

Report by the *Bundesforschungsanstalt für Ernährung*
[Federal Nutrition Research Institute]
BFE-R—99-01

Food Irradiation
Fifth German Conference

Editors:
M. Knörr, D.A.E. Ehlermann and H. Delincée

Conference on November 11 & 13, 1998
BFE, Karlsruhe

Bundesforschungsanstalt für Ernährung
Karlsruhe

1999

Translated from the German by Public Citizen, Washington, D.C.
February 2001

Genotoxicity of 2-dodecylcyclobutanone

Henry Delincée, Beatrice-Louise Pool-Zobel and Gerhard Rechkemmer

Institut für Ernährungsphysiologie der Bundesforschungsanstalt für Ernährung
[Institute for Nutritional Physiology of the Federal Nutrition Research Institute]
Haid-und-Neu-Str. 9, D-76131 Karlsruhe

Summary

In the treatment of foods containing fat with ionizing radiation — for example, the irradiation of chicken or hamburger to kill pathogens such as *Salmonella spp.* or *E. coli* O.157:H7 — a range of lipolytic digestion products are generated, among them the group of 2-alkylcyclobutanones. These compounds contain the same number (n) of carbon atoms as their precursor fatty acids, whereby a hydrocarbon chain with n-4 carbon atoms is attached to ring position 2 of the cyclobutanone. In this way, 2-dodecylcyclobutanone is generated from palmitic acid. Up to the present day, cyclobutanones have not been found in non-irradiated foods. Therefore, it is important to examine the toxic or genotoxic potential of cyclobutanones in the context of discussions about the safety of irradiated foods

In this study, *in vivo* experiments were conducted on rats, which received two different doses of 2-dodecylcyclobutanones by way of pharyngeal probe. After 16 hours, colon cells were isolated from the rat and analyzed for DNA damage by means of the comet assay.

No cytotoxic effects were detected in the trypan blue vitality test. When the “% tail intensity” or the “tail moment” was used in the comet assay for quantitative analysis, the values obtained with an experimental group that received a low concentration of 2-dodecylbutanone (1.12 mg/kg body weight) were similar to those of the control group, which was administered 2% dimethyl sulfoxide. Slight but significant DNA damage was observed in the experimental group that received the higher concentration of 2-dodecylcyclobutanone (14.9 mg/kg body weight). Further studies are needed to clarify the relevance of these results to an evaluation of risk from the consumption of irradiated foods.

Introduction

Of late there has been growing interest in the treatment of foods with ionizing radiation. The irradiation can help improve the hygienic quality of the food and prevent diseases that otherwise could be caused by consumption of foods contaminated with parasites or pathogenic microorganisms. Furthermore, the irradiation of certain foods facilitates an improvement in the storage life and reduces the spoilage rate [Diehl, 1995]. A growing number of countries have approved the use of ionizing radiation for numerous products [Anon., 1998]. Within the EU, one can expect harmonization of the legal regulations of the member states with regard to foods and food components treated with ionizing radiation. As a first step, irradiation of dried aromatic herbs and spices is to be permitted in all EU nations. This development is based in part on the positive evaluation of the procedure by the World Health Organization. In a 1992 position

