Genetic Toxicology Studies of SALATRIM Structured Triacylglycerols. 2. Lack of Genetic Damage in *in Vitro* Mammalian Cell Assays and the *in Vivo* Micronucleus Assay

Johnnie R. Hayes,*,† Colette J. Rudd,‡ Jon C. Mirsalis,‡ James P. Bakke‡, Richard A. Winegar,‡ and Hemalatha Murli§

Bowman Gray Technical Center, Reynolds Boulevard, Winston-Salem, North Carolina 27102, SRI International, 333 Ravenswood Avenue, Menlo Park, California 94025, and Hazleton Washington, 9200 Leesburg Pike, Vienna, Virginia 22182

The SALATRIM family of triacylglycerols differs from other fats in the ratio of short-chain fatty acids (SCFA) to long-chain fatty acids (LCFA) and in that stearic acid is the major LCFA. These fats have caloric availability values (4.5–6 kcal/g) lower than that of corn oil (9 kcal/g). SALATRIM 23CA Lot A014, a typical SALATRIM fat, was tested in *in vitro* mammalian cell genotoxicity assays including the chromosomal aberration, unscheduled DNA synthesis, and HPRT mammalian cell mutagenesis assays. Corn oil also was tested as a reference fat. Both the SALATRIM fat and corn oil were negative in the three assays. SALATRIM 234CA lot A019 and SALATRIM 234CS lot A018 were tested in the *in vivo* bone marrow micronucleus assay. Rats received these SALATRIM fats or corn oil at 10% (w/w) in the diet for 13 weeks. Both fats were negative in this assay. The data confirm the prediction that SALATRIM fats lack genotoxic potential.

INTRODUCTION

Members of the SALATRIM family of structured triacylglycerols are typical triacylglycerols composed of fatty acids esterified to a glycerol backbone. The unique characteristic of this family of fats is the triacylglycerols contain a preponderance of stearic, acetic, propionic, and/or butyric acids. Triacylglycerols that contain one stearic acid and two short-chain fatty acids (SCFA) predominate among the mixed triacylglycerols that comprise these fats (Softly et al., 1994). Because of the limited absorption of stearic acid compared to certain other fatty acids (Benzonana and Desnuelle, 1968; Carey et al., 1983; Finley et al., 1994; Gacs and Barltrop, 1977; Hashim and Babayan, 1978; Jensen et al., 1982) and the fewer carbons available for energy production from the SCFA, these fats have lower caloric availabilities (4.5–6 kcal/g) (Finley et al., 1994) than fats such corn oil (9 kcal/g). SALATRIM fats are produced by interesterification among high-stearate fats, such as hydrogenated canola oil and hydrogenated soybean oil, and triacetin, tripropionin, and tributyrin. Because the natural precursor fats contain mixed triacylglycerols, SALATRIM fats contain small quantities of other fatty acids such as oleic and palmitic, among others. SALATRIM fats can have different physical and functional characteristics because the ratios of the SCFA can be varied.

On the basis of structure/activity relationships and review of the scientific literature, it can be predicted that SALATRIM fats should not interact with DNA to produce genetic changes. To test this hypothesis, a number of short-term *in vitro* genetic toxicology assays have been completed. It has been previoiusly reported that a series of six SALATRIM fats that varied in the ratio of specific SCFA to LCFA and varied in the source for the LCFA esterified to glycerol was negative in the *Salmonella*/microsome reverse mutation assay (Hayes and Riccio, 1994). These previous studies used five *Salmonella* tester strains (TA98, TA100, TA1535, TA1537, and TA1538), tested with and without metabolic activation. This paper extends those data by testing a representative SALATRIM fat in a series of *in vitro* mammalian cell assays including (1) the chromosomal aberration assay; (2) the unscheduled DNA synthesis (UDS) assay; and (3) the hypoxanthine–guanine phosphoribosyltransferase (HPRT) mammalian cell gene mutation assay. Also, the *in vivo* genetic toxicology of two SALATRIM fats was determined by the bone marrow micronucleus assay using rats fed SALATRIM fats at 10% (w/w) of the diet for at least 13 weeks. Corn oil was used as a reference control to represent a typical dietary fat in both the *in vitro* and *in vivo* studies and to control for the high fat content of the 10% SALATRIM diet in the *in vivo* studies.

The studies reported here used SALATRIM 23CA lot A014 in the *in vitro* mammalian cell studies as a representative of SALATRIM fats. SALATRIM 23CA lot A014 was produced from triacetin, tripropionin, and hydrogenated canola oil, with triacetin being the predominant source of the SCFA. As a result, acetic acid is the predominant short-chain fatty acid and occurs in the vast majority of triacylglycerols that comprise this fat. The *in vivo* study used SALATRIM 234CA lot A019 and SALATRIM 234CS lot A018 as typical SALATRIM fats. SALATRIM 234CA lot A019 was produced from triacetin, tripropionin, tributyrin, and hydrogenated canola oil. This results in a relatively random distribution of acetic, propionic, and butyric acids among the SCFA esterified to glycerol. SALATRIM 234CS lot A018 was similar except the precursor fat was hydrogenated cottonseed oil. This resulted in a fat similar to SALATRIM 234CA lot A019 with stearic acid still the predominant LCFA but with a slightly different distribution of other LCFA. The use of these three SALATRIM fats results in testing a fat containing a preponderance of a single short-chain fatty acid (acetic acid), fats containing all three SCFA (acetic, propionic, and butyric acid), and SALATRIM fats pro-

* Author to whom correspondence should be addressed.
† Bowman Gray Technical Center.
‡ SRI International.
§ Hazleton Washington.

0021-8561/94/1442-0521$04.50/0

duced from different precursor fats. The choice of these particular SALATRIM fats enables the major compositional variables within the SALATRIM family of triacylglycerols to be evaluated.

The *in vitro* genetic toxicology assays used in this study provide data on a number of mechanisms of genetic damage including clastogenesis (chromosomal aberrations), mutagenesis (HPRT), and DNA damage (UDS). These assays use mammalian cells as opposed to the previous studies with bacterial cells (Hayes and Riccio, 1994). Also, an *in vivo* genetic toxicology assay, the rat bone marrow micronucleus assay, was used. Use of this assay allows all mechanisms associated with digestion, absorption, distribution, metabolism, and excretion to be functional in the whole animal. The hypothesis to be tested in these studies was that the SALATRIM fats do not possess potential to produce genetic damage in mammalian cells in culture and in the intact animal.

