Detection of 2-Alkylcyclobutanones, Markers for Irradiated Foods, in Adipose Tissues of Animals Fed with These Substances

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MS 01-491: Received 31 December 2001/Accepted 12 May 2002

ABSTRACT

Laboratory rats received a freshly prepared drinking fluid containing 0.005% 2-tetradecyl- or 2-tetradeceny1-cyclobutanones daily for 4 months. These two compounds were recovered in the adipose tissues of the animals that consumed them. Less than 1% of the 2-alkycyclobutanones ingested daily were excreted in the feces. In addition, our data indicate that 2-alkycyclobutanones are able to cross the intestinal barrier, to enter into the bloodstream, and to be stored in the adipose tissue of an animal. However, the amounts of these substances detected in the adipose tissues and in the feces were much smaller than the amounts ingested.

2-Alkylcyclobutanones are four-membered ring compounds that are generated from triglycerides present in foods treated with ionizing radiation. These compounds contain the same number (n) of carbon atoms as their fatty acid precursors and n – 4 carbon atom alkyl side chains in position 2. The formation of a four-membered ring starting from a triglyceride requires a significant energy contribution during a very short period and at a precise site of the molecule. These conditions are met during the interaction of high-energy radiation with matter (commonly called radiation processing). Crone et al. (2, 3) demonstrated that 2-dodecylcyclobutanone, produced by the irradiation of palmitic acid, was absent from the nonirradiated foods they studied and was not observed in these foods either with heating or after storage (under reduced pressure, air or CO2). These authors suggested that the 2-alkylcyclobutanones were formed specifically by irradiation, and until now, these compounds have been considered unique radiolytic products.

More recently, Ndiaye et al. (11) investigated the presence of other saturated 2-alkylcyclobutanones (2-hexyl-, 2-octyl-, 2-decyl-, 2-dodecyl-, and 2-tetradecyl-cyclobutanones) in irradiated and nonirradiated foods. These authors could not invalidate the unique radiolytic-product character of the 2-alkylcyclobutanones. Indeed, no formation of 2-alkylcyclobutanones was detected in various nonirradiated foods either after 1 month of storage at 4 or 20°C or in emulsions of standard triglycerides (tricaprin, trilaurin, tripalmitin, and tristearin) subjected to various physical processes, including microwave, heating, UV irradiation, sonication, and high-pressure treatment.

However, the presence of 2-methylcyclobutanone was detected by Nishimura et al. (12) in the latex of Hevea brasiliensis. According to these authors, 2-methylcyclobutanone would result from the cyclization of isoprene after the ultrasound treatment realized during the analysis. However, this assumption was not confirmed by Ndiaye et al. (11), since no formation of 2-alkylcyclobutanone was observed after the ultrasound treatment of a mixture of synthetic triglycerides.

The toxicological potential of 2-alkylcyclobutanones has not been systematically evaluated. Preliminary studies have indicated slight genotoxic effects of 2-dodecylcyclobutanone in both in vitro (4) and in vivo (5) studies. In a recent study on the potential toxicity of various 2-alkylcyclobutanones, some toxic effects were observed by Marchioni et al. (9).

Until now, no data have been available concerning the presence of 2-alkylcyclobutanones in tissues of animals that have eaten irradiated foods. Therefore, the aim of the present study was to determine whether the presence of 2-(tetradec-5′-enyl)-cyclobutanone and 2-tetradecyclobutanone (produced, respectively, from oleic and stearic acids), the most common 2-alkylcyclobutanones found in irradiated foods, can be detected in the adipose tissues of rats that have consumed these compounds.

