JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

2-Dodecylcyclobutanone Does Not Induce Mutations in the *Escherichia coli* Tryptophan Reverse Mutation Assay

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Like thermal processing, ionizing radiation can break molecular bonds and induce the formation of chemicals not found in the unprocessed product. Irradiation of foods containing palmitic acid can lead to the formation of 2-dodecylcyclobutanone (2-DCB). In this study, the *Escherichia coli* tryptophan reverse mutation assay was used to evaluate the capacity of 2-DCB to induce mutations. *E. coli* tester strains WP2 (pkM101) and WP2 *uvrA* (p*K*M101), with and without exogenous metabolic activation, were exposed to 0, 0.05, 0.1, 0.5, and 1 mg/well 2-DCB using the Miniscreen version of the assay. 2-DCB did not induce mutations in the *E. coli* tryptophan reverse mutation assay. These results are in agreement with negative results obtained in short-term and long-term genetic toxicology tests of irradiated food products.

KEYWORDS: Ionizing radiation; cyclobutanones; 2-DCB; mutations; food

INTRODUCTION

Safety testing of irradiated foods has included over 1000 long-term and short-term toxicology tests that have been reviewed by international regulatory agencies (*1*−*3*). Review of those studies has determined irradiated foods to be safe for human consumption. Ionizing radiation, like other food processing technologies, can produce changes in food chemistry. Exposure of fat containing foods to ionizing radiation leads to the formation of a class of compounds known as the alkylcyclobutanones, which are not detectable in nonirradiated food products (*4*, *5*). Cleavage of the acyl-oxygen bonds of palmitic acid by ionizing radiation can lead to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid with an alkyl group in the second ring position and named 2-dodecylcyclobutanone (2-DCB) (*6*). 2-DCB is produced in trace quantities (≤0.1 μg 2-DCB/g fat) in irradiated meats (*4*, *5*, *7*, *8*).

In recent work using purified 2-DCB in the comet assay (*9*−*11*), it was reported that 2-DCB induced DNA strand breaks in rodent and human intestinal cells and raised the possibility that the compound was a weak genotoxin. The authors themselves discussed the equivocal nature of the results, the limitations of the test system used, and cautioned against misinterpretation of the results, including interpretation of the data to infer that 2-DCB is a carcinogen (*9*−*11*). Scientific review of those results by international regulatory agencies indicated that the reports of 2-DCB-induced genotoxicity could not be supported based on the data and were contradictory in nature to the negative results obtained in both short-term and long-term genetic toxicology studies using irradiated foods (*12*, *13*). Unfortunately, some groups have misinterpreted those studies (*9*−*11*) and erroneously claimed them as proof that irradiated foods are carcinogenic (*14*).

The *Escherichia coli* tryptophan reverse mutation (Trp) assay, developed in 1976 by Green and Muriel (*15*), reports the ability of test compounds to induce reversion of the *trpE65* mutation in *E. coli* from auxotrophy to prototrophy. Reversion of the *trpE65* mutation can occur via a number of genetic pathways (*16*). The *E. coli* Trp assay, as is the *Salmonella* mutagenicity test, is accepted as a validated short-term genotoxicity test by international regulatory agencies (*17*). In this work, the ability of 2-DCB to induce mutations in the *E. coli* Trp reverse mutation assay (*E. coli* Trp assay) was examined.

MATERIALS AND METHODS

Strains. *E. coli* Trp reversion strains WP2 (pKM101) and WP2 *uvrA* (pKM101) were purchased from Moltox, Inc. (Boone, NC). Upon receipt, the tester strains were tested for Trp dependence, ampicillin resistance, UV sensitivity, and spontaneous reversion frequency (*15*, *17*). The strains were propagated on Vogel−Bonner Minimal medium supplemented with 25 μg/mL Trp and stored at 0−2 °C for up to 1 week before use in assays (*15*, *17*). Strains were grown, from single colonies, in 100 mL of sterile nutrient broth in 500 mL baffled Erlenmeyer flasks (37 °C, 150 rpm) for approximately 16 h for use in the assays.

Media and Media Components. Nutrient broth was obtained from Difco, Inc. (Sparks, MD). Vogel−Bonner salts were obtained from Moltox, Inc. Sodium chloride, glucose, and Trp were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Sterile six well microtiter plates (35 mm diameter) (Corning, Inc., Corning, NY) were prepared by dispensing 5 mL of sterile minimal agar per well (*18*−*20*). Sterile top agar was melted using a microwave oven and cooled to 45 °C in a heated water bath, and filter-sterilized Trp was added to a final concentration of 1 μg/mL (*15*, *17*).