MATERIALS AND METHODS

Reagents. Aroclor 1254-induced rat liver S-9 preparations (supernatant obtained from centrifugation of homogenized liver at 9000*g*) from Fischer 344 rats were obtained from Molecular Toxicology, Inc., Annapolis, MD. Acetone (CAS No. 67-64-1) was obtained from Mallinckrodt, Paris, KY. Cyclophosphamide (CP) (CAS No. 50-18-0), 3-methylcholanthrene (3-MC) (CAS No. 56-49-5), and ethyl methanesulfonate (EMS) (CAS No. 62-50-0) were obtained from Sigma Chemical Co., St. Louis, MO. Methyl methanesulfonate (MMS) (CAS No. 66-27-3) was obtained from Aldrich Chemical Co., Milwaukee, WI, as was 2-(acetylamino)fluorene (2-AAF) (CAS No. 53-96-3).

Test Materials. SALATRIM 23CA lot A014, SALATRIM 234CA lot A019, and SALATRIM 234CS lot A018 were produced by Nabisco Foods Group, East Hanover, NJ. The corn oil used in these studies was a commercially available brand. SALATRIM 23CA lot A014 was used in the *in vitro* studies, and SALATRIM 234CA lot A019 and SALATRIM 234CS lot A018 were used in the *in vivo* studies.

Chemical Analysis of Test Fats. Total fatty acid profiles of SALATRIM 23CA lot A014, SALATRIM 234CA lot A019, and SALATRIM 234CS lot A018 were conducted at EPL Bio-Analytical Services, Inc. (EPL-BAS), Decatur, IL. Fatty acid profiles for fatty acids at or above C12:0 were obtained by saponification of the fat with methanolic sodium hydroxide followed by esterification with methanolic boron trifluoride. Methyl esters were quantified by gas chromatography. For fatty acids less than C12:0, saponification was identical to the longer chain fatty acids and quantification was by gas chromatography of the protonated acids without formation of the methyl esters. Free fatty acid concentration (by titration) and peroxide analysis were conducted by Nabisco Foods Group, East Hanover, NJ, and EPL-BAS. Free fatty acid titration was done according to AOCS Official Method Ca 5a-40 (AOCS, 1990a) and peroxide analysis by AOCS Official Method Cd 8-53 (AOCS, 1990b).

Chromosomal Aberration Assay. Chinese hamster ovary cells (CHO: ETCC CCL 61 CHO-KL, proline-requiring) were used for the *in vitro* cytogenetic assay (Galloway et al., 1985). Cells were grown in a 5% CO_2 atmosphere at 37 °C in McCoy's 5a medium with 15% fetal bovine serum (FBS), 2 mM 1-glutamine, and 1% penicillin–streptomycin solution. This medium was used during exposure of the test fats to the cells without metabolic activation. McCoy's 5a medium with 2.5% FBS (containing 2 mM L-glutamine and 1% penicillin–streptomycin) was used during exposure of the test fats to the cells with metabolic activation.

The metabolic activation system was prepared immediately before use and contained 1 part S-9 preparation to 9 parts McCoy's 5a medium with 2.5% FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin solution. Cofactors were 24 mg of NADP and 45 mg of sodium isocitrate per milliliter of S-9 preparation.

In the absence of metabolic activation, the fats and 0.01 mM deoxybromouridine (BrdU) were added to the cell culture and incubated at 37 °C for 8 h. The cells were washed and then incubated in fresh medium containing 0.01 mM BrdU for 13.5 h. When metabolic activation was used, the cultures were exposed to the fats for 2 h at 37 °C, washed, and then incubated in fresh medium containing 0.01 mM BrdU for 6–8 h. After incubation in BrdU, colchicine was added to the cultures at 0.4 μg/mL followed by incubation for 2.5 h at 37 °C.

The cells were harvested and suspended in 5 mL of 0.075 M KCl. After 15 min at 37 °C, 1 mL of Carnoy's fixative [absolute methanol/glacial acetic acid (3:1 v/v)] was added. The cells were centrifuged and then suspended in 5 mL of fixative. After 5 min, the process was repeated. Slides were prepared, air-dried, and stained in 3% Giemsa for 20 min, rinsed in deionized water, and passed through xylene, and then coverslips were mounted. The mitotic index was evaluated on the basis of at least 1000 cells per flask. Fifty cells per flask were evaluated for chromosomal aberrations, resulting in a total of 100 cells evaluated per dose. The number of cells with chromosomal damage in the test fat groups and positive control group were compared to the concurrent solvent control by Fischer's exact test (Gad and Weil, 1991). A test material would be considered positive if there was a statistically significant ($p \leq 0.05$) increase in the frequency of cells with chromosomal damage and if this increase was dose-related.

A preliminary dose range study to determine cytotoxicity and optimal cell fixation time was done before the definitive study was initiated. Doses of 8.0, 40.0, 200.0, 500.0, and 1000 μg/mL were tested. Acetone was used as the solvent for both SALATRIM 23CA lot A014 and corn oil. The high dose was limited by the low solubility of the fats in the assay medium. On the basis of the results of the range-finding study, doses of 250, 500, and 1000 μg/mL for the SALATRIM fat and 500, 750, and 1000 μg/mL for corn oil were used for the definitive study and a harvest time of 8–10 h was selected.

Hepatocyte Unscheduled DNA Synthesis Assay. The basic methodology for the assay followed that developed by Williams (1977). Fischer 344 rats were anesthetized with sodium pentobarbital (60 mg/kg). Livers were perfused with collagenase and the resulting hepatocytes harvested. Cell viability during these studies ranged between 86% and 94.8%. Approximately 300 000 viable cells/mL was added to six-well culture dishes containing coverslips. The cell medium used for attachment was Williams Medium E supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin sulfate, and 10% fetal bovine serum. The cellular preparation was incubated for 1.5–2.0 h at 37 °C in a 5% CO_2 atmosphere to allow the cells to adhere to the coverslips. The UDS assay was repeated twice. The first assay was to determine cytotoxicity and any UDS response; the second assay was to confirm the results of the first. Cultures were simultaneously exposed to concentrations of test fat and 10 μCi/mL [^{3}H]thymidine (specific activity 80 Ci/mmol) for approximately 19 h at 37 °C. The fats were assayed at doses ranging from 0 to 1000 μg/mL. The high dose was limited by the insolubility of the fats in the culture medium.

