

Reaction of Cholesterol 5,6-Epoxides with Simulated Gastric Juice

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Cholesterol 5 α ,6 α -epoxide (α -epoxide) and cholesterol 5 β ,6 β -epoxide (β -epoxide) were individually suspended in simulated gastric juice (pH 1.2) at 37 C, and their reaction was followed by gradient high performance liquid chromatography (HPLC) with flame ionization (FID) detection. Both epoxides reacted rapidly in the aqueous acid medium. The α -epoxide formed 6 β -chlorocholestane-3 β ,5 α -diol (α -chlorohydrin) and 5 α -cholestane-3 β ,5,6 β -triol (triol), while the β -epoxide formed 5 α -chlorocholestane-3 β ,6 β -diol (β -chlorohydrin) and triol. The isomeric chlorohydrins reacted further to form the triol. In mildly alkaline aqueous medium, each chlorohydrin reverted to the epoxide from which it was formed. The data suggest that both epoxides, which have been reported to have adverse health effects in animals, would be largely hydrolyzed in the stomach and to the triol, which also has been reported to have biological activity. The data further suggest that residual chlorohydrins surviving stomach residence can be expected to revert to epoxide in the more alkaline intestinal environment. *Lipids* 23, 761-765 (1988).

It is well-recognized that the exposure of cholesterol to oxidizing conditions gives rise to oxidation products (1), and many of these have been identified and now can be measured with some precision (2). A few of the major products of cholesterol oxidation have been reported to have adverse biological effects (3-12), and their implication in cardiovascular disease has been of concern. The possible adverse health effects of cholesterol oxidation products are highly relevant to food safety, because many cholesterol-containing foods are subjected to oxidizing conditions during various stages of processing, storage and/or preparation. Indeed, the problem may extend to foods that contain plant sterols, compounds that are closely related chemically to cholesterol and can be expected to give similar oxidation products, but this aspect has been studied only superficially.

The cholesterol oxidation products that have greatest relevance to food safety are the 5 α ,6 α -epoxide, which has been reported to be carcinogenic and mutagenic (11) and the 3 β ,5 α ,6 β -triol, which has been cited as being cytotoxic (6,9,10). The 5 β ,6 β -epoxide also has recently been reported to be toxic (13), and because it is hydrated to form the triol at a faster rate than the 5 α ,6 α -epoxide (14), its presence also must be considered pertinent to the food safety perspective.

The presence of cholesterol oxidation products in animal-derived foods has been reported with increasing

frequency in recent years. Products that were reported to contain these cholesterol derivatives include heated tallow (15,16), dried egg preparations (17-21), butter and other dairy products (17,20,22-24), and other foods (17, 20,25,26). The documented existence of the 5,6-epoxides and of the triol in a large variety of foods raises the question of their fate upon ingestion.

Cholesterol 5 α ,6 α -epoxide (α -epoxide) reacts with hydrochloric acid to give 6 β -chlorocholestane-3 β ,5 α -diol (α -chlorohydrin) (27,28). Similarly, cholesterol 5 β ,6 β -epoxide (β -epoxide), on reaction with hydrochloric acid, yields 5 α -chlorocholestane-3 β ,6 β -diol (β -chlorohydrin) (28-31). Both chlorohydrins might be formed when mixtures of the isomeric cholesterol 5,6-epoxides are ingested. This paper reports a study of the reaction of the cholesterol epoxides with simulated gastric juice (pH 1.2) at 37 C.

EXPERIMENTAL

Materials and reagents. Cholesterol, 99+%, and α -epoxide were purchased from Sigma Chemical Co. (St. Louis, MO) and 5-cholestene-3 β ,25-diol was purchased from Research Plus, Inc. (Bayonne, NJ). β -Epoxide was prepared from cholesterol via 5 α -cholestane-3 β ,5,6 β -triol (triol) (32) and the corresponding triacetate (33) by the method of Chicoye et al. (34). Dulbecco's Modified Eagle Medium (high glucose) was purchased from Flow Laboratories (McLean, VA).

