Cholesterol Oxidation in Heated Lard Enriched with Two Levels of Cholesterol[1]

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Cholesterol oxidation in lard containing two levels of added cholesterol was monitored using capillary gas-chromatography. Loss of cholesterol and formation of cholesterol oxidation products (COPs) were measured. Lard samples with 10 times (Test I) and 2 times (Test II) the amount of cholesterol originally found in each batch of lard were heated at 180°C for 10 hr a day for 240 and 160 hr, respectively. Cholesterol steadily decreased throughout the heating period in both tests. Cholesterol loss followed a first-order reaction rate, with a rate constant (k) of -1.18×10^{-3} h^{-1} for Test I and -9.45×10^{-3} h^{-1} for Test II. The COPs accumulated during both heating tests. But the amount of COPs formed did not total the amount of cholesterol lost. During heating, thermal degradation of cholesterol likely occurred, and those products were not detected. During cooling, hydroperoxides formed, which further oxidized into the COPs that were detected. The 7-ketocholesterol and 5α,6α-epoxycholesterol were the predominant COPs formed. The isomeric 7α-and 7β-hydroxycholesterols also accumulated in the heating tests. The 3β,5α,6β-cholestantriol was found in very small amounts and the 25-hydroxycholesterol was not detected.

KEY WORDS: Cholesterol oxidation, oxidized sterols, sterol oxidation.

Cholesterol readily undergoes oxidation to produce a variety of reaction products. Some of the cholesterol oxidation products (COPs) have been shown to be cytotoxic, atherogenic, mutagenic and carcinogenic when ingested by laboratory animals. Earlier experimental results were summarized by Smith (1). More recently, Addis (2) and Sevanian and Peterson (3) reported the health implications of these COPs. The impact of cholesterol oxide consumption is recognized by the Food and Drug Administration (FDA) in the United States as a potential public health issue (4). The development of new and improved gas chromatographic (GLC), high performance liquid chromatographic (HPLC) and GC-mass spectrometric (GC-MS) methods has greatly enhanced the research in this area in the past few years (5).

In a recent publication, Addis (2) reviewed many of these new GLC, HPLC and GC-MS methods. Among the methods described, one measured one or two oxides as evidence of cholesterol oxidation (6); other methods separated and quantified a host of major COPs (7,8). In addition, Maerker and Unruh (9) made use of semi-preparative HPLC to separate cholesterol, triglycerides and other lipids from COPs, thereby enriching the COP fraction. That fraction was later analyzed and quantified by direct on-column capillary GLC with or without trimethylsilyl ether (TMS) derivatization. Nourooz-Zadeh and Appelqvist (10) purified and enriched their lipid extracts with a Sep-Pak silica cartridge, a Lipidex-5000 column and an anion-exchange column. Fractions containing different COPs were collected, TMS-derivatized and analyzed by nonpolar capillary GLC.

The presence of COPs in processed foods has been reported, and the findings were reviewed by Maerker (5). Many of the COPs have been identified in dried or processed foods (7,10–15).

The generation of COPs during deep-fat frying was first suggested by Ryan *et al.* (16). Since then, researchers have tried to identify and quantify the COPs produced under these conditions in tallow (17–19), bacon rind fried at 200°C (15), and butter (20). Park and Addis (18) noticed that nearly equal amounts of 7-ketocholesterol and 5α,6α-epoxide were found in fats heated at 135°C, whereas 7-ketocholesterol was predominant at 165°C. In their study, only small peaks were observed at the retention site of COPs when tallow was heated at 190°C. Researchers also detected noticeable losses of cholesterol at these high temperatures. Ryan *et al.* (16) discovered a marked decrease in the size of the cholesterol spot on TLC plates, concomitant with the formation of COPs. Csiky (20) reported a 4% loss of cholesterol after heating butter for 5 min at 180°C. Park and Addis (17) noted that about 40–45% of cholesterol was lost after 200 hr of heating at 190°C or 300 hr at 155°C. Bascoul *et al.* (19) detected a loss of 25% cholesterol after 60 hr of commercial frying.

The new objectives of the current study were to determine the loss of cholesterol and the formation of cholesterol oxide products in lard during heating at 180°C.