After exposure, the cultures were washed with fresh medium and swelled in 1% sodium citrate. Cells were fixed in 1:3 glacial acetic acid/ethanol and washed in deionized water. The coverslips were mounted on slides and dipped in Kodak NTB-2 photographic emulsion and exposed at −20 °C for 7 days before development. After development, the cells were stained with 1% methyl-green pyronine Y, dried, and evaluated. Quantitative autoradiographic grain counting was performed on at least 30 morphologically unaltered cells in a randomly selected field. The higher count from two nuclear-sized areas in the most heavily labeled cytoplasmic areas adjacent to the nucleus was subtracted from the nuclear count to give the net grains per nucleus. The percentage of cells in repair (% IR) (cells in repair were those showing at least 5 net grains/nucleus) indicated the response throughout the liver culture. For each experiment, 90 cells per dose were scored. Three slides per dose were scored. The average net grains per nucleus and % IR were calculated for each dose.

A test material was considered positive if the mean net grain count was greater than 5 net grains/nucleus. A material was considered negative if the net grains per nucleus was less than zero and the % IR was less than 10% for all dose groups.

HPRT Assay. The HPRT assay used Chinese hamster ovary (CHO) CHO-K1 cells obtained from the American Type Culture

Collection and was similar to that described by Li and Shimizu (1983). CHO cells were grown in Ham's F12 medium with 31 μg/mL penicillin, 50 μg/mL streptomycin sulfate, and 5% heat-inactivated fetal bovine serum (F12/5). When metabolic activation was employed, the final serum concentration was reduced to 0.8%. The medium used for cloning contained 10% fetal bovine serum and 0.3% purified agar. The selective cloning medium contained 30 μM 6-thioguanine (TG).

The metabolic activation mixture consisted of 5% S-9 preparation, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 30 mM KCl, 5 mM glucose 6-phosphate, 4 mM NADP, and 50 mM sodium phosphate buffer. One milliliter of this mixture was added to 4 mL of Ham's F12 containing 1% fetal bovine serum. This was added to the cell culture to yield a final S-9 concentration of 1%.

Stock solutions of SALATRIM 23CA lot A014 and corn oil were made in acetone such that the acetone never exceeded 1% of the culture. Although the test material was soluble in the acetone, turbidity was noted in the cultures, indicating solubility had been exceeded. Positive controls were EMS (200 μg/mL of culture) without metabolic activation and 3-MC (5 μg/mL of culture) with metabolic activation.

Preliminary experiments were done to determine cytotoxicity and aid in selection of the dose range. The definitive study was done in duplicate using duplicate cultures for each replicate. Six SALATRIM doses, 31.25–1000 μg/mL were selected for both studies. Corn oil doses were 327.7–1000 μg/mL.

Cytotoxicity was determined by detaching the cells from the culture flask with 0.05% trypsin–0.02% EDTA. A Model 2F Coulter counter was used to determine cell numbers, and an aliquot containing at least $1–2 \times 10^6$ cells was added to 20 mL of F12/5 to determine phenotypic expression. The remaining cell suspension was diluted in approximately 35 mL of medium so that 500 cells were plated into two Petri dishes. After incubation for 14–17 days, cell colonies were determined. Survival was expressed as the cloning efficiency (CE) relative to the solvent control.

Phenotypic expression of induced mutants was conducted by incubating the cells for 7 days. Cells were subcultured every 2–3 days by trypsinizing the flask, counting the cells, and replating 2×10^6 cells. After the expression period, approximately 3×10^6 cells from each culture were seeded in 100 mL of cloning medium supplemented with TG for selection of resistant cells. Approximately 600 cells were seeded in 100 mL of TG-free medium to determine the percentage of viable cells. The cells were incubated for 14–17 days and cell colonies counted with an ARTEK colony counter. Mutant frequency (ratio of mutant cells to nonmutant cells) was calculated by dividing the number of resistant colonies by the number of unselected viable colonies. The test results are considered positive if a dose-related increase in the number of mutant colonies occurs and the mutant frequencies of duplicate cultures treated with one or more concentrations of the test article are at least 3 times the average of those from the solvent control cultures.

***In Vivo* Rat Micronucleus Assay.** The in-life and bone marrow smear preparation portions of the study were part of a 13-week subchronic toxicity study conducted at Hazleton Wisconsin, Inc., Madison, WI, and the micronucleus assay portion of the study was conducted at Hazleton Washington, Vienna, VA. Detailed information on the experimental design and methods for the subchronic study can be found in the paper for the subchronic toxicology portion of that study (Hayes et al., 1994). The test fats used in the study were SALATRIM 234CA lot A019 and SALATRIM 234CS lot A018. A corn oil control group was used to control for the high fat content of the diets.

Groups of 20 male and 20 female Crl:CD BR VAF/Plus rats from Charles River Laboratories, Portage, MI, were exposed to either of the two SALATRIM fats or corn oil at 10% (w/w) of the diet for at least 13 weeks. SALATRIM fats and corn oil were mixed separately with NIH-07 Rat and Mouse Ration 5018 (Purina Mills, Inc., St. Louis, MO) and fed *ad libitum*. Animal husbandry complied with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86-23, 1985). At necropsy, duplicate bone marrow slides for each rat were prepared for clinical pathology. One set of unstained bone marrow slides was shipped to Hazelton Washington for the micronucleus assay.