All solvents used were "distilled in glass grade" and for high performance liquid chromatography (HPLC) were degassed by vacuum filtration through a 0.2 μ m filter. Water was double-deionized, glass distilled. Thin layer chromatography (TLC) plates, Silica Gel G and GHL (250 μ), were purchased from Analtech (Newark, DE).

Simulated gastric juice was prepared by diluting a solution of conc. HCl (0.82 ml, 12 M) and 0.411 g NaCl in distilled, deionized water to a total volume of 100 ml. The solution had a pH of 1.22. Phosphate buffer (pH 7.44) was a solution of monobasic potassium phosphate (KH₂PO₄, 0.5905 g) and dibasic sodium phosphate (Na₂HPO₄, 2.130 g) in 500 ml water.

PROCEDURES

Preparation of chlorohydrins: 6 β -chlorocholestane-3 β ,5 α -diol (3) (α -chlorohydrin). α -Epoxide (1) (501 mg) in a glass stoppered 500 ml Erlenmeyer flask was dissolved in ether (230 ml), and conc. HCl (125 ml) was added with cooling. The stoppered flask was allowed to stand at room temperature for 18 hr with magnetic stirring. After addition of 50 ml water and 50 ml ethyl acetate, the mixture was neutralized with 50% aqueous NaOH with cooling in ice to a pink phenolphthalein endpoint. The ether layer was separated, and the aqueous phase was extracted with 2 \times 100 ml ethyl acetate. The combined organic phases were dried over anhydrous Na₂SO₄ and distilled on a rotary evaporator under aspirator vacuum to a dry residue (590 mg). The product was purified by semi-preparative HPLC using hexane/2-propanol (100:3, v/v).

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Abbreviations: α -Chlorohydrin, 6 β -chlorocholestane-3 β ,5 α -diol; α -epoxide, cholesterol 5 α ,6 α -epoxide; β -chlorohydrin, 5 α -chlorocholestane-3 β ,6 β -diol; β -epoxide, cholesterol 5 β ,6 β -epoxide; triol, 5 α -cholestane-3 β ,5,6 β -triol; FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

5 α -Chlorocholestane-3 β ,6 β -diol (4) (β -chlorohydrin). The method used to prepare the β -chlorohydrin from the β -epoxide (2) was identical to that used for the α -chlorohydrin.

Reaction of epoxides with simulated gastric juice: cholesterol 5 α ,6 α -epoxide (1). To a 100 ml three-neck flask, equipped with magnetic stirring bar and a thermometer to which a thermoregulator was attached, 75.0 ml of simulated gastric juice was added. The flask was set on a stirring hot plate, and the temperature of the solution was regulated at 37.0 ± 0.5 C with vigorous agitation. α -Epoxide (21.03 mg) dissolved in 2-propanol (1000 μ L) was injected slowly (3 min) below the surface of the stirred aqueous solution by means of a 500 μ L syringe, resulting in a cloudy suspension. Aliquots (5 ml) were withdrawn from the reaction mixture immediately after the injection was completed and at periodic intervals thereafter by use of a volumetric pipette. Each aliquot was placed in a 20 ml screw-top vial containing 5 ml of ethyl acetate and 1 ml of a solution of internal standard (568 μ g 5-cholesten-3 β , 25 diol in 1 ml ethyl acetate). Vigorous shaking of the aliquot mixture was followed by phase separation and additional extractions (3×5 ml ethyl acetate) of the aqueous layer. Combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2 . The dry residue was dissolved in 30 μ L 2-propanol, and the resulting solution was diluted with 1000 μ L hexane in preparation for HPLC analysis.

Cholesterol 5 β ,6 β -epoxide (2). The procedure used was the same as that used for the α -epoxide.

Reaction of chlorohydrins with simulated gastric juice: 6 β -chlorocholestane-3 β ,5 α -diol (3) (α -chlorohydrin). The α -chlorohydrin was somewhat less soluble in 2-propanol than the α -epoxide, and 1500 μ L 2-propanol were required to dissolve 24 mg of the α -chlorohydrin. Otherwise, the procedure described for the reaction of α -epoxide with simulated gastric juice was followed in detail.