statement, WHO stated that “foods that have been treated with ionizing radiation and produced according to good manufacturing practice (GMP) are to be regarded as safe in terms of health and satisfactory from the perspective of nutritional physiology.” Numerous studies and animal feeding experiments, as well as experiments on volunteer test subjects, support this conclusion [WHO, 1994]. Taking account of the studies available to date, a new expert committee concluded in 1997 that “even irradiation of foods with high doses (> 10 kGy) may be judged safe and satisfactory in terms of nutrition” [WHO, 1997, 1998]. In recent years, there has also been increasing interest in analytical techniques to determine whether a product has been irradiated [Delincée, 1998]. For example, a research team in Northern Ireland has determined that certain lipolytic digestion products — namely, the 2-alkylcyclobutanones [LeTellier and Nawar, 1972] — might be products that are unique to irradiation and therefore hold great promise as markers of irradiation treatment [Stevenson et al., 1990, Stevenson, 1996]. As a result of irradiation, the acyl-oxygen bond in triglycerides is cleaved, with formation of 2-alkylcyclobutanones with the same number of carbon atoms as the initial fatty acid and with the alkyl group in ring position 2. For example, 2-dodecylcyclobutanone and 2-tetradecylcyclobutanone are formed from palmitic acid and stearic acid, respectively. Although 2-methylcyclobutanone has been identified following ultrasound treatment of *Hevea brasiliensis* latex, for example [Nishimura et al., 1977], cyclobutanones have not yet been detected in non-irradiated foods [Stevenson, 1996]. However, since cyclobutanones do occur in irradiated foods — for example, at levels of 0.3-0.6 µg 2-dodecylcyclobutanone/g fat/kGy in chickens [Stevenson et al., 1990, 1993; Boyd et al., 1991; Crone et al., 1992 a, b, 1993; Stevenson, 1996] — it is necessary to characterize their potentially toxic features and undertake a risk evaluation.

In this study, the so-called “comet assay,” a new test procedure that detects DNA damage in individual cells by means of microgel electrophoresis, has been employed as the toxicological test procedure [McKelvey-Martin et al., 1993; Fairbairn et al., 1995]. Rat colon cells¹, tissue in which tumors can be generated under certain nutritional conditions, were used as the target cells.

¹ [Translator’s note] The German “Dickdarm” used here can be translated “large intestine” or “colon” (the latter a segment of the former); “colon” has been translated since the authors used the unmistakable “Kolon” in the same context in the “Summary.”

Materials and Methods

Materials

The test substance, 2-dodecylcyclobutanone (2-DCB) was synthesized according to the specifications of Boyd et al. (1991).

In vivo experiment

Male Sprague-Dawley rats (≈ 250 g) were obtained from Charles River Wiga GmbH (D-97633 Sulzfeld) and kept under the usual conditions. The rats were randomly divided into 4 groups. Two groups of six animals each received 2-DCB via pharyngeal probe: the first group received 1.12 mg/kg body weight (BW), the second group 14.9 mg/kg BW. A group of three animals served as negative control, and received the solvent of 2-DCB, namely 2% dimethyl sulfoxide (DMSO) in physiological sodium chloride solution (5 ml/kg BW). The fourth group with three animals was employed as positive control, and received 15 mg 1,2-dimethylhydrazine (DMH)/kg BW (dissolved in physiological sodium chloride solution, 5 ml/kg BW). The feeding and treatment regimen employed here has been described (Pool-Zobel et al., 1996). After 16 hours of exposure — which was determined to be the optimal period of time for the formation of DNA damage in colon cells caused by DMH and measurable by the comet assay [Pool-Zobel, 1996] — the colon was removed from the rats and the colon cells isolated by means of enzymatic digestion [Brendler-Schwaab et al., 1994].

Cytotoxicity

The potential cytotoxicity of 2-DCB to the cells of the colon was checked with the aid of the trypan blue vitality test, a rapid and simple method to differentiate between living and non-living cells [Pool et al., 1990; Pool-Zobel et al., 1994].

Comet assay

DNA damage to the colon cells was determined by means of single-cell microgel electrophoresis (comet assay) [Pool-Zobel et al., 1994; Pool-Zobel and Leucht, 1997]. For each data point, 50 cells per slide and 3 slides per determination were analyzed. The evaluation was carried out on a fluorescence microscope with the image processing system of Perceptive Instruments (Halstead, Great Britain). The DNA distribution in the comet was calculated as “% tail intensity” and “tail moment” — the latter a product of the proportion of DNA in the tail and the length of the comet tail [Fairbairn et al., 1995]. With more severe damage to the DNA, the proportion of DNA in the tail, and hence also the “% tail intensity” and “tail moment,” increase.

Determination of the quantity of substance administered

Two different concentrations of 2-DCB were selected. The low concentration was meant to model radiation pasteurization (e.g. with 3 kGy), while the higher concentration was intended to represent radiation sterilization (60 kGy).