MATERIALS AND METHODS

Chemicals. The 2-alkylcyclobutanone standards, 2-undecylcyclobutanone (2-uDCB), 2-(tetradec-5′-enyl)-cyclobutanone (2-
tDeCB), and 2-tetradecylcyclobutanone (2-tDCB), were synthesized according to the method of Miesch et al. (10). Carbon dioxide of 99.9999% purity with a 140-atom (14,200-kPa) helium headspace was purchased from Air Product and Chemicals (Allentown, Pa.). The tert-butyl methyl ether was a Merck (Darmstadt, Germany) product. n-Hexane of technical quality was distilled from calcium hydride (Lancaster Synthesis, Morecambe, UK). Its purity was checked by gas chromatography, Silica gel of 63 to 200 μm (70 to 230 mesh) (Merck) was heated at 100°C overnight, allowed to cool, and deactivated with the addition of ultrapure water at a proportion of 4 ml of water (Milli Q, Millipore, Bedford, Mass.) to 100 g of silica. Florisil PR (60 to 100 mesh; Aldrich, Saint-Quentin, France) was heated at 550°C for 5 h and deactivated with the addition of distilled water at a proportion of 20 ml of water to 100 g of Florisil. These two adsorbents were stored in stoppered flasks in a desiccator at room temperature for a maximum of 1 week. Sodium sulfate was purchased from SDS (Peypin, France), heated at 650°C for 5 h, and allowed to cool in a desiccator before it was used. The Hydromatrix (Varian, Palo Alto, Calif.) was washed for 6 h with n-hexane under reflux before it was used.

**Animals and diets.** The experiments were conducted according to the National Research Council’s guidelines for the use and care of laboratory animals (authorization no. 67-49 of the French Ministry of Agriculture). Male Wistar rats (n = 18) weighing 260 to 270 g were housed under standardized conditions (22°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes per h) and fed a standard diet with free access to water. The experiments were conducted according to the National Research Council’s guidelines for the use and care of laboratory animals (authorization no. 67-49 of the French Ministry of Agriculture). Male Wistar rats (n = 18) weighing 260 to 270 g were housed under standardized conditions (22°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes per h) and fed a standard diet with free access to water. The rats were randomly divided into three groups (six rats per group) that received the same isonenergetic diet (UAR A04, Villemoisson/Orge, France) ad libitum. This nonirradiated diet contained 16% proteins such as casein and fish protein, 60% carbohydrates such as wheat starch, 3% lipids such as soya and fish oil, 6% salt mixture, and 1% vitamin mixture. The fatty acid composition of the diet was as follows: 2.6 mg of palmitic acid per g, 0.5 mg of stearic acid per g, 8 mg of oleic acid per g, and 14.5 mg of linoleic acid per g.

At 5:00 p.m. daily, the two experimental groups received a freshly prepared aqueous solution of either 2-tDCB or of 2-tDeCB at a concentration of 0.005% (wt/vol) in 1% ethanol as a drinking fluid. The control group received 1% ethanol. Fluid consumption was recorded daily, and the average consumption of 2-tDCB or of 2-tDeCB was about 1 mg per rat per day. Body weight was determined once a week throughout the experimental period (4 months).

The feces of each rat were collected during the last 3 days of the experimental period (between 8:00 and 8:30 a.m.) and immediately frozen at −80°C. Samples from each rat were pooled in order to obtain a minimal amount of 10 g for analysis. Adipose tissues, essentially epididymal and inguinal fat pads, were collected from sacrificed rats and stored frozen at −80°C until chemical analysis, which was performed in triplicate.