Table 1. Total Fatty Acid Profile for SALATRIM 23CA Lot A014[a]

fatty acid name	fatty acid designation	wt %
stearic (octadecanoic)	C18:0	57 ± 1
acetic	C2:0	21.1 ± 0.1
propionic	C3:0	2.58 ± 0.03
palmitic (hexadecanoic)	C16:0	2.37 ± 0.04
arachidic (eicosanoic)	C20:0	1.50 ± 0.03
behenic (docosanoic)	C22:0	0.668 ● 0.007
oleic (9-octadecenoic)	C18:1	0.572 ± 0.005
lignoceric (tetracosanoic)	C24:0	0.335 ± 0.001
linoleic (9,12-octadecadienoic)	C18:2	0.066 ± 0.001
lauric (dodecanoic)	C12:0	0.007 ± 0.000

[a] Data represent the mean ± standard deviation for triplicate determinations.

Upon arrival, the slides were fixed in methanol and stained with acridine orange. A bone marrow slide from each of the 20 rats per group (with the exception of the SALATRIM 234CS lot A018 group, where only 18 slides were available) was analyzed using fluorescent microscopy. One thousand polychromatic erythrocytes per rat were scored for micronuclei. Identification of micronuclei was based upon the criteria of Schmid (1976). The scoring unit was the micronucleated cell, not the number of micronuclei. Therefore, a cell that contained more than one micronucleus was scored as a single micronucleated cell. The frequency of micronucleated cells was expressed as percent micronucleated cells based upon the total number of polychromatic erythrocytes scored. The normal frequency of micronucleated erythrocytes for this strain of rat is 0.0–0.4%.

Statistical analysis of the data was by analysis of variance on the square root arcsine transformation. Tukey's Studentized range test (Sokal and Rohlf, 1981) was used to determine statistical significance ($p \leq 0.05$) from the corn oil control group. Analyses were conducted separately for each SALATRIM-treated group and each sex combination. The criteria for a positive response was a significant increase in micronucleated polychromatic erythrocytes compared with the corn oil control group. If no increase was found, the test fats were considered negative in this assay.

RESULTS

Chemical Characterization of Test Fats. The total fatty acid profile of SALATRIM 23CA lot A014 is presented in Table 1. As expected, acetic acid is the predominant SCFA followed by propionic acid. Stearic acid is the predominant LCFA. Because this SALATRIM was made from hydrogenated canola oil, small quantities of other LCFA are present. Table 2 presents the fatty acid profile of SALATRIM 234CA lot A019. Again, the fatty acid profile of this hydrogenated canola oil derived SALATRIM was predictable. Approximately equal concentrations of acetic, propionic, and butyric acids were found to be esterified to glycerol, and the predominant LCFA was stearic. As seen in Table 3, SALATRIM 234CS lot A018 was similar to SALATRIM 234CA lot A019 with respect to the SCFA and in that stearic acid is the predominant LCFA. Differences between these two SALATRIM fats occur in the minor LCFA because hydrogenated cottonseed oil was used as the fat source in the production of SALATRIM 234CS lot A018 instead of the hydrogenated canola used to produce SALATRIM 234CA lot A019. The free fatty acid concentration and peroxide values of these three SALATRIM fats are presented in Table 4. As expected, the free fatty acid concentrations and peroxide values for these fats are low.

Chromosomal Aberration Assay. The SALATRIM fat appeared to be soluble in the culture medium for the

Table 2. Total Fatty Acid Profile for SALATRIM 234CA Lot A019[a]

fatty acid name	designation	wt %
stearic (octadecanoic)	C18:0	46.802 ± 5.213
butyric	C4:0	11.71 ± 0.56
propionic	C3:0	9.12 ± 0.36
acetic	C2:0	8.22 ± 0.17
palmitic (hexadecanoic)	C16:0	3.059 ± 0.333
arachidic (eicosanoic)	C20:0	2.077 ± 0.126
oleic (9-octadecenoic)	C18:1	0.927 ± 0.076
behenic (docosanoic)	C22:0	0.601 ± 0.046
lignoceric (tetracosanoic)	C24:0	0.297 ± 0.023
linoleic (9,12-octadecadienoic)	C18:2	0.055 ± 0.004
myristic (tetradecanoic)	C14:0	0.053 ± 0.004
lauric (dodecanoic)	C12:0	<0.014

[a] Data represent the mean ± standard deviation for triplicate determinations.

Table 3. Total Fatty Acid Profile for SALATRIM 234CS Lot A018[a]

fatty acid name	designation	wt %
stearic (octadecanoic)	C18:0	39.848 ± 0.453
palmitic (hexadecanoic)	C16:0	11.663 ± 0.134
butyric	C4:0	10.67 ± 0.85
propionic	C3:0	7.84 ± 1.64
acetic	C2:0	6.82 ± 2.34
myristic (tetradecanoic)	C14:0	0.503 ± 0.028
arachidic (eicosanoic)	C20:0	0.245 ± 0.009
behenic (docosanoic)	C22:0	0.106 ± 0.005
lignoceric (tetracosanoic)	C24:0	0.075 ± 0.005
oleic (9-octadecenoic)	C18:1	0.251 ± 0.026
linoleic (9,12-octadecadienoic)	C18:2	0.068 ± 0.004
lauric (dodecanoic)	C12:0	0.024 ± 0.001

[a] Data represent the mean ± standard deviation for triplicate determinations.

Table 4. Free Fatty Acid Concentration and Peroxide Value Analyses for Three SALATRIM Fats[a,b]

	SALATRIM 23CA lot A014	SALATRIM 234CS lot A018	SALATRIM 234CA lot A019
free fatty acid (wt %)	0.86 ± 0.01	0.19 ± 0.03	0.07 ± 0.01
peroxide value (mequiv/kg)	0.3 ± 0.1	0.87 ± 0.04	0.33 ± 0.05

[a] Data represent the mean ± standard deviation for triplicate determinations. [b] Data for SALATRIM 23CA lot A014 were generated at EPL Bio-Analytical Services, Inc., and data for SALATRIM 234CS lot A018 and SALATRIM 234CA lot A019 were generated at Nabisco Foods Group.

chromosomal aberration assay at a concentration of 40 μg/mL but produced a suspension at higher concentrations. The pH of the high dose in the medium was approximately 7.0. The preliminary dose range/cytotoxicity study used a dose range of 0–1000 μg/mL and was limited by the lack of solubility of the fat. Corn oil behaved in a manner similar to that of SALATRIM 23CA lot A014 and was tested using the same criteria. No significant cell cycle delay or reduction in the mitotic index was noted with either corn oil or the SALATRIM fat. There was a slight increase in the number of cells in metaphase 1 at a SALATRIM dose of 1000 μg/mL and at corn oil doses of 40 and 200 μg/mL, but these were not significant on the basis of assay criteria. Therefore, for the definitive assay, a dose range of 0 (solvent control), 250, 500, and 1000 μg/mL was chosen for the SALATRIM and 500, 750, and 1000 μg/mL for corn oil.