For the radiation pasteurization (3 kGy) of fresh chicken, we assumed formation of ≈ 1.5 μg of 2-DCB/g fat. Since palmitic acid represents only about 1/5 of the fatty acids in chicken, the total quantity of cyclobutanones was roughly projected to be 5 times as great. If one assumes at the same time that all of the fat that a person consumes is irradiated (according to the DGE²-

² DGE = Deutsche Gesellschaft für Ernährung = German Nutrition Association

Nutrition Report 1996, a man weighing 70 kg consumes an average of 104 g fat/day, or 1.49 g fat/kg BW), this would lead to a 2-DCB content of $1.5 \mu\text{g} \times 5 \times 1.49 = 11.2 \mu\text{g}$ of 2-DCB/kg BW.

With a safety factor [Classen et al., 1987] of 10 for individual differences, and an additional factor of 10 to account for differences between various species (here, rat/human), the expected no-effect level (NOEL) for radiation pasteurization lies at

$$11.2 \mu\text{g} \times 10 \times 10 = 1.12 \text{ mg 2-DCB/kg BW.}$$

Similarly, one would expect a NOEL of

$$20 \mu\text{g} \times 5 \times 1.49 \times 10 \times 10 = 1.49 \text{ mg 2-DCB/kg BW}$$

for the radiation sterilization (60 kGy) of frozen chicken. This calculation is based on formation of $\approx 20 \mu\text{g}$ of 2-DCB/g fat for radiation sterilized (60 kGy), frozen (-46°C) chicken [Crone et al., 1992a].

Results

The trypan blue vitality test did not reveal any cytotoxic effects on the colon cells from the 2-DCB that was administered. The vitality of the treated cells was on the same order of magnitude ($\approx 90\%$) as the cells of the negative control group, which were treated with DMSO alone.

On the other hand, DNA damage from 2-DCB was observed in the comet assay. In the evaluation of the comets, both as “% tail intensity” and as “tail moment,” the DNA damage exceeded that found in the negative control group. In the group of six animals that received the lower concentration of 1.12 mg 2-DCB/kg BW, two of the animals exhibited increased DNA damage, while four of the animals exhibited values like those of the control group (Fig. 1a).

When the results of the experimental group animals were combined, there was no significant difference relative to the negative control group (Fig. 1b). At the higher concentration of 14.9 mg 2-DCB/kg BW, an increased level of DNA damage was also detectable in the group, relative to the negative control group (Fig. 1b). While the increase in DNA damage is slight compared to the positive control group, which received DMN as alkylating agent, one must recall that the latter is a strong and specific rat colon carcinogen.

