6 animals/dose; 1000 polychromatic erythrocytes/animal).

For the *analysis of chromosome aberrations* – performed according to the conventional technique (Schwarzacher and Wolf, 1974) – cocoa or theobromine were administered in the above-mentioned manner 24 h before the bone-marrow cells were collected (6 animals/dose; 300 metaphases/animal). In this test and in the SCE test, colchicine (Demecolcin, SERVA, Heidelberg) was injected s.c. at a dose of 1 mg/kg 2 h before the animals were killed.

Ames test. Overnight cultures of TA1535, TA100, TA1537, TA1538 and TA98 of *Salmonella typhimurium* were used in the mutation assay. The Salmonella/mammalian microsome mutagenicity test was carried out by the standard plate-incorporation technique described by Ames et al. (1975). Each plate was inoculated with 0.1 ml of the bacterial tester strain, 0.1 ml of test solution and 0.5 ml of S9 mix (containing 0.1 ml of S9 fraction/ml of mix), if required. As a positive control, 2-aminoanthracene was used. The post-mitochondrial supernatant fraction (S9) was

prepared from the pooled livers of 3 male Sprague–Dawley rats pretreated with a single i.p. injection of Aroclor 1254 (500 mg/kg) 5 days before the killing. For the Ames test, cocoa powder free of fat was dispersed in distilled water and autoclaved for 10 min at 121 °C. Theobromine was dissolved in hot distilled water.

Theobromine (puriss.) and caffeine (puriss.) were supplied by Merck, Darmstadt. Freshly imported unroasted and roasted (chocolate liquor) cocoa from the same lot (origin, Ivory Coast) was obtained from a commercial roastery. Cocoa free of fat was obtained by fat extraction with light petroleum. Extraction of theobromine was performed with chloroform.

Results and discussion

Unroasted cocoa given at doses in the range of 0.1–0.6 g/animal caused a dose-dependent elevation of SCEs. Roasted cocoa (chocolate liquor) yielded nearly identical results (Table 1). This indicates that the roasting process did not influence the number of SCEs/cell. Cocoa from the same lot was also tested after extraction

TABLE 1

RESULTS OF THE SCE TEST ON COCOA AND THEOBROMINE IN CHINESE HAMSTERS, BONE-MARROW CELLS

Dose/animal		0.1 g	0.2 g	0.4 g	0.6 g
Control	3.81 ± 0.07	–	–	–	–
Cocoa butter		–	–	–	3.82 ± 0.09
Unroasted cocoa		4.39 ± 0.04	5.07 ± 0.07	5.89 ± 0.11	6.80 ± 0.07
Roasted cocoa (chocolate liquor)		4.41 ± 0.08	4.98 ± 0.11	5.90 ± 0.08	6.81 ± 0.09
Roasted cocoa, fat-free		5.13 ± 0.21	6.07 ± 0.13	7.88 ± 0.07	10.01 ± 0.16
Roasted cocoa, fat- and theobromine-free		–	–	–	4.15 ± 0.11
Dose/animal	2.5 mg	5 mg	10 mg	15 mg	20 mg
Theobromine	4.78 ± 0.11	5.16 ± 0.18	6.74 ± 0.07	7.86 ± 0.11	9.05 ± 0.20
Theobromine + caffeine (6:1)	4.77 ± 0.18	5.11 ± 0.09	6.54 ± 0.05	7.35 ± 0.11	9.50 ± 0.50

Numbers indicate SCEs/cell: total mean value and standard deviation of the mean values/animal.

of fat (the fat portion was 47%). The elevation of the number of SCEs above the control level was now about twice as high, compared with the values obtained with chocolate liquor (Table 1). Cocoa butter did not cause an alteration of the number of SCEs. In agreement with other investigators who use the SCE test, we considered the test results as positive when the numbers of SCEs/cell reached at least 1.5 times those of the control (> 5.7 SCEs/cell corresponding to $P < 0.0001$, t test). For the fat-free cocoa sample, a positive result in the SCE test was obtained when the animals had received about 5 g of cocoa/kg of body weight via stomach tube within 3 h. When we gave 20% of cocoa in the diet, SCE values were slightly elevated, but the number of SCEs/cell did not reach 1.5 times those of the control. If only cocoa (100%) was given during 3–4 days, as tested in a preliminary assay, animals lost on the average 2.3 g of body weight/day. A subsequent mutagenicity test would not have been realistic.