In the absence of metabolic activation, no major changes were noted in the mitotic index with either SALATRIM 23CA lot A014 or corn oil, indicating a lack of cytotoxicity (Table 5). The positive control (MMS) used in the absence of metabolic activation produced the expected positive response. The percent cells with abnormal chromosomes was analyzed statistically and did not differ from the solvent control with either fat tested. There were no increases in either the percent cells with chromatid deletion/exchange or the number of chromosomal structural aberrations per cell with either fat. Therefore, the SALATRIM fat and corn oil were not clastogenic in the absence of metabolic activation and did not present evidence of cytotoxicity.

The chromosomal aberration data for corn oil and SALATRIM 23CA lot A014 in the presence of metabolic activation are presented in Table 6. The doses used in the presence of metabolic activation were identical to those used in the absence of metabolic activation. The positive control (CP) used in the presence of metabolic activation produced the expected response. There was no change in the mitotic index with either fat compared to the solvent control. Neither fat produced increases in the percent cells with abnormal chromosomes or in any of the other criteria for chromosomal damage. Therefore, both SALATRIM 23CA lot A014 and corn oil were neither clastogenic nor cytotoxic in the chromosomal aberration assay in the presence of metabolic activation.

Induction of Unscheduled DNA Synthesis in Isolated Hepatocytes. The results from the unscheduled DNA synthesis assay for corn oil are presented in Table 7. The positive control (2-AAF) increased the net nuclear grain counts and the percent of hepatocytes undergoing DNA repair. There was no evidence of increases in these variables with corn oil when compared with the solvent control.

Unscheduled DNA synthesis data for SALATRIM 23CA lot A014 are presented in Table 8. As with corn oil, the SALATRIM fat presented no evidence of increased UDS. According to the criteria for this assay, neither fat induced UDS in isolated hepatocytes under the conditions of the assay.

HPRT Mammalian Cell Gene Mutation Assay. Results of the HPRT assay with and without metabolic activation for corn oil are shown in Table 9. Both positive controls gave the expected results, validating the assay. Corn oil did not alter either the relative cloning efficiency or the mutation frequency of the cells. On the basis of the criteria for the assay, corn oil did not induce mutagenesis in this assay.

Results of the HPRT assay, with and without metabolic activation, for SALATRIM 23CA lot A014 are presented in Table 10. The positive controls responded in the expected manner, and the SALATRIM fat presented no evidence of mutagenic potential under the conditions of the assay.

***In Vivo* Rat Bone Marrow Micronucleus Assay.** Data from the *in vivo* micronucleus assay are presented in Table 11. Because these data were collected from a 13-week subchronic toxicity study, a positive control for micronuclei formation was not included. However, a control for the high fat content of the diet (10% corn oil treatment group) was included. Comparison of the data for the two SALATRIM fats with that from the corn oil group indicated that neither SALATRIM fat increased the incidence of micronucleated polychromatic erythrocytes. Therefore, the SALATRIM fats were considered to be negative in the assay.

DISCUSSION

A number of studies using bacterial mutagenicity assays have addressed the genetic toxicology of dietary fats

Table 5. Chromosomal Aberrations in CHO Cells Treated with Corn Oil or SALATRIM 23CA Lot A014 in the Absence of Metabolic Activation[a,b]

treatment	dose (μg/mL)	mitotic index (%)	cells with abnormal chromosomes (%)	cells with chromatid deletions/exchanges[c] (%)	structural aberrations per cell
methyl methanesulfonate	110	0.9	99*	0/0	4.95
acetone (solvent control)	0	9.5	3	2/0	0.02
corn oil	500	13.8	1	1/0	0.01
corn oil	750	10.8	2	1/0	0.06
corn oil	1000	9.6	2	2/0	0.02
methyl methanesulfonate	20	4.7	18*	13/5	0.22
acetone (solvent control)	0	5.9	4	3/0	0.04
SALATRIM	250	4.8	2	1/0	0.01
SALATRIM	500	5.0	4	3/0	0.06
SALATRIM	1000	4.4	4	3/0	0.05

[a] The corn oil and SALATRIM studies were not run concurrently. [b] Data marked with an asterisk (*) are significantly different ($p \leq 0.05$) from the solvent control by Fisher's exact test. [c] Data represent the percentages based upon scoring 100 cells per experimental point; deletions are presented first, exchanges presented last.

Table 6. Chromosomal Aberrations in CHO Cells Treated with Corn Oil or SALATRIM 23CA Lot A014 in the Presence of Metabolic Activation[a,b]

treatment	dose (μg/mL)	mitotic index (%)	cells with abnormal chromosomes (%)	cells with chromatid deletions/exchanges[c] (%)	structural aberrations per cell
cyclophosphamide	110	0.7	62.5*	50/11.1	1.64
acetone (solvent control)	0	6.3	5	5/0	0.07
corn oil	500	6.5	3	1/0	0.03
corn oil	750	5.9	7	5/0	0.15
corn oil	1000	4.7	5	4/0	0.05
cyclophosphamide	50	0.4	26*	16.2/6.1	0.42
acetone (solvent control)	0	3.6	4	3/0	0.06
SALATRIM	250	3.9	0	0/0	0.00
SALATRIM	500	3.9	2	2/0	0.03
SALATRIM	1000	5.1	3	3/0	0.03

[a] The corn oil and SALATRIM studies were not run concurrently. [b] Data marked with an asterisk (*) are significantly different ($p \leq 0.05$) from the solvent control by Fisher's exact test. [c] Data represent the percentages based upon scoring 100 cells per experimental point; deletions are presented first, exchanges presented last.