Fig. 1a Effect on Individual Animals

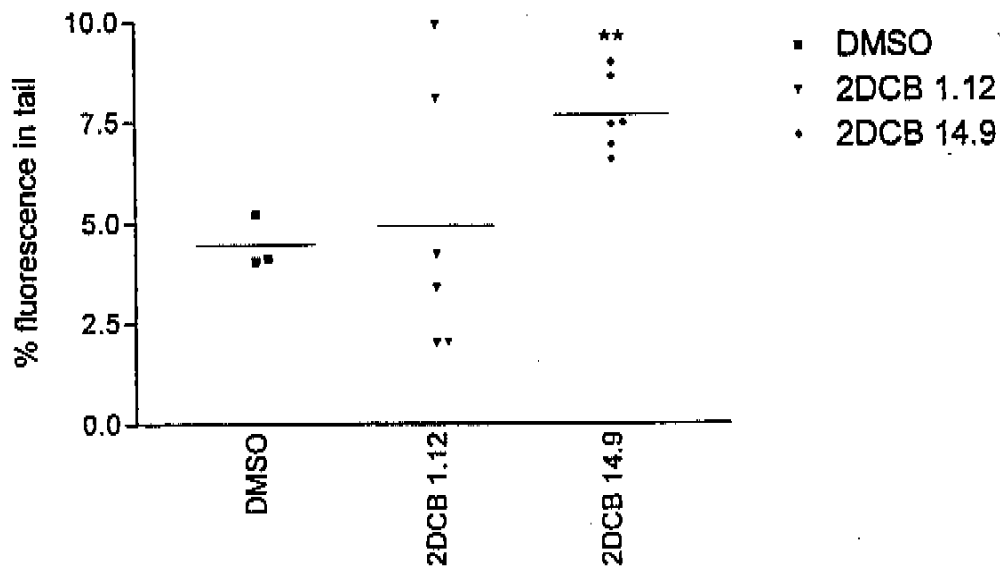
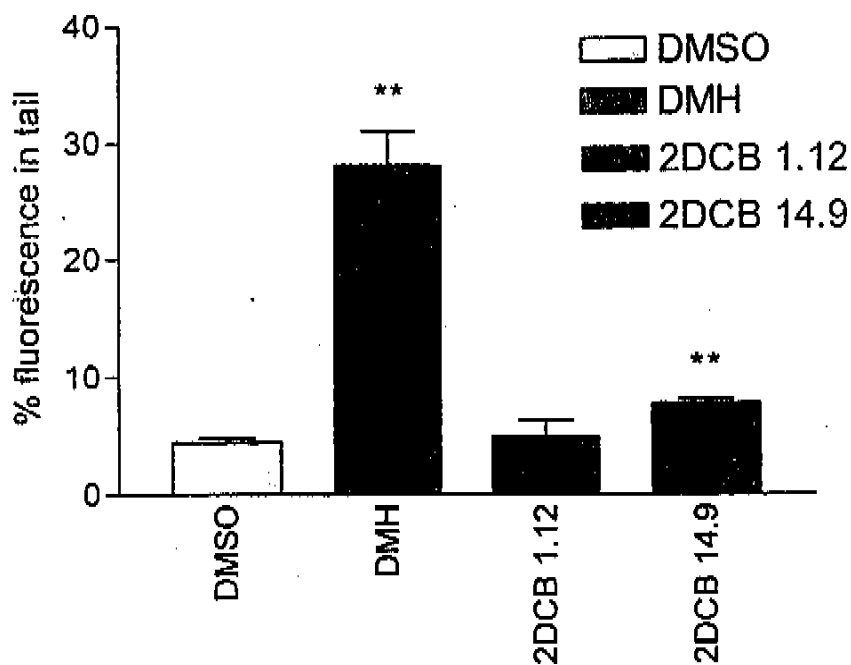


Fig. 1b Effect on Groups of Animals



Figures 1a, b DNA single-strand breaks in rat colon cells from the action of 2-dodecylcyclobutanone and DMSO, or DMH. Administered with pharyngeal probe 16 hours before isolation of the colon. (** $p < 0.01$ significantly different from the negative control with DMSO; unpaired, two-sided Student's t-test, $n = 3-6$).

Discussion

Initial *in vitro* experiments with 2-dodecylcyclobutanone, which at various concentrations was applied to rat colon cells as well as colon cells from human biopsies, have shown that 2-DCB leads to DNA damage [Delincée and Pool-Zobel, 1998]. Although the concentrations of 2-DCB that were used, ranging from 0.30 — 1.25 mg/ml, are large in comparison to the expected consumption of μg quantities of 2-DCB, further clarification is needed to determine whether these results are relevant to the safety of irradiated foods.

The *in vivo* experiments that were just conducted likewise show DNA damage to colon cells at higher concentrations of 2-DCB. Of course, one must keep in mind that not every instance of DNA damage proves to be a precursor to damage severe enough to generate a tumor, or leads to mutations in tumor-relevant genes. Furthermore, possible DNA repair processes and other cytotoxic events, for instance apoptosis, play a role before lesions become manifest and cell degeneration is initiated.