Looking for the causes of the phenomena observed in the SCE test, we checked theobromine-free (chloroform-extracted) cocoa and found no increase of SCE values (Table 1). Theobromine, a dimethylxanthine, in the dose range of 2.5–20 mg/animal, caused an increasing dose response (Fig. 1 and Table 1). The test results were positive with 6 mg (or more) of theobromine/animal. The combined application of theobromine + caffeine (at a ratio 6:1, as occurring in cocoa) produced the same SCE values as theobromine alone (Fig. 1). We conclude that theobromine is responsible for the elevated SCE values after cocoa administration, but we cannot exclude the possibility that cocoa contains other chloroform-soluble mutagenic compounds.

In the micronucleus test, all results obtained after administration of cocoa (roasted and fat-free, 0.6 g/animal) were negative. When the chromosome aberration test was used, cocoa (same dose) did not exhibit clastogenic properties.

In the micronucleus test, theobromine caused highly positive results (8.83 ‰ micronucleated polychromatic erythrocytes: 3.01 ‰ in the controls; $P < 0.01$, Wilcoxon rank test) but only at a dose of 2 × 40 mg/animal (Table 2), whereas this

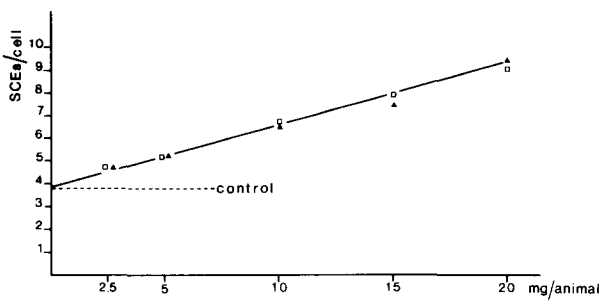

Fig. 1. Induction of SCEs. Chinese hamster, bone-marrow cells. □, theobromine; ▲, theobromine + caffeine (6:1).

TABLE 2

MICRONUCLEUS TEST

Chinese hamsters (bone-marrow cells), $n = 6$/dose. Number of micronucleated polychromatic erythrocytes/1000 (min.–max.).

Control	3.0 (1– 4)
2 × 20 mg theobromine	3.8 (3– 7)
2 × 30 mg theobromine	3.7 (3– 5)
2 × 40 mg theobromine	8.8 (8–10)

compound was negative in the chromosome aberration test at 1 × 40 mg (Table 3). Single higher doses than 40 mg theobromine/animal were toxic.

Table 4 shows the results of the Ames test. Cocoa incubated with *Salmonella typhimurium* strains TA1535, TA100, TA1537, TA1538 and TA98 did not show any mutagenic effect at concentrations of 5.0, 2.5, 1 and 0.5%. At concentrations exceeding 5.0%, cocoa had an antibacterial activity. Likewise, theobromine, up to the highest concentration still soluble of 0.25% (2.5 mg/ml), gave no evidence of mutagenicity in *Salmonella typhimurium* with or without metabolic activation.

The lack of agreement between the micronucleus and the chromosomal assays raises the question, in which cell cycle were the cells scored? Treatment times in the 3 mammalian tests with cocoa or theobromine were all in similar ranges (30, 26 and 24 h). A more likely explanation is the difference in applied dose. The test procedure of the micronucleus test requires 2 applications (2 × 40 mg theobromine) of the test compound, whereas in the chromosome aberration test 1 × 40 mg theobromine was used. Negative results in both tests with cocoa (0.6 g cocoa contains distinctly less theobromine than 40 mg!) and positive results in the SCE test indicate the higher sensitivity of the latter test system. Of course the different genetic end-points of the tests should also be taken into account.

According to Timson (1975), theobromine causes chromosome abnormalities in plant cells and in mammalian cells in culture. Similar observations in cultured

TABLE 3

CHROMOSOME ABERRATION TEST

Chinese hamsters (bone-marrow cells), $n = 6$/dose; 300 cells/animal. Numbers indicate gaps and breaks in 1800 cells.