MATERIALS AND METHODS

Lard. Distilled lard was purchased from the Meat Laboratory (Iowa State University, Ames, IA) and a distilled and deodorized lard was obtained from Hormel (Austin, MN) for use as the heating media. The original cholesterol content of each lard type was determined by using the colorimetric method of Searcy and Bergquist (21) as modified by Reitmeier and Prusa (22). The lard from the Meat Laboratory, which was used in Test I, was found to have 95 mg cholesterol/100 g lard, whereas that from Hormel (used in Test II) had 70 mg/100 g lard.

Chromatographic standards. Cholesterol (99%) was

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purchased from Sigma Chemical Company (St. Louis, MO). Duplicate gas-chromatographic determinations were carried out to ensure its purity. The cholesterol was used as a standard in GLC analyses and also was added to the lard to enhance the accuracy of tracing cholesterol oxidation. Cholesterol oxide standards, 5,6α-epoxy-5α-cholestane-3β-ol(α-epoxide), 3β-hydroxycholest-5-en-7-one (7-keto), and 5α-cholestane-3β,5,6β-triol (triol) were purchased from Sigma Chemical Company. Cholest-5-ene-3β, 7α-diol (7α-OH), cholest-5-ene-3β, 7β-diol (7β-OH) and cholest-5-ene-3β, 25-diol (25-OH) were purchased from Steraloids, Inc. (Wilton, NH). To quantify and account for the losses during sample preparation and chromatographic analysis, 5α-cholestane (Sigma Chemical Co.) was added before saponification as an internal standard.

Heating studies: Test 1. Lard samples (600 g) spiked with ten times (i.e., 950 mg cholesterol/100 g lard) the amount of cholesterol originally present in the lard were heated in Fry Baby Containers at 180±5°C for 10 hr a day for 25 days. The temperature of each individual fryer was controlled by a rheostat. Sample aliquots were taken at time zero and at the end of each day. Samples were stored under N_2 in Telfon-capped tubes at −10°C until analyzed.

Heating studies: Test 2. Deodorized lard samples (500 g) spiked with two times (i.e., 140 mg cholesterol/100 g lard) the amount of cholesterol originally present in the lard were heated at 180±5°C for 10 hr a day for 16 days.

Sample preparations for cholesterol oxidation analysis: Cold saponification and extraction. The method of Park and Addis (8) was followed with some modifications. Because of the high initial cholesterol level in our lard samples, a sample size of 0.1 g was used instead of the 0.2-g sample suggested in the original method. A 30-mg aliquot of 5α-cholestane was added to each sample as an internal standard. The samples were layered with N_2 and cold saponified in 1.0 N methanolic KOH for 20 hr. The saponified samples were mixed with deionized water and extracted three times with diethyl ether. The extracts were pooled and back-washed with 0.5 N methanolic KOH. Park and Addis (8,17) recommended washing the extracts containing the unsaponifiables two times with deionized water. We found it difficult to remove the residual methanol after only two washings. But, by increasing the washing step to three or more times, the residual solvent was successfully removed at 35°C. The end point of the washing step was determined by testing the neutrality of the wash water. Such precautions were essential because the silylating reagents that were added at the next step are sensitive to any residual alcohols and moisture.

Quantification of sterols by capillary column GLC. For derivatization of the sterols into their corresponding trimethylsilyl (TMS) ethers, the dried ether extracts were redissolved directly into Sylon BTZ (Supelco Inc., Bellefonte, PA) instead of into pyridine, to avoid further dilution of the COPs. This step was necessary because the great initial cholesterol amounts in the current study required 150 mL of the silylating reagents instead of the 50 mL used in the original method of Park and Addis (8).

A Varian Aerograph series 3300 Gas Chromatograph (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector was used with a DB-1 capillary column (15 m × 0.25 mm, 0.1 m film thickness; J&W Scientific, Inc., Rancho Cordova, CA) to quantify the TMS ether sterols. Peak areas of eight of these sterols were measured by using the internal-standard procedure of a Hewlett Packard (HP) 3390A reporting integrator. The assignments of the peaks were checked visually due to the close proximities of some unknown peaks to the COP peaks. The cholesterol and COP peaks, in order of increasing retention time (R_T), were: 5α-cholestane, cholesterol, 7α-OH, α-epoxide, 7β-OH, triol, 7-keto and 25-OH. Because helium rather than hydrogen was used as the carrier gas, the total elution time for the products was slightly longer than that of Park and Addis. The reported temperature programming was used (3°C/minute at 80–250°C), but a holding period at 250°C was added to accomodate the longer elution time. To confirm the identities of the eight sterols, the DB-1 GLC column was installed in a Finnigan 4500 GC-MS (Finnigan Corp., Sunnyvale, CA). The fragmentation patterns of the eight peaks, partly identified by their R_T, were compared with those found in the sterol standard solution.