In addition, the quantity of 2-DCB that was administered here is to be regarded as very high. A projection shows that the concentration of 14.9 mg/kg BW in humans corresponds to consumption of more than 800 radiation-sterilized (60 kGy) broiler chickens. This comparison raises the question of whether the safety factors must in fact be 10 x 10. With several food ingredients (e.g. selenium), this concept would lead to deficiency symptoms, since the amount required in rats, for example, is about 25% of the toxic dose [Classen et al., 1987]. With lower safety factors, and hence lower test concentrations of 2-DCB, there would no longer be any detectable DNA damage.

It should be mentioned once again that in many animal feeding experiments with irradiated foods in which it is known that cyclobutanone was also in the feed, no evidence has been found to indicate an injury from irradiated foods that have been consumed. Typical in this regard is the Raltech study in the USA [Thayer et al., 1987], in which several generations of mice and dogs were fed with radiation-sterilized chicken. This study also included nutrition-physiological, teratological and genotoxic experiments on various species of animal.

In each case, it is necessary to check the relevance of the results that have been obtained. It is striking that the variation in observations is much greater at the low dose than the high dose, which in the latter case entails statistical significance. This must also be clarified.

Conclusion

High concentrations of 2-dodecylcyclobutanone lead to DNA damage in colon cells that is detectable with the comet assay. The requisite concentrations are very much higher than those that can be reached through the consumption of irradiated foods that contain fat. The results urge caution, and should provide impetus for further studies.

Note of thanks

We thank Ms. R. Lambertz and Mr. M. Knoll for their excellent technical assistance. We also thank Dr. C. H. McMurray (The Department of Agriculture for Northern Ireland, Belfast, United Kingdom) for providing the 2-dodecylcyclobutanone, and the International Consultative Group on Food Irradiation (ICGFI) for partial financial support.

Bibliography

- Anon. (1998) Supplement, Clearance of item by country. *Food and Environmental Protection Newsletter (IAEA, Vienna)*, 1 (2 Supplement), 1-17.
- Boyd, D.R., A.V.J. Crone, J.T.G. Hamilton, M.V. Hand, M.H. Stevenson and P.J. Stevenson (1991) Synthesis, characterization, potential use of 2-dodecylcyclobutanone as a marker for irradiated chicken. *J.Agric.Food Chem.* 39, 789-792.
- Brendler-Schwaab, S.Y., P. Schmezer, U. Liegibel, S. Weber, K. Michalek, A. Tompa and B.L. Pool-Zobel (1994) Cells of different tissues for *in vitro* and *in vivo* studies in toxicology: Compilation of isolation methods. *Toxic. in Vitro*, 8, 1285-1302.
- Classen, H.-G., P.S. Elias und W.P. Hammes (1987) *Toxikologisch-hygienische Beurteilung von Lebensmittelinhalts- und -zusatzstoffen sowie bedenklicher Verunreinigungen*. Paul Parey, Berlin und Hamburg.
- Crone, A.V.J., J.T.G. Hamilton and M.H. Stevenson (1992a) Detection of 2-dodecylcyclobutanone in radiation-sterilized chicken meat stored for several years. *Int. J. Food Sci. Technol.* 27, 691-696.
- Crone, A.V.J., J.T.G. Hamilton and M.H. Stevenson (1992b) Effect of storage and cooking on the dose response of 2-dodecylcyclobutanone, a potential marker for irradiated chicken. *J.Sci.FoodAgric.* 58, 249-252.
- Crone, A.V.J., M.V. Hand, J.T.G. Hamilton, N.D. Sharma, D.R. Boyd and M.H. Stevenson (1993) Synthesis, Characterisation and Use of 2-Tetradecylcyclobutanone Together with Other Cyclobutanones as Markers for Irradiated Liquid Whole Egg. *J.Sci.Food Agric.* 62, 361-367.
- Delincée, H. (1998) Detection of food treated with ionizing radiation. *Trends Food Sci. & Technol.*, 9, 73-82.
- DGE (1996) Ernährungssituation in der Bundesrepublik Deutschland. *Ernährungsbericht 1996*, DGE, Frankfurt /M., p 43.
- Diehl, J.F. (1995) *Safety of irradiated foods*, 2nd ed., Marcel Dekker, New York.
- Fairbairn, D.W., P.L. Olive and K.L. O'Neill (1995) The comet assay: a comprehensive review. *Mutation Res.* 339, 37-59.
- LeTellier, P.R. and W.W. Nawar (1972) 2-Alkylcyclobutanones from radiolysis of triglycerides. *Lipids*, 1, 75-76.
- McKelvey-Martin, V.J., M.H.L. Green, P. Schmezer, B.L. Pool-Zobel, M.P. De Méo and A. Collins (1993) The single cell gel electrophoresis assay (comet assay): A European review. *Mutation Res.* 288, 47-63.
- Nishimura, H., R.P. Philp, M. Calvin (1977) Lipids of *Hevea brasiliensis* and *Euphorbia coerulescens*. *Phytochemistry*, 16, 1048-1049.
-