5 α -Chlorocholestane-3 β ,6 β -diol (4) (β -chlorohydrin). The procedure used was identical to that described for the reaction of the α -epoxide, except that 24 mg of β -chlorohydrin was dissolved in 600 μ L 2-propanol for injection in the reaction flask. The β -chlorohydrin appeared to be much less soluble in the aqueous phase than either of the epoxides or the α -chlorohydrin and formed large particles in the water layer and deposits on the outside of the injection needle.

Measurement of stability of the α -chlorohydrin (3) and the β -chlorohydrin (4) in Dulbecco's Modified Eagle Medium (high glucose), pH 7.35. The procedure described for the reaction of the α -epoxide with simulated gastric juice at 37 C was followed in detail, except that the aqueous medium was Dulbecco's Modified Eagle Medium (high glucose) rather than simulated gastric juice. Each chlorohydrin (~ 24 mg) was injected into the medium in a separate experiment, and product analysis was performed as described.

Measurement of the stability of α -chlorohydrin (3) in phosphate buffer, pH 7.44. α -Chlorohydrin (21.1 mg) in 2-propanol (1500 μ L) was injected into 75 ml of phosphate buffer at 36 C, and product isolation and measurement were carried out as described above for the reaction of α -epoxide in simulated gastric juice.

Gas chromatography (GC). Analyses were performed as described (14).

Liquid chromatography (HPLC). The instrumentation used, including a flame ionization HPLC detector, has been described (35). Normal phase separations were performed on a 3.9 mm \times 30 cm, 10 μ m μ -Porasil column (Waters Associate, Framingham, MA) at ambient temperature. Separations of the four compounds of interest were achieved with a gradient solvent system at a total flow rate of 1.5 mL/min. The mobile phase consisted of solvent A, which was hexane/2-propanol (100:3, v/v), and solvent B, which was 2-propanol. At injection and for 10 min thereafter, the mobile phase was 100% A. The solvent composition then was changed linearly over a period of 10 min to 50% A and 50% B, stayed at that composition for five min and then changed linearly over a period of five min to 100% A to reach the starting point for the next analysis.

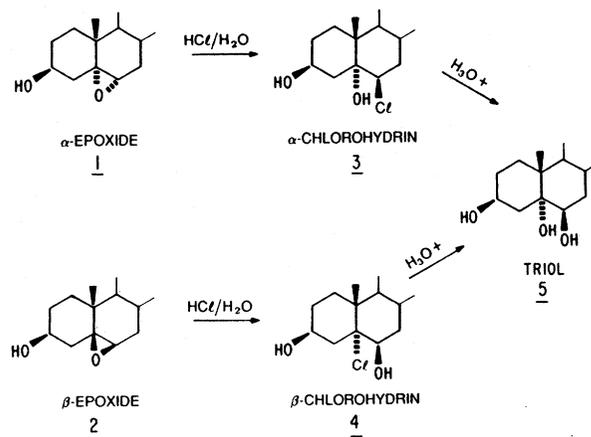
Thin layer chromatography. Before use, plates were washed by development with chloroform:methanol (2:1, v/v) and activated overnight in an air oven at 110 C. Spotted samples were developed with benzene:ethyl acetate:acetic acid (60:40:1, v/v/v). Air-dried plates were sprayed lightly with 50% sulfuric acid, placed on an unheated hot plate and gradually warmed to produce maximum color display of the cholesterol oxidation products, followed by complete charring at 220 C.

RESULTS AND DISCUSSION

Addition of hydrogen chloride to α -epoxide (1) and to β -epoxide (2) gave α -chlorohydrin (3) and β -chlorohydrin (4), respectively (Scheme 1).

Observation of the progress of the reaction in simulated gastric juice required analytical means to distinguish between the three expected components of these mixtures and to measure their concentrations. The components of interest were the epoxide and its corresponding chlorohydrin as well as triol (5).