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10.1021/jf030348o
Published on Web 09/12/2003

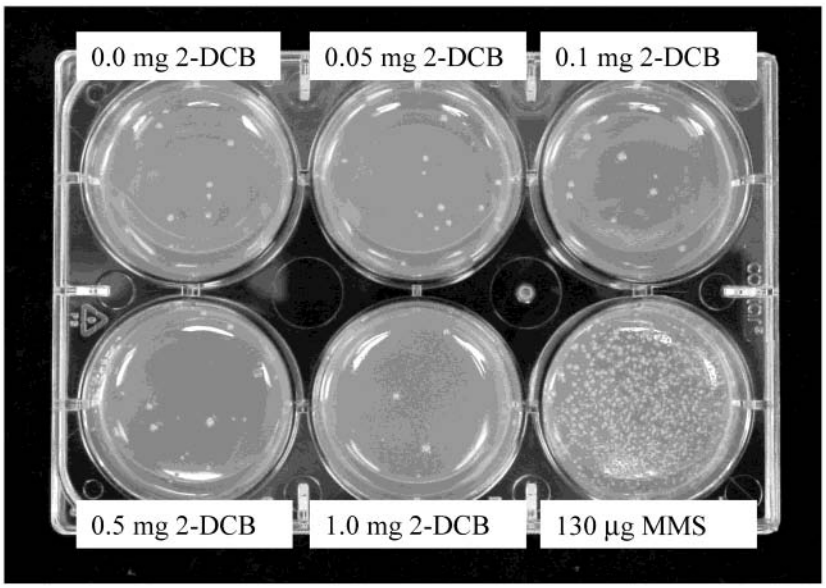

Figure 1. Mictotiter plate setup for testing of 2-DCB in the *E. coli* Trp reverse mutation assay. Three test plates were utilized per culture, WP2 (pKM101) or WP2 *uvrA* (pKM101), tested ($n = 3$). Three independent cultures were tested per strain. Test compound concentrations included the negative (solvent) control, 0.05 mg of 2-DCB, 0.1 mg of 2-DCB, 0.5 mg of 2-DCB, and positive control (130 μg of MMS or 33 μg of 2-AA) per well.

Table 1. Induction of Mutation in the *E. coli* Trp Reverse Mutation Assay with or without Exogenous Metabolic Activation (5% S9 Fraction) by 2-DCB

		2-DCB/Trp$^+$ revertant colonies per well[a]					control
strain	S9 fraction (%)	0 mg	0.05 mg	0.10 mg	0.50 mg	1.00 mg	2-AA or MMS
WP2 (pKM101)	0	4.11 ± 0.99	5.55 ± 1.64	7.56 ± 1.75	4.78 ± 1.33	5.22 ± 1.57	146 ± 14.7
WP2 (pKM101)	5	2.11 ± 0.11	1.89 ± 0.29	1.89 ± 0.48	1.11 ± 0.40	1.67 ± 0.19	32.4 ± 0.73
WP2 *uvrA* (pKM101)	0	8.11 ± 2.73	6.33 ± 3.23	7.00 ± 2.52	9.00 ± 2.65	9.44 ± 3.23	141 ± 9.45
WP2 *uvrA* (pKM101)	5	9.22 ± 1.37	8.44 ± 2.50	9.44 ± 2.50	8.67 ± 0.69	9.22 ± 0.69	116 ± 2.67

[a] The number of *Trp*$^+$ revertant colonies per well represents the mean of three independent cultures ($n = 3$) followed by the standard error of the mean for those values.

Chemicals. The positive control compound methyl methanesulfonate (MMS) (CAS No. 66-27-3) was obtained from Sigma-Aldrich, Inc. The positive control compound 2-aminoanthracene (2-AA) (CAS No. 613-13-8) was obtained from Moltox, Inc. 2-DCB (CAS No. 35493-46-0), analytical testing grade (>95%) for detection of irradiated foods by regulatory agencies, was obtained from Sigma-Aldrich, Inc. 2-DCB was suspended in dimethyl sulfoxide as previously described by Delincee and Pool-Zobel (*9*), as were the MMS and 2-AA positive control compounds. 2-DCB concentrations used in the assay were 1.0, 0.5, 0.1, and 0.05 mg/well. MMS was used at 130 μg/well while 2-AA was used at 33 μg/well. MMS does not require metabolic activation, while 2-AA requires metabolic activation (S9 fraction) and acts as a control for S9 fraction function. Microtiter plate set up is shown in **Figure 1**.