Table 7. Assay for the Induction of Unscheduled DNA Synthesis Assay with Corn Oil

			preliminary assay		replicate assay	
treatment	control concn	corn oil concn (μg/mL)	NG[a]	% IR[b]	NG	% IR
control/medium			−9.3 ± 1.8	1	−15.0 ± 0.2	1
control/acetone	1%		−12.1 ± 1.4	1	−15.8 ± 3.1	1
2-AAF	3.0 μg/mL		27.4 ± 3.1	97	18.9 ± 1.5	97
corn oil		0.5	NS[c]		NT[d]	
corn oil		1.0	NS		NT	
corn oil		5.0	−11.1 ± 0.7	0	−16.3 ± 2.1	0
corn oil		10.0	−14.5 ± 1.5	0	−16.0 ± 1.5	0
corn oil		25.0	−12.3 ± 1.4	2	−14.2 ± 1.4	1
corn oil		50.0	−11.6 ± 0.7	2	−17.4 ± 3.7	0
corn oil		100.0	−14.1 ± 0.7	0	−11.8 ± 2.3	2
corn oil		250.0	−12.1 ± 2.9	0	−15.0 ± 1.1	1
corn oil		500.0	−10.9 ± 1.1	1	−12.0 ± 2.0	0
corn oil		1000.0	−14.3 ± 0.4	0	−10.6 ± 1.7	0

[a] Data represent the mean nuclear grain counts (NG) ± the standard error of the mean. [b] % IR, percentage of cells in repair. [c] NS, not scored at this concentration. [d] NT, not tested at this concentration.

Table 8. Assay for the Induction of Unscheduled DNA Synthesis with SALATRIM 23CA Lot A014

			preliminary assay		replicate assay	
treatment	control concn	SALATRIM concn (μg/mL)	NG[a]	% IR[b]	NG	% IR
control/medium			−11.8 ± 1.2	2	−12.7 ± 2.0	2
control/acetone	1%		−13.2 ± 3.6	3	−13.4 ± 3.4	4
2-AAF	0.5 μg/mL		NS[c]		NS	
2-AAF	3.0 μg/mL		18.6 ± 1.9	80	39.3 ± 14.4	90
SALATRIM		0.5	NS		NT[d]	
SALATRIM		1.0	NS		NT	
SALATRIM		5.0	−8.7 ± 1.7	2	−12.7 ± 2.0	4
SALATRIM		10.0	−15.7 ± 1.1	1	−14.0 ± 2.2	0
SALATRIM		25.0	−16.7 ± 2.3	2	−14.0 ± 2.0	2
SALATRIM		50.0	−13.5 ± 4.4	3	−13.4 ± 2.3	1
SALATRIM		100.0	−13.1 ± 0.7	2	−12.8 ± 2.0	0
SALATRIM		250.0	−13.2 ± 2.1	0	−14.4 ± 2.2	1
SALATRIM		500.0	−14.1 ± 3.3	1	−14.7 ± 2.0	2
SALATRIM		1000.0	−14.4 ± 1.1	1	−13.4 ± 2.9	0

[a] Data represent the mean nuclear grain counts (NG) ± the standard error of the mean. [b] % IR, percentage of cells in repair. [c] NS, not scored at this concentration. [d] NT, not tested at this concentration.

(Hageman et al., 1990, 1991; Van Gastel et al., 1984; Taylor et al., 1983; Kensese et al., 1989; Hayes and Riccio, 1994). These studies have indicated there is no genetic toxicology associated with common dietary fats unless they have either been significantly oxidized or subjected to high heat for prolonged time periods. The mutagenicity associated with abused fats apparently results from hydroperoxide formation at the double bonds of unsaturated fatty acids. Hayes and Riccio (1994) demonstrated that corn oil and six SALATRIM fats were not mutagenic with five *Salmonella* strains, with and without metabolic activation.

A number of the chemical components of SALATRIM and other triacylglycerols have been shown to have little or no potential for genotoxicity. Glycerol has been evaluated for genotoxicity using a wide range of genotoxicity assays. Doolittle et al. (1988) tested glycerol in the Ames assay and a number of mammalian cell assays including the sister chromatid exchange, the chromosomal aberration assay, HPRT gene mutation, and unscheduled DNA synthesis in isolated rat hepatocytes. Others have tested glycerol in a number of genetic toxicology assays (Cotruvo et al., 1977; Shimuzu et al., 1985; Ishidate et al., 1984), and the combined data indicate that glycerol is without genotoxic potential.

The short-chain fatty acid components of SALATRIM have been tested for their genotoxic potential by a number

Table 9. Results of the HPRT Mammalian Cell Mutation Assay of Corn Oil with and without Metabolic Activation[a]

	without metabolic activation		with metabolic activation	
dose (μg/mL)	rel cloning efficiency (% of control)	mutant freq (per 10^6 cells)	rel cloning efficiency (% of control)	mutant freq (per 10^6 cells)
corn oil				
0	100	10	100	16
327.7	91	11	88	24
409.6	98	10	97	17
512	104	9	90	20
640	81	12	84	21
800	81	5	83	21
1000	91	6	79	15
EMS[b]				
200	77	198		
3MC[c]				
5			79	189

[a] Data represent the mean of two cultures with the exception of the 0 dose (solvent control), which represents the mean of triplicate cultures. [b] Ethyl methanesulfonate; positive control for the assay without metabolic activation. [c] 3-Methylcholanthrene; positive control for the assay with metabolic activation.

Table 10. Results of the HPRT Mammalian Cell Mutation Assay of SALATRIM 23CA Lot A014 with and without Metabolic Activation[a]

	without metabolic activation		with metabolic activation	
dose (μg/mL)	rel cloning efficiency (% of control)	mutant freq (per 10^6 cells)	rel cloning efficiency (% of control)	mutant freq (per 10^6 cells)
SALATRIM				
0	100	15	100	10
31.3	96	7	94	7
62.5	100	18	84	6
125	89	11	93	10
250	90	17	97	15
500	78	12	69	9
1000	86	17	75	10
EMS[b]				
200	56	170		
3MC[c]				
5			79	170

[a] Data represent the mean of two cultures with the exception of the 0 dose (solvent control), which represents the mean of triplicate cultures. [b] Ethyl methanesulfonate; positive control for the assay without metabolic activation. [c] 3-Methylcholanthrene; positive control for the assay with metabolic activation.