**Analytical procedure.** The adipose tissue of rats (ca. 20 g) and feces samples (ca. 10 g) were finely ground, mixed (50:50) with anhydrous sodium sulphate, placed in a cellulose thimble (inside diameter 33 mm, length 100 mm; Schleicher & Schuell GmbH, Dassel, Germany) and extracted for 6 h with n-hexane in a 125-ml Soxhlet apparatus (4 cycles per h) (Verlabo 2000, Strasbourg, France). The extracts were concentrated to dryness with a rotary evaporator at 0.22 atm (22 kPa) and at 30°C. All of the hexane extract from each feces sample was analyzed by the Florisil cleanup procedure described in official protocol EN 1785 (1). The hexane extracts from the adipose tissue were purified by supercritical carbon dioxide. Two grams of the extracted lipids was placed in a 10-ml-internal-volume stainless steel cylindrical extraction cell (inside diameter, 10 mm; length, 128 mm) on a ~1-g Hydromatrix bed. Two hundred microliters of an n-hexane solution of 2-undecylcyclobutanone (1 mg/ml) (internal standard) was added to the sample. The remaining empty volume of the extraction cell was filled with about 1.5 g of Hydromatrix. The Suprex (Pittsburgh, Pa.) Prepmaster supercritical extractor used was equipped with an Accutrap collector consisting of an automated flow control heated restrictor connected to a 5-ml-internal-volume stainless steel cylindrical solid trap (inside diameter, 10 mm; length, 65 mm; maintained at room temperature) containing 3 g of deactivated silica (rinsed beforehand with 15 ml of n-hexane and dried immediately prior to extraction with a stream of nitrogen [99.995% purity; Air Liquide, Paris, France] for 2 s and at a pressure of 4 atm [405 kPa]), with the trap itself being connected to a test tube (liquid trap) containing 5 ml of n-hexane. The extraction cell was placed in the oven vertically, and the carbon dioxide passed through it (from bottom to top) at a flow rate of 4 ml/min (150 atm [15,200 kPa], 80°C, 60 min). The Accutrap collector was equipped with a high-pressure pump, making it possible to elute (at room temperature) the compounds retained on the silica trap with 15 ml of n-hexane at 2 ml/min and then, after the removal of the liquid trap, with 55 ml of a mixture of n-hexane and tert-butyl methyl ether (99.1 vol/vol), of which only the last 40 ml was preserved and concentrated at 40°C under a nitrogen stream to 200 μl.

**Gas chromatography.** The extracts were analyzed with a Varian chromatograph (type 3400) coupled with a mass-sensitive detector (Saturn 2000, Varian) operating in the electron impact mode (emission current, 10 μA; axial modulation voltage, 4.0 V; auto gain control, 20,000 ions; trap, manifold, and transfer line temperatures, 150, 40, and 270°C, respectively; electron-multiplier voltage, 1,800 V). The mass spectra were recorded between 50 and 350 m/z, the gas chromatograph was fitted with an OV-20-MS capillary column (Ohio Valley, Marietta, Ohio) of 60 m with an inside diameter of 0.25 mm and a 0.10-μm stationary phase (20% diphenyl and 80% dimethylpolysiloxane). The SPI cooled injector’s initial temperature (50°C, held for 0.1 min) was followed by a 230°C/min increase to a final temperature 240°C, which was held till the end of the column temperature program. This program consisted of 60°C for 2 min followed by an 8°C/min increase to a final temperature of 300°C, which was held for 15 min. The injection volume was 1 μl. The carrier (with a flow rate of 1 ml/min) was helium (99.9995% purity; Air Liquide). The 2-alkylcyclobutanones were quantified by studying the chromatograms derived from mass spectra data (m/z = 95 + 98 for 2-tDeCB; m/z = 98 for 2-tDCB).

**RESULTS AND DISCUSSION**

At the end of the 4-month treatments, body weight gain values were similar for the rats of the three groups. These values were 569 ± 14 g (mean ± standard error) for the control group, 577 ± 19 g for the group receiving 2-tDCB, and 588 ± 21 g for the group treated with 2-tDeCB.

2-Alkylcyclobutanones in the body fat of rats. The chromatograms of body fat extracts obtained from samples taken from animals receiving 2-tDCB (Fig. 1c) showed a chromatographic peak whose retention time and mass spectrum corresponded with those of the 2-tDCB standard. In the same way, the chromatograms of the extracts obtained from samples taken from animals consuming 2-tDeCB (Fig.
of animals that had consumed 2-tDCB or the 2-tDeCB were identical to those of the corresponding standards. This finding indicates that some of these molecules could go through the entire intestinal tract without being modified. The quantities of 2-alkylcyclobutanones recovered in the fecal matter (3 g) per day were approximately 1.5 and 3 µg of 2-tDeCB and 2-tDCB, respectively. This result shows that the amounts of 2-alkylcyclobutanones recovered each day from rat feces were of the same order of magnitude as those recovered from the whole body fat of the animals fed for 4 months. These amounts, however, corresponded to only 0.1 and 0.3%, respectively, of the amount consumed by the animals daily. This finding clearly indicates either that most of these compounds were stored in parts of the animal other than fat or that these compounds underwent rapid metabolic transformation. Elliot et al. (6) proposed a conversion of 2-dodecylcyclobutanone to 5-dodecyl-oxolan-2-one (or γ-palmitolactone) when 2-dodecylcyclobutanone was inoculated into the body of a rabbit. On the other hand, Hamilton et al. (7) also observed the formation of this γ-palmitolactone when 2-dodecylcyclobutanone was stored for a few months at 4°C as a hexane solution. Vajdi et al. (14) reported the presence of long-chain γ and δ lactones in beef irradiated at a very high dose. These authors proposed an oxidative