Statistical analysis. The rate constants of cholesterol disappearance and COP formation were calculated from the first-order rate plots by using the linear regression model. Data were tested by the analysis of variance (ANOVA) by using the general linear models procedure of the Statistical Analysis System (SAS) computer program (23). The rate constants for cholesterol loss in Test I and Test II (cholesterol added at 10 times and 2 times, respectively) were compared by using Student's *t*-test.

RESULTS AND DISCUSSION

For many years, the detection and quantification of COPs were hampered by technical difficulties (2,4). There was a great need for the development of more sensitive and more reliable methods. Recently, there has been a sudden increase in method development for COP analysis, but few interlaboratory evaluations of those methods have occurred. Such evaluations are essential to providing additional confidence that the methods are useful. In the current study, Park and Addis' (8) capillary GLC method was used, which they adapted for analyzing COPs in heated tallow (17,18). Some slight modifications of the procedure were necessary. Because of differences in the chromatographic set-ups, the gas chromatographic conditions were readjusted to successfully resolve cholesterol and the six COPs. The recovery of COPs in the presence of lard through the cold saponification and extraction procedures was checked by using the recovery procedure described by Park and Addis (17). When an internal standard was added before sample preparation, the recoveries of five of the COPs were 90% and greater, with the recovery of triol at about 70%. Park and Addis (17) reported recovery in tallow close to 100%, except for triol recovery, which was about 85%.

In the present study, lard was heated intermit-

tently at 180°C for up to 240 hr, with two different levels of added cholesterol. Park and Addis (17,18) heated tallow at 135, 150, 165, 180 and 190°C for up to 376 continuous hours. The results from studies by Park and Addis and from our laboratory were compared.

Loss of cholesterol in heated lard. In both Tests I and II, the cholesterol content decreased steadily during the heating time (see Fig. 1A). In Test I, with lard containing 10 times the original amount of cholesterol, the cholesterol content dropped during 240 hr of heating from an average initial amount of about 8000 ppm to a final amount of about 6500 ppm. In Test II, with lard containing two times the original amount of cholesterol, the cholesterol content dropped during 160 hr of heating from an average of 1900 ppm to a final amount of 430 ppm.

Figure 2B shows the percentage retention of cholesterol in Test II. By using an expanded scale, this Figure more clearly shows the reduction in rate of cholesterol loss at about 100 hr, when about 70% of the initial substrate was consumed. A pseudo first-order reaction rate was suspected. The natural logarithm of the amount of cholesterol was plotted against hours of heating and the resulting coefficient of variance (R^2) was 0.97 for Test II. The cholesterol loss during Test I was more erratic than in Test II, resulting in a smaller R^2 of 0.86. The rate constant (k) of cholesterol loss was -1.35×10^{-3} hr^{-1} in Test I and -8.85×10^{-3} in Test II. Student's *t*-test showed these slopes to be significantly different from each other ($p<0.0001$). Only about 19% of the original cholesterol was lost at 240 hr of heating in Test I, compared with 70% cholesterol loss at 100 hr of heating in Test II. By heating the oil in Test I for a much longer time, additional destruction of the cholesterol might have occurred, with the loss eventually leveling off at around 70%. Perhaps what is observed in the current results of Test I is just the very beginning of an expanded curve.