Table 11. Percent Micronucleated Polychromatic Erythrocytes in Bone Marrow of Rats Fed Diets Containing 10% SALATRIM 234CA Lot A019, SALATRIM 234CS Lot A018, and Corn Oil for 13 Weeks[a,b]

treatment	males (% MN/1000 PCE)[c]	females (% MN/1000 PCE)	combined male and female (% MN/1000 PCE)
corn oil	0.18 ± 0.11	0.17 ± 0.13	0.17 ± 0.12
SALATRIM 234CA lot A019	0.25 ± 0.17	0.17 ± 0.11	0.21 ± 0.14
SALATRIM 234CS lot A018	0.21 ± 0.17	0.12 ± 0.12	0.16 ± 0.14

[a] Data represent the mean ± standard deviation for 18–20 rats per sex per group. [b] Data significantly different from the corn oil control ($p \leq 0.05$) are noted with an asterisk (*). [c] MN, micronucleated cells; PCE, polychromatic erythrocytes.

of investigators. Acetic acid was negative in bacterial mutagenicity assays and in *Saccharomyces cerevisiae* (Cotruvo et al., 1977; Ishidate et al., 1984; Litton Bionetics, 1975; McCann et al., 1975; McCarroll et al., 1981). Acetic acid produced no evidence of chromosomal damage in studies by Ishidate et al. (1984) but was noted to produce some damage by Brusick (1986), who attributed the damage to a pH effect. Propionic acid was found to be negative in bacterial mutagenicity assays (Litton Bionetics, 1976; Basler, 1987). A positive result in the *Escherichia coli* DNA repair assay was noted by Basler (1987). Propionic acid did not produce activity in the sister chromatid exchange assay (Basler, 1987). Butyric acid does not appear to have been as thoroughly tested in genotoxicity assays as acetic and propionic acids. Some evidence of DNA damage in human HeLa cells and chick fibroblasts has been reported (Hagopian et al., 1977).

Taken together, the reported studies of the genotoxic potential of fats and the chemical moieties comprising the SALATRIM family of triacylglycerols indicate SALATRIM fats should have little or no potential for genotoxicity. A previous study indicated no evidence of mutagenic potential for SALATRIM fats in a bacterial test system (Hayes and Riccio, 1994). The studies reported here have extended the previous study by using mammalian cell *in vitro* assays that measure different genotoxicity mechanisms. The low solubility of fats limits the maximal dose that can be tested in the *in vitro* genotoxicity assays. Because of this limitation and to test the genotoxic potential of the SALATRIM fats in a whole animal model, an *in vivo* genotoxicity assay using rats was conducted.

SALATRIM 23CA lot A014 did not produce chromosomal aberrations, indicating a lack of clastogenic potential. This SALATRIM fat failed to produce evidence of mutagenic potential in a mammalian cell line, in agreement with its lack of mutagenicity in bacteria. No evidence of DNA damage was detected with this fat when assessed by unscheduled DNA synthesis.

The *in vitro* studies were extended to an *in vivo* assay measuring the ability of two additional SALATRIM fats to produce micronucleated polychromatic erythrocytes in bone marrow. This *in vivo* study is significant because the two SALATRIM fats were fed to rats at high doses (10% of the diet) for a prolonged period (13 weeks). This study is also significant because all mechanisms of digestion, absorption, metabolism, distribution, and excretion were operational in the intact animal. Micronucleated polychromatic erythrocytes did not differ between the SALATRIM fats and the corn oil control, and all of the data were within the range of historical control data for this assay. Therefore, the data from the *in vivo* genetic toxicology assay confirm the data from the *in vitro* genetic toxicology assays.

These studies were done to test the hypothesis, based upon structure/activity relationships and review of the scientific literature, that members of the SALATRIM family of structured triacylglycerols should have no capability of interacting with mammalian cell DNA to produce genetic changes. The previous studies of SALATRIM fats using bacterial test systems (Hayes and Riccio, 1994) and the studies reported here using *in vitro* mammalian cell cultures and an *in vivo* rat study support the conclusion that SALATRIM fats should possess no genotoxic potential.

LITERATURE CITED

AOCS. Free Fatty Acids. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed.; Firestone, D., Ed.; AOCS: Champaign, IL, 1990a; Method Ca 5a-40.

AOCS. Peroxide Value. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed.; Firestone, D., Ed.; AOCS: Champaign, IL, 1990b; Method Cd 8-53.

Basler, A.; Von der Hude, W.; Scheutwinkel, M. Screening of the Food Additive Propionic Acid for Genotoxic Properties. *Food Chem. Toxicol.* **1987**, *25*, 287–290.

Benzonana, G.; Desnuelle, P. Action of Some Effectors on the Hydrolysis of Long Chain Triglycerides by Pancreatic Lipase. *Biochim. Biophys. Acta* **1968**, *164*, 47–58.

Brusick, D. Genotoxic Effects in Cultured Mammalian Cells Produced by Low pH Treatment Conditions and Increased Ion Concentrations. *Environ. Mutagen.* **1986**, *8*, 879–886.

Carey, M. C.; Small, D. M.; Bliss, C. M. Lipid Digestion and Absorption. *Annu. Rev. Physiol.* **1983**, *45*, 651–677.

Cotruvo, J. A.; Simmon, V. F.; Spanggord, R. J. Investigation of Mutagenic Effects of Products of Ozonation Reactions in Water. *Ann. N.Y. Acad. Sci.* **1977**, *298*, 124–140.

Doolittle, D. J.; Lee, D. A.; Lee, C. K. The Genotoxic Activity of Glycerol in an *In Vitro* Test Battery. *Food Chem. Toxicol.* **1988**, *26*, 631–635.

Finley, J. W.; Klemann, L. P.; Leveille, G. A.; Otterburn, M. S.; Walchak, C. G. Caloric Availability of SALATRIM in Rats and Humans. *J. Agric. Food Chem.* **1994**, one of several papers in this issue.