Analysis of 2-alkylcyclobutanones in rats feces. Because of the limited lipid content of the feces, these samples were analyzed by the EN 1785 procedure (1). For this procedure, only 200 mg of lipids was necessary. Once again, the analyzed fecal matter contained only the 2-alkylcyclobutanone that had been consumed by the animals (Fig. 2). No 2-alkylcyclobutanone was detected in the fecal samples from control animals receiving only a 1% ethanol aqueous solution in their drinking fluid. The retention times and mass spectra of the compounds detected in the fecal matter

1b) showed a peak whose retention time and mass spectrum corresponded to those obtained with the 2-tDeCB standard. The samples from control animals contained no traces of these two compounds (Fig. 1a). These results indicate clearly that the detected 2-alkylcyclobutanones were not a result of food contamination. Although the quantities of 2-tDCB and 2-tDeCB consumed were identical, the amount of 2-tDeCB found in the adipose tissues of the animals was approximately four times as low as the amount of 2-tDCB (Table 1) providing evidence either that less of this monounsaturated compound is stored or that it is more rapidly metabolized by rats.

These results show that 2-alkylcyclobutanones were able to cross the intestinal barrier, to enter the bloodstream, and to be stored in the adipose tissues of the animals. Assuming that a rat has a body fat mass of approximately 30 g, the total quantities of 2-tDeCB and 2-tDCB accumulated could reach 2 and 9 µg, respectively, for rats consuming about 1 mg of 2-tDCB and 2-tDeCB daily throughout the 4-month experimental period. These amounts of 2-alkylcyclobutanones present in body fat are extremely low (10⁻⁵ times the total quantity consumed) (Table 1). Thus, it is clear that these compounds were extensively metabolized by the animals and, as shown later, were also eliminated in the feces.

### Table 1. Concentrations of 2-tDeCB and 2-tDCB in adipose tissues of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>2-tDeCB (µg/g of fat)ᵃ</th>
<th>2-tDCB (µg/g of fat)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-tDeCB</td>
<td>0.07 (0.03)</td>
<td>ND</td>
</tr>
<tr>
<td>2-tDCB</td>
<td>ND</td>
<td>0.31 (0.08)</td>
</tr>
</tbody>
</table>

ᵃ Mean of three measurements (standard deviation in parentheses).
ᵇ ND, not detected.
process to explain the formation of these lactones in fatty acids and triglycerides. Obviously, these lactones may also occur via oxidation of the radiation-induced 2-alkylcyclobutanones. Thus, one may assume that the oxidation of 2-alkylcyclobutanones also occurs in animal tissues.

The amount of 2-tDeCB found in the fecal samples remained approximately twice as small as that of 2-tDCB (Table 2). It seems that this monounsaturated compound is more easily degraded than its saturated alkyl side-chain counterpart.

This is the first report demonstrating that 2-alkylcyclobutanones, specific markers for food irradiation, were present in the tissues of nonirradiated animals fed with these substances. Indeed, until now, 2-alkylcyclobutanones have been detected only in irradiated triglycerides or in irradiated foods (8, 13). These compounds were also found in the fecal matter excreted by rats. The amounts of these compounds detected in the adipose tissue and in the feces of the rats remained very low compared with the high amounts of 2-alkylcyclobutanones ingested by these animals. Nevertheless, our results indicate that 2-alkylcyclobutanones were not totally stored nor excreted, but either were stored in other parts of the organism or underwent metabolic transformation. Further studies are therefore needed to determine the metabolic breakdown products of 2-alkylcyclobutanones and to establish the pathophysiological consequences of the stored 2-alkylcyclobutanones.

ACKNOWLEDGMENTS

This work was supported in part by INTERREG II, Upper Rhine Centers Southern programme (project no. 3.171). The authors thank Françoise Gossé for excellent technical assistance.

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