	Gaps	Chromatid breaks
Control	9	5
1 × 20 mg theobromine	7	3
1 × 30 mg theobromine	9	5
1 × 40 mg theobromine	8	6

TABLE 4

MUTAGENICITY TESTING OF COCOA AND THEOBROMINE WITH THE SALMONELLA/MICROSOME ASSAY

Test substance	Conc. (%)	Microsomal activation	Number of his$^+$ revertant colonies per plate[a]				
			TA1535	TA100	TA1537	TA1538	TA98
Roasted cocoa fat-free	5.0	–	16	67	3	9	19
		+	12	63	4	11	28
	2.5	–	13	86	4	10	17
		+	9	78	7	14	20
	1.0	–	17	85	6	12	17
		+	11	68	9	20	18
	0.5	–	13	89	7	12	18
		+	12	81	9	25	29
Theobromine	0.25	–	11	83	5	10	12
		+	11	74	7	18	23
	0.1	–	12	91	5	9	17
		+	11	81	6	19	22
	0.01	–	12	78	7	14	14
		+	11	76	12	28	19
	0.001	–	15	83	4	10	13
		+	11	76	6	22	21
Negative control		–	14	86	5	12	16
		+	12	77	7	23	25
2-Aminoanthracene	0.002	–	248	860	47	328	250
		+					

[a] The results for each compound are an average of 2 independent experiments, each using 3 plates per concentration.

human cells have been reported by Weinstein et al. (1975). Rather scanty evidence suggests that theobromine is not mutagenic in mammals. Weinfeld and Christman (1953) and Cornish and Christman (1957) assumed that the ability to demethylate theobromine accounts for non-mutagenicity in mammals and man. Our positive test results with theobromine in the SCE test in vivo and also in the micronucleus test do not support this assumption.

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References

Allen, J.W., C.F. Shuler, R.W. Mendes and S.A. Latt (1977) A simplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodeoxyuridine tablets, Cytogenet. Cell Genet., 18, 231–237.

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347–364

Commoner, B., A.J. Vithayathil, P. Colara, S. Naiv, P. Madyastha and G.C. Cuca (1978) Formation of mutagens in beef and beef extract during cooking, Science, 201, 913–916.

Cornish, H.H., and A.A. Christman (1957) A study of the metabolism of theobromine, theophylline and caffeine in man, J. Biol. Chem., 228, 315–323.

Matsumoto, T., D. Yoshida, S. Mizusaki and H. Okamoto (1978) Mutagenicity of the pyrolysates of peptides and proteins, Mutation Res., 56, 281–288.

Nagao, M., M. Honda, Y. Seino, T. Yahagi and T. Sugimura (1977) Mutagenicities of smoke condensates and the charred surface of fish and meat, Cancer Lett., 2, 221–226.

Renner, H.W. (1979) Zur Bewertung des In-vivo-SCE-Tests, dargestellt an verschiedenen Zytostatika, Arzneim. Forsch./Drug Res., 29 (II) 1871–1875.

Schmid, W. (1973) Chemical mutagen testing on in vivo somatic mammalian cells, Agents and Actions, 3, 77–85.

Schwarzacher, H.G., and U. Wolf (Eds.) (1974) Methods in Human Cytogenetics, Springer, Berlin, p. 78.

Spingarn, N.E., and J.H. Weisburger (1979) Formation of mutagens in cooked foods, I. Beef, Cancer Lett., 7, 259–264.

Sugimura, T. (1978) Let's be scientific about the problem of mutagens in cooked food, Mutation Res., 55, 149–152.

Sugimura, T., and M. Nagao (1979) Mutagenic factors in cooked foods, Crit. Rev. Toxicol., (August) 189–209.

Timson, J. (1975) Theobromine and theophylline, Mutation Res., 32, 169–177.

Weinfeld, H., and A.A. Christman (1953) The metabolism of caffeine and theophylline, J. Biol. Chem., 200, 345–355.

Weinstein, D., M. Mauer, M.L. Katz and S. Kazmer (1975) The effect of methylxanthines on chromosomes of human lymphocytes in culture, Mutation Res., 31, 57–61.

Zoumas, B.L., W.R. Kreiser and R.A. Martin (1980) Theobromine and caffeine content of chocolate products, J. Food Sci., 45, 314–316.