The α -epoxide and its chlorohydrin were not resolvable by TLC with any of the several developing solvents tried. They gave single peaks by isocratic, normal phase HPLC and by capillary GC with or without prior silylation. Gradient, normal-phase HPLC gave excellent resolution of the three compounds of interest and the internal standard, 25-hydroxycholesterol (Fig. 1), with the use of a



SCHEME 1

REACTION OF CHOLESTEROL EPOXIDES WITH GASTRIC JUICE

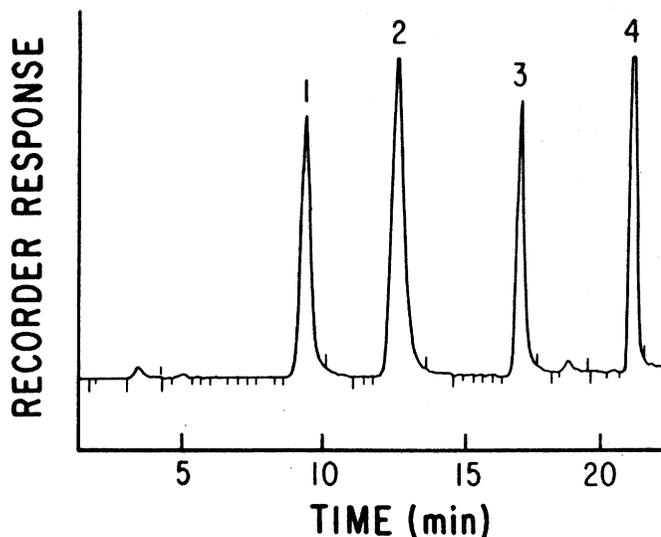


FIG. 1. Gradient HPLC of a four component mixture with FID detection (see Experimental). Peaks: (1) 5-cholestene-3 β , 25 diol, (2) cholesterol 5 α ,6 α -epoxide, (3) 6 β -chlorocholestane-3 β , 5 α -diol, (4) 5 α -cholestane-3 β ,5,6 α -triol.

FID. The initial mobile phase was hexane/2-propanol (100:3, v/v) the polarity of which was increased linearly with 2-propanol. Response factors and linearity of response over a concentration range of 100 μ g-0.39 μ g per 100 μ L were determined as reported (35) and permitted measurement of the relative concentrations of the components in the mixture from their area counts.

The β -epoxide and its chlorohydrin were readily resolved by TLC but not by GC. The gradient, normal-phase HPLC procedure developed for the α -epoxide and derived compounds was used without change to analyze mixtures of β -epoxide, β -chlorohydrin and triol, again with 25-hydroxycholesterol as internal standard.

Human gastric juice contains a variety of organic and inorganic constituents. The current study was confined to a simulated gastric juice consisting of an aqueous solution of hydrochloric acid and sodium chloride that approximates the concentration of these components in stimulated human gastric juice (36). A comparison of the cited composition and that of the present study is shown in Table 1.

Injection of the substrates (either of the two epoxides or either of the two chlorohydrins) into the vigorously stirred simulated gastric juice at 37 C was carried out slowly to minimize particle size of the resulting suspensions. Nevertheless, individual suspended particles were clearly visible and did not appear to change during the course of the reaction. Concentrations of the substrate in 75 ml reaction medium were chosen so that injection of 100 μ L of an extract of each 5 ml aliquot produced a sizable signal on the HPLC-FID detector.

In early experiments, each 5 ml aliquot removed from the reaction mixture was immediately neutralized with concentrated sodium hydroxide to a phenolphthalein endpoint before the mixture was extracted with ethyl acetate. It was discovered, however, that this led to erroneous product compositions. Because the chlorohydrins are highly sensitive to base that converts them to epoxides, fleeting

TABLE 1

Composition of Gastric Juice

Component	Simulation of this study ^a	Simulated human gastric juice ^b
HCl	99.6 mM	0-135 mM
Cl ⁻	170 mM	131-170 mM
Na ⁺	70.3 mM	19-70 mM
pH	1.22	1.2-2.0

^aSolution in double-deionized water.

^bRef. 36.