Exogenous Metabolic Activation. The S9 fraction from Aroclor 1254-induced rats was obtained from Moltox, Inc., as was NADPH regeneration system components A and B. The S9 fraction (5% solution) was prepared immediately before performance of the assays (*21*, *22*). The S9 fraction solution was maintained on ice during the assay procedure.

Assay Procedure. Because of the expense of 2-DCB (>$12 000 per gram), the microtiter plate-based miniscreen assay (*18*−*20*), which requires reduced amounts of test compound, was utilized. In short, 500 μL of top agar (45 °C), 20 μL of test compound (solvent alone, solvent with 2-DCB, or solvent with positive control compound), 20 μL of overnight culture, and 100 μL of 5% S9 solution (if required) were combined, mixed by vortexing, and dispensed into the well of a microtiter plate that contained 5 mL of minimal agar. After the top agar solidified for 1 h, the plates were incubated at 37 °C for 2 days and the colonies per well were scored using a calibrated AccuCount 1000 colony counter (AccuCount, Inc., Gainesville, VA). Cytotoxicity was determined by examination of bacterial lawn turbidity in the top agar, following the 2 day incubation (*15*, *21*), which is standard for the bacterial reverse mutation assays.

Statistical Analysis. Three replicate microtiter plates were used per bacterial tester strain culture. Each experiment was performed independently three times. Data were analyzed using the statistics package of Microsoft Excel Office 2000 (Microsoft, Inc. Redmond, WA).

RESULTS AND DISCUSSION

2-DCB (with or without exogenous metabolic activation) did not induce reversion of the *trpE* mutation in tester strains WP2 (pKM101) or WP2 *uvrA* (pKM101) as determined by analysis of variance and Students *t*-test. ($n = 3$, $\alpha = 0.05$) (**Table 1**). No effect on viability was observed by examination of the bacterial lawn turbidity in the top agar. Results for the negative control (solvent) and positive controls (130 μg/well MMS or 33μg/well 2-AA) were consistent with historical data (*18*−*20*). The concentration of 1 mg/well 2-DCB in the miniscreen assay is the equivalent of the maximum allowed concentration (5 mg/plate) in the standard plate incorporation assay (*15*, *18*).

These results are in contrast to studies (*9*−*11*) in which 2-DCB was reported as a potential genotoxin, due to a weak response in the comet assay, which measures increases in DNA strand breakage as its end point. Because the comet assay is not validated for detection of weak mutagens and can produce "false positive" results when cell viability is reduced (*13*, *22*) and the high 2-DCB concentrations (1.49 mg/kg/bw or ≥1.25 mg/mL) used (*9*−*11*), the authors cautioned against misinter-

pretation of the results of those studies. A review of the reports by the European Commission Scientific Committee on Food and Health Canada (*12*, *13*) did not support the claim of genotoxity for 2-DCB for reasons including: "The genotoxicity of 2-ACB's has not been established by the standard genotoxicity assays...". The reviews (*12*, *13*) also noted the disparity between short-term and long-term genetic toxicology studies using irradiated meats vs the equivocal results obtained with 2-DCB using the comet assay.

There is a considerable body of research pertaining to the testing of food additives, and food processing-induced compounds, in bacterial reverse mutation assays. Mutagenic activity in thermally processed foods has been well-established (*24*−*26*). A number of these studies have confirmed the mutagenicity of cooked meats and their fats (*27*−*29*). In contrast, irradiated meats have tested negative in bacterial reverse mutation assays (*1*, *2*). In the study presented herein, the maximum allowable concentration of 2-DCB, a compound produced by radiolysis of fat-containing foods did not induce mutations in the *E. coli* Trp reverse mutation assay. The negative results obtained in this study are consistent with short-term and long-term genetic toxicology tests using irradiated foods, which included multigenerational/multispecies long-term feeding studies using diets of irradiated foods, conducted over a 40 year period (*1*−*3*).

Future work will include testing of 2-DCB with and without exogenous metabolic activation in the *Salmonella* mutagenicity test, which is typically used in combination with the *E. coli* Trp reverse mutation assay. The yeast del assay, an intrachromosomal recombination (chromosome aberration) assay that responds to DNA strand breaks as its inducer, but does not produce false positive results due to cytotoxicity, will also be utilized.

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Received for review May 9, 2003. Revised manuscript received July 31, 2003. Accepted August 3, 2003. Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

JF030348O