Park and Addis (17) noted that cholesterol loss ceased in two heated tallow samples when 40–45% of the initial cholesterol was gone. It took 300 hr of heating at 155°C or 200 hr of heating at 190°C to arrive at that point. In Test II of the current study, it is likely that the intermittent heating resulted in more and quicker cholesterol loss as compared with the continuous heating of tallow reported by Park and Addis (17,18). The intermittent heating and cooling allows more oxygen to be introduced into the frying medium due to the increased solubility of oxygen at lower temperatures and results in greater oxidation of its components. Kimura *et al.* (24) and Bergstrom and Wintersteiner (25) reported that an aqueous cholesterol mixture arrived at an apparent final state where consumption of cholesterol and formation of COPs were suspended after more than 70% of the initial substrate was consumed. They suggested that the accumulation of COPs in the reaction media may have changed the micellar structure, resulting in the cessation of cholesterol oxidation. Although the researchers documented the slowing down of cholesterol loss (24,25), none tried to fit their data to a pseudo first-order reaction rate.

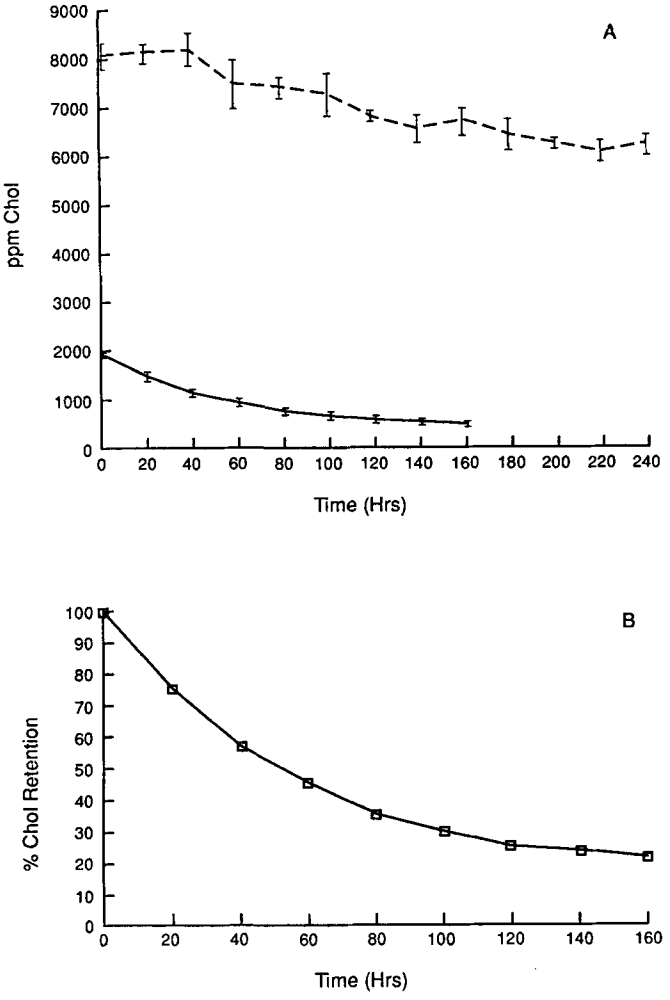

FIG. 1. (A) The amount of cholesterol loss in lard with 10 times added cholesterol in Test I (upper line); and 2 times added cholesterol in Test II (lower line). (B) Percentage cholesterol retention in lard with 2 times added cholesterol in Test II (□). Data shown are mean % S.D. (n=4).

Formation of cholesterol oxidation products in heated lard. Figure 2 represents a typical chromatogram of heated lard with the addition of two times the original amount of cholesterol (Test II). Of the seven compounds studied, five were detected in this test (cholesterol, 7α-OH, α-epoxide, 7β-OH and 7-keto), and the corresponding peaks are labelled. The 5α-cholestane was the internal standard. Although baseline separations were achieved among all of the five compounds observed, the initial cholesterol peak covered up the 7α-OH peak that followed it when cholesterol was added at 10 times the original level (Test I). As the cholesterol content decreased with heating, the 7α-OH peak gained resolution from the cholesterol peak. This condition occurred after about 80 hr of heating. In Test II, the resolution of 7α-OH was apparent at time zero.