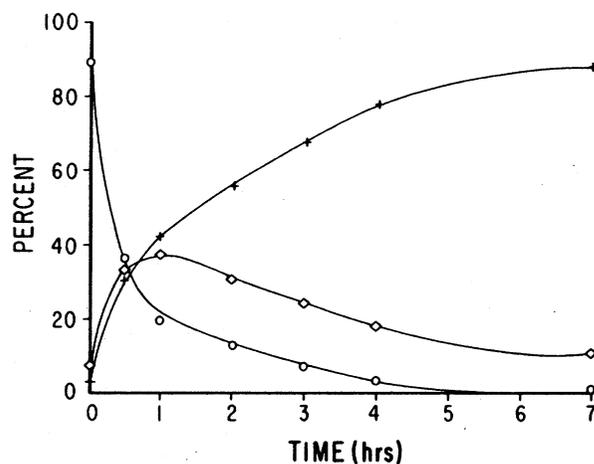


FIG. 2. Reaction of cholesterol 5 α ,6 α -epoxide (α -epoxide) in simulated gastric juice at 37 C. (O) α -epoxide, (□) α -chlorohydrin, (+) triol.

local concentrations of base during the neutralization procedure gave unrealistically high concentrations of epoxides and low concentrations of chlorohydrins in these aliquots of the reaction mixture. The same sensitivity to base accounts for the presence of low concentrations of α -epoxide in the synthesized α -chlorohydrin where a neutralization step also is involved (see Experimental). The β -chlorohydrin is somewhat less sensitive to base-catalyzed dehydrohalogenation, so the synthesized β -chlorohydrin could be obtained free of contaminating β -epoxide. In the experiments reported here, neutralization with base before extraction was avoided (see Experimental).

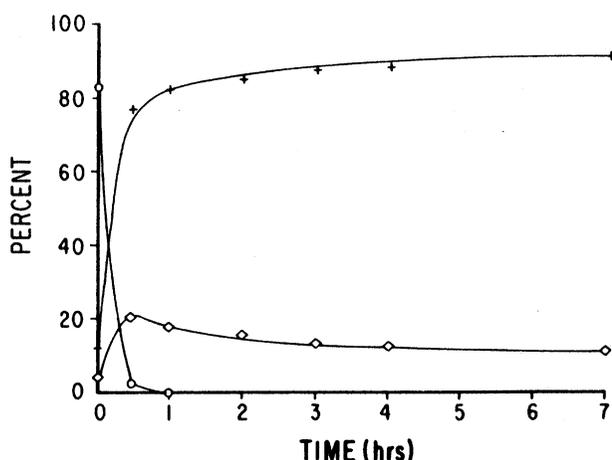
The course of the reaction of the α -epoxide in simulated gastric juice is shown in Figure 2.

During the first two hr, the α -epoxide concentration decreased to less than 15%, and after four hr its concentration was less than 4%. Most foods are considered to have a residence time of two to four hr in the stomach (37). While the α -epoxide concentration decreased, the α -chlorohydrin concentration reached a maximum after about one hr and then decreased. Meanwhile the triol concentration increased steadily. The simultaneous decrease of the α -epoxide and the α -chlorohydrin accompanied by a steady increase in triol led to the expectation that a substantial portion of the α -chlorohydrin reacted further in the acid, aqueous medium to form triol. To test this

TABLE 2

Hydrolysis of α -Chlorohydrin and β -Chlorohydrin in Simulated Gastric Juice at 37 C

Time (hr)	α -Chlorohydrin			β -Chlorohydrin		
	Chlorohydrin (%)	Epoxide (%)	Triol ^a (%)	Chlorohydrin (%)	Epoxide (%)	Triol ^a (%)
0.0	93.4	3.0	3.6	99.2	—	0.8
0.5	69.3	4.3	26.4	96.7	—	3.3
1.0	51.6	3.3	45.1	92.8	—	7.2
2.0	30.6	2.2	67.2	89.0	—	11.0
3.0	18.2	1.3	80.5	85.4	—	14.6
4.0	16.0	0.9	83.1	84.3	—	15.7
7.0	6.9	0.5	92.6	78.6	—	21.4

^aThe same triol is formed from both chlorohydrins.FIG. 3. Reaction of cholesterol 5 β ,6 β -epoxide (β -epoxide) in simulated gastric juice 37 C. (O) β -epoxide, (□) β -chlorohydrin, (+) triol.

hypothesis, α -chlorohydrin was suspended in simulated gastric juice at 37 C. Results are shown in Table 2. It is apparent from these data that the α -chlorohydrin was indeed converted rather rapidly to triol. The small amount of α -epoxide that was present in the starting material as an impurity, was also mostly converted to triol. This latter conversion probably occurs via the α -chlorohydrin and directly, as well.