Gacs, G.; Barltrop, D. Significance of Ca-Soap Formation for Calcium Absorption in the Rat. *Gut* **1977**, *18*, 64–68.

Gad, S.; Weil, C. S. Hypothesis Testing: Categorical and Ranked Data. *Statistics and Experimental Design for Toxicologists*; CRC Press: Boca Raton, FL, 1991; pp 53–69.

Galloway, S. M.; Bloom, A. D.; Resnick, M.; Margolin, B. H.; Nakamura, F.; Archer, P.; Zeiger, E. Development of a Standard Protocol for *In Vitro* Cytogenetic Testing with Chinese Hamster Ovary Cells: Comparison of Results for 22 Compounds in Two Laboratories. *Environ. Mutagen.* **1985**, *7*, 1–51.

Hageman, G.; Hermans, R.; Ten Hoor, F.; Kleinjans, J. Mutagenicity of Deep-Frying Fat, and Evaluation of Urine Mutagenicity After Consumption of Fried Potatoes. *Food Chem. Toxicol.* **1990**, *28*, 75–80.

Hageman, G.; Verhagen, H.; Schutte, B.; Kleinjans, J. Biological Effects of Short-Term Feeding to Rats of Repeatedly Used Deep-Frying Fats in Relation to Fat Mutagen Content. *Food Chem. Toxicol.* **1991**, *29*, 689–698.

Hagopian, H. K.; Riggs, M. G.; Swartz, L. A.; Ingram, V. M. Effect of n-Butyrate on DNA Synthesis in Chick Fibroblast and HeLa Cells. *Cell* **1977**, *12*, 855–860.

Hashim, S. A.; Babayan, V. K. Studies in Man of Partially Absorbed Dietary Fats. *Am. J. Clin. Nutr.* **1978**, *31*, S273–S276.

Hayes, J. R.; Riccio, E. S. Genetic Toxicology Studies of SALATRIM Structured Triacylglycerols: 1. Lack of Mutagenicity in the *Salmonella*/Microsome Reverse Mutation Assay. *J. Agric. Food Chem.* **1994**, one of several papers in this issue.

Hayes, J. R.; Wilson, N. H.; Pence, D. H.; Williams, K. D. Subchronic Toxicity Studies of SALATRIM Structured Triacylglycerols in Rats. 3. Triacylglycerols Composed of Stearate, Acetate, Propionate, and Butyrate. *J. Agric. Food Chem.* **1994**, one of several papers in this issue.

Ishidate, M., Jr.; Sofuni, T.; Yoshikawa, K.; Hayashi, M.; Nohmi, T.; Sawada, M.; Matsuoka, A. Primary mutagenicity screening of food additives currently used in Japan. *Food Chem. Toxicol.* **1984**, *22*, 623–636.

Jensen, R. G.; Clark, R. M.; deJong, F. A.; Hamosh, M.; Liao, T. H.; Mehta, N. R. The Lipolytic Triad: Human Lingual, Breast Milk, and Pancreatic Lipases: Physiological Implications of Their Characteristics in Digestion of Dietary Fats. *J. Pediatr. Gastroenterol. Nutr.* **1982**, *1*, 243–255.

Kensese, S. M.; Teng, J. I.; Smith, L. L. Mutagenic Lipid Peroxides from Edible Oils. *Teratog. Carcinog. Mutagen.* **1989**, *9*, 133–145.

Li, A. P.; Shimizu, R. W. A Modified Agar Assay for the Quantitation of Mutation at the Hypoxanthine Guanine Phosphoribosyl Transferase Gene Locus in Chinese Hamster Ovary Cells. *Mutat. Res.* **1983**, *111*, 365–370.

Litton Bionetics. "Mutagenic Evaluation of Compound, FDA 74-3 Sodium Acetate 3-H_2O, NF Granular"; U.S. Department of Commerce, NTIS No. PB-254 514; National Technical Information Service: Springfield, VA, 1975.

Litton Bionetics, "Mutagenic Evaluation of Compound, Compound FDA 75-62 Propionic Acid"; U.S. Department of Commerce, NTIS No. PB-266-879; National Technical Information Service: Springfield, VA, 1976.

McCann, J.; Choi, E.; Yamasaki, E.; Ames, B. N. Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 5135–5139.

McCarroll, N. E.; Piper, C. E.; Keech, B. H. An *E. coli* Microsuspension Assay for the Detection of DNA Damage Induced by Direct-Acting Agents and Promutagens. *Environ. Mutagen.* **1981**, *3*, 429–444.

Schmid, W. The Micronucleus Test for Cytogenetic Analysis. In *Chemical Mutagens: Principles and Methods for Their Detection*; Hollander, A., Ed.; Plenum: New York, 1976; Vol. 4, pp 31–53.

Shimuzu, H.; Suzuka, Y.; Takemura, N.; Goto, S.; Matsushita, H. The Results of Microbial Mutation Test for Forty-Three Industrial Chemicals. *Jpn. J. Ind. Health* **1985**, *27*, 400–419.

Softly, B.; Huang, A.; Finley, J.; Templeman, G.; Otterburn, M. Comparison of the Composition of thre SALATRIM Family of Edible Fats. *J. Agric. Food Chem.* **1994**, one of several papers in the issue.

Sokal, R. R.; Rohlf, J. J. *Biometry*; Wilson, J., Cotter, S., Eds.; Freeman: New York, 1981.

Taylor, S. L.; Berg, C. M.; Shoptaugh, N. H.; Traisman, E. Mutagen Formation in Deep-Fat Fried Foods as a Function of Frying Conditions. *J. Am. Oil Chem. Soc.* **1983**, *60*, 576–580.

Van Gastel, A.; Mathur, R.; Roy, V. V.; Rukmini, C. Ames Mutagenicity Test of Repeatedly Heated Edible Oils. *Food Chem. Toxicol.* **1984**, *22*, 403–405.

Williams, G. M. Detection of Chemical Carcinogens by Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures. *Cancer Res.* **1977**, *37*, 1845–1851.

Received for review August 2, 1993. Accepted October 28, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, January 1, 1994.