Figure 3A shows the amounts of 7α-OH, α-epoxide, 7β-OH and 7-keto formed in Test I, and Figure 3B shows the amounts of the same four COPs plus triol formed in Test II. In general, Test I yielded slightly more COPs than did Test II, likely because of the greater amount of cholesterol present in Test I. But

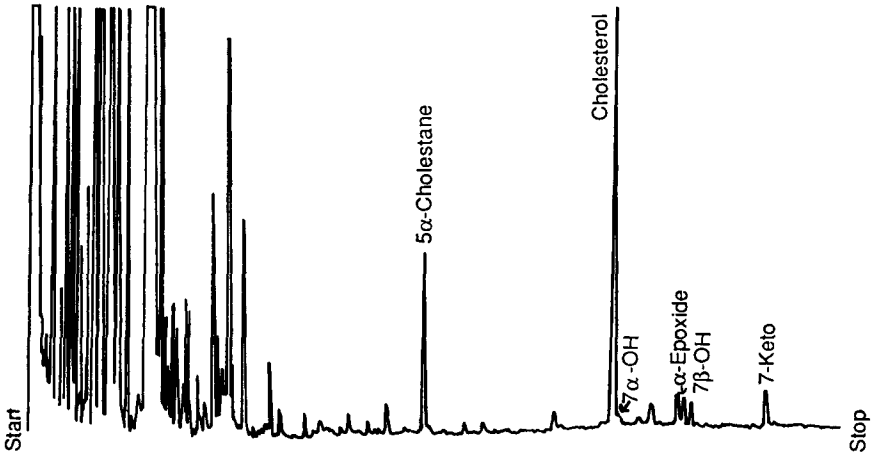

FIG. 2. A typical capillary gas chromatogram of a heated lard sample.

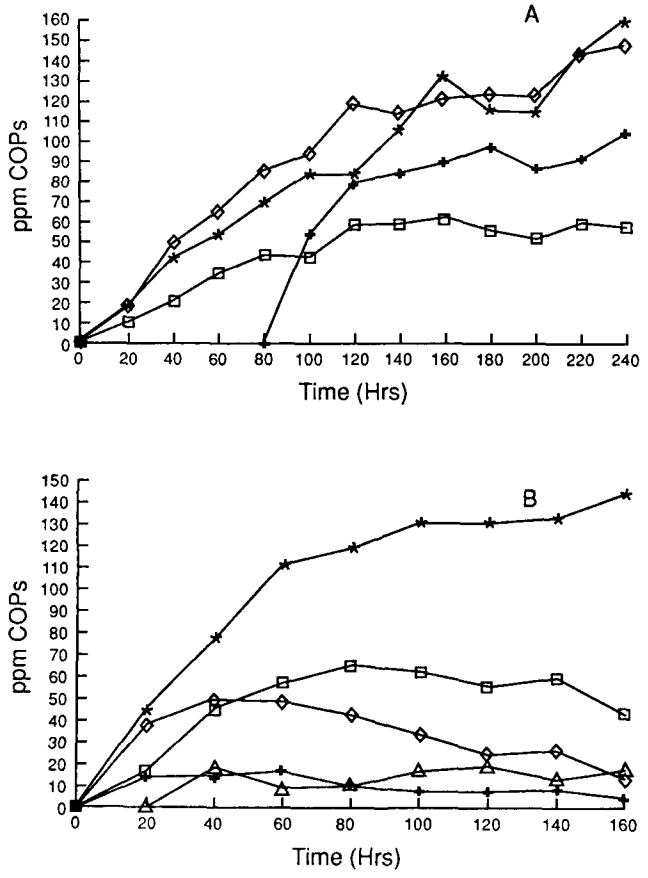

FIG. 3. COP formation in lard with (A) 10 times added cholesterol in Test I, and (B) 2 times added cholesterol in Test II. The COPs measured are: (□) α-epoxide, (+) 7α-OH, (◇) 7β-OH, (*) 7-keto and (Δ) triol.

the greater amount of COPs in Test I did not represent the almost five-fold difference in initial cholesterol contents between Tests I and II. A similar trend was noted earlier for cholesterol loss between the two tests. When the rates of COP formation from Tests I and II were compared, the slopes from the first-order plots of 7α- and 7β-OH were significantly different ($p<0.05$). The differences between these two heating tests were not so much with the amount of COP accumulation as with the overall trend at the end of the heating period. In Test I, the amounts of all COPs were still increasing at 160 and 240 hr of heating. In Test II, the COPs (except 7-keto) were experiencing either a steady state (triol and 7α-OH) or had achieved a maximum amount and were decreasing (α-epoxide and 7β-OH) at 160 hr of heating.