Exposure of the β -epoxide to the action of simulated gastric juice at 37 C caused a very rapid disappearance of epoxide from the mixture, as shown in Figure 3. The β -chlorohydrin peaked at only 1/2 hr into the experiment after it had reached the 20% level and then tapered off gradually, while the triol concentration rose steeply during the first hour. These data seem to indicate that much of the β -epoxide is converted directly to triol without the intermediacy of the β -chlorohydrin. The rapid hydration of the β -epoxide at pH 5.5 had been observed and measured earlier (14). Exposure of the β -chlorohydrin to simulated gastric juice at 37 C (Table 2) confirmed that the hydrolysis of the β -chlorohydrin was much slower than that of the α -chlorohydrin.

TABLE 3

Stability of Chlorohydrins in Growth Medium (pH 7.35)^a at 37 C

Time (hr)	Epoxide (%)		Chlorohydrin (%)		Triol ^b (%)	
	α	β	α	β	α	β
0.0	4.6	0.7	93.6	98.9	1.8	0.4
0.5	28.5	5.5	69.4	93.4	2.1	1.1
1.0	32.4	7.3	65.7	91.2	1.9	1.5
2.0	33.6	10.1	64.5	87.5	1.9	2.4
7.0	38.2	16.9	59.3	78.4	2.5	4.7
24.0	46.8	32.1	50.3	59.5	2.9	8.4

^aDulbecco's Modified Eagle Medium (high glucose).^bThe same triol is formed from both chlorohydrins.

TABLE 4

Stability of α -Chlorohydrin in Growth Medium (pH 7.35) and in Phosphate Buffer (pH 7.44), 37 C

Time (hr)	α -Epoxide (%)		α -Chlorohydrin (%)		Triol (%)	
	pH 7.35	pH 7.44	pH 7.35	pH 7.44	pH 7.35	pH 7.44
0.0	4.6	8.5	93.6	90.6	1.8	0.9
0.5	28.5	33.2	69.4	63.5	2.1	3.2
1.0	32.4	32.9	65.7	64.4	1.9	2.7
2.0	33.6	37.6	64.5	59.9	1.9	2.5
4.0	36.2	39.3	61.5	58.2	2.3	2.5
7.0	38.2	42.2	59.3	54.9	2.5	2.9
24.0	46.8	47.3	50.3	49.8	2.9	2.9

Stability of the isomeric chlorohydrins to mildly alkaline aqueous media is of concern, if their physiological activity is to be measured. Each of the chlorohydrins separately was placed into Dulbecco's Modified Eagle Medium (high glucose), which had a measured pH of 7.35. Results are shown in Table 3. It is clear that both chlorohydrins were converted to their respective epoxides at a very significant rate in this mildly alkaline medium. The conversion rate of the α -chlorohydrin appeared to be somewhat more rapid than that of the β -chlorohydrin, and surprisingly, a small amount of the latter was hydrolyzed to triol. Because this growth medium is a complex mixture of organic and inorganic ingredients, it was desirable to test the question whether dehydrohalogenation of the chlorohydrins was due to pH or possibly due to one or more of the components of the medium. Some of the α -chlorohydrin was suspended in phosphate buffer (pH 7.44) at 37 C, and the composition of the mixture was analyzed. The results are shown in Table 4 where the data at pH 7.35 (growth medium) and pH 7.44 (phosphate buffer) are compared. The similarity of the data in the two media confirms that the effect is due to pH rather than an ingredient of the growth medium. The comparison also serves as an indication of the reproducibility of the analytical procedure used in these experiments.

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