- Pool, B.-L., S.Y. Brendler, U.M. Liegibel, A. Tompa and P. Schmezer (1990) Employment of adult mammalian primary cells in toxicology: *In vivo* and *in vitro* genotoxic effects of environmentally significant N-nitrosodialkylamines in cells of the liver, lung and kidney. *Environm. Mol. Mutagen.* **15**, 24-35.
- Pool-Zobel, B.-L. (1996) *In vitro / ex vivo* studies using primary cells of tumor target tissues for studying aspects of cancer prevention. In: *The Scientific Bases of Cancer Chemoprevention* (C. Maltoni, M. Soffritti and W.Davis, eds.) pp. 111-117. Elsevier, Amsterdam.
- Pool-Zobel, B.L. and U. Leucht (1997) Induction of DNA damage in human colon cells from biopsies by suggested risk factors of colon cancer. *Mutation Res.* **375**, 105-115.
- Pool-Zobel, B.L., N. Lotzmann, M. Knoll, F. Kuchenmeister, R. Lambertz, U. Leucht, H.-G. Schröder and P. Schmezer (1994) Detection of Genotoxic Effects in Human Gastric and Nasal Mucosa Cells Isolated from Biopsy Samples. *Environmental and Molecular Mutagenesis*, **24**, 23-45.
- Pool-Zobel, B.-L., C. Neudecker, I. Domizlaff, S. Ji, U. Schillinger, C. Rumney, M. Moretti, I. Vilarini, R. Scassellati-Sforzolin and I. Rowland (1996) Lactobacillus- and Bifidobacterium-Mediated Antigenotoxicity in the Colon of Rats. *Nutr. & Cancer*, **26**, No.3, 365-380.
- Stevenson, M.H. (1996) Validation of the Cyclobutanone Protocol for Detection of Irradiated Lipid Containing Foods by Interlaboratory Trial. In: *Detection Methods for Irradiated Foods - Current Status* (C.H. McMurray, E.M. Stewart, R. Gray and J. Pearce, eds.), pp. 269-284. Royal Society of Chemistry, Cambridge, UK.
- Stevenson, M.H., A.V.J. Crone and J.T.G. Hamilton (1990) Irradiation detection. *Nature*, **344**, 202-203.
- Stevenson, M.H., A.V.J. Crone, J.T.G. Hamilton and C.H. McMurray (1993) The use of 2-dodecylcyclobutanone for the identification of irradiated chicken meat and eggs. *Radiat.Phys.Chem.* **42**, 363-366.
- Thayer, D.W., J.P. Christopher, L.A. Campbell, D.C. Ronning, R.R. Dahlgren, G.M. Thomson and E. Wierbicki (1987) Toxicology studies of irradiation-sterilized chicken. *J. Food Prot.* **50**, 278-288.
- WHO (1994) *Safety and nutritional adequacy of irradiated foods*. WHO, Geneva.
- WHO (1997) Food Irradiation - Sky's The Limit. Press Release WHO/68, 19. Sept. 1997.
- WHO (1998) Food Safety. Joint FAO/IAEA/WHO Study Group on High Dose Irradiation. *Weekly Epidemiological Record* **73** (3), 9-11.