Smith (1) and later Maerker (5) summarized the current knowledge on the formation of the commonly found COPs. It is thought that the autoxidation of cholesterol proceeds via an initial formation of the epimeric 7α- and 7β-hydroperoxides (7-OOHs), which later reduce to their corresponding alcohols with both epimers dehydrating to 7-keto. In addition to the homolysis of the peroxide bonds previously described, however, reactions such as isomerization of the axial α-OOHs and α-OHs to the corresponding equatorial β-OOHs and β-OHs (both 5- and 7-isomers) and the disproportionation of 7-OOHs to equal molar concentrations of 7α-OH, 7β-OH and 7-keto complicate the final proportion of each COP in an oxidized mixture. Furthermore, heating a frying medium results in the formation of free fatty acids, which reduces the pH and causes the hydrolysis of α- and β-epoxides to triols.

In our tests, the amount of total COPs formed (480 and 320 ppm in Tests I and II, respectively) was not equal to the amount of cholesterol loss (1550 and 1470 in Test I and II, respectively). There may be many other degradation products formed for which analyses were not done or that were lost through volatilization. Park and Addis (8,17) also proposed that COP degradation was the cause for the small amounts of COP accumulation in tallow heated at 180 and 190°C.

Reaction rates for formation of COPs. The formation of most commonly found COPs in heated lard samples, like that of the cholesterol degradation, can be explained by a pseudo first-order reaction rate. In

both tests, the correlation coefficients of 7α-OH, 7β-OH, α-epoxide and 7-keto from the first-order rate plots varied from 0.52 to 0.97, but all had significance levels of $p \leq 0.05$. Triol was found only in Test II, and it was found in the least amounts among all the COPs quantified. It did not follow a first-order rate plot, possibly due to the small amounts found and the difficulties associated with integrating a small peak. Maerker and Bunick (26) also found that first-order reaction rates described the hydrolysis of α- and β-epoxides at pH 5.5 and 80°C. They reported rate constants of 13.3 and 5.27×10^{-7} sec^{-1} for β- and α-epoxide, respectively. In the current study, only α-epoxide formation was followed, and the rate constants were 2.6 and 2.0×10^{-6} sec^{-1} for Test I and II, respectively. Park and Addis (17,18) reported that the formation of 7-keto seemed proportional to heating time at 155°C and suggested that its formation may follow zero-order kinetics, but they did not report any rate constants. In our studies, the first-order rate constants for 7-keto were 2.9 and 1.9×10^{-6} sec^{-1} for Test I and II, respectively.

Another important parameter observed in the current study, as well as by other researchers, was that the β-isomer of 7-OHs accumulated to a greater extent than did the α-isomer, likely because of the presence of less steric hindrance in the formation of the former isomer. In Test I, the α/β ratio was 1:1.3–1.8. In Test II, it was 1:2.7–4.3. The α/β ratios of the 7-OHs remained fairly constant throughout the heating in Test I (for those samples where the 7α-OH peak was sufficiently separated from the cholesterol peak), but increased initially, followed by a decrease in Test II, indicating possible preferential degradation of 7β-OH as indicated by Smith (1) and Maerker (5).

A wide variety of α/β ratios is reported in the literature, ranging from 1:8–11 (27) in the autoxidation mixture of cholesterol to 1:1–23 in commercial spray-dried egg products (28). Nourooz-Zadeh and Appelqvist (10) suggested that differences in the relative proportions of the epimeric 7-OHs and the isomeric epoxides in several skim milk samples may reflect differences in the storage conditions. In a model system carried out by Maerker and Bunick (26), the ratio of α/β epoxides remained constant at alkaline pHs even though the level of accumulation increased with heating at 80°C. In acidic pHs, this trend did not hold, possibly because β-epoxides were preferentially hydrolyzed to triol.

The accumulation of COPs in a high-temperature, cholesterol-rich food system is a dynamic one, depending upon the amount of cholesterol present, the treatment of the oil (intermittent or continuous heating) and the severity of the heat treatment. Further studies where food is actually fried in the fat would contribute to practical information about the accumulation of COPs in such a system. On the other hand, adding pure COPs individually to heating media such as pure triolein would allow one to monitor the rate of COP formation in a model system. Information from both types of studies would provide further understanding of the occurrence of COPs in our foods.

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