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Sterol Metabolism

XXXVII. On the Oxidation of Cholesterol by Dioxygenases[1]

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The oxidation of cholesterol by plant and mammalian dioxygenases yielding cholesterol 7α- and 7β-hydroperoxides has been demonstrated. Cholesterol oxidation is coupled to the oxygenation of polyunsaturated fatty acid esters by soybean lipoxygenase, to the reduction of hydrogen peroxide catalyzed by horseradish peroxidase, and to the oxidation of NADPH by the NADPH-dependent microsomal lipid peroxidation system of rat liver. The initially formed epimeric cholesterol 7-hydroperoxides are transformed in each case to the commonly encountered corresponding 7-alcohol and 7-ketone derivatives. These dioxygenase transformations thus mimic in detail the radiation-induced free radical oxidation of cholesterol by molecular oxygen. Electronically excited (singlet) molecular oxygen is not implicated in these transformations.

INTRODUCTION

Our demonstration of the rearrangement of cholesterol 20α-hydroperoxide[2] by bovine and murine adrenal cortex mitochondrial enzymes anaerobically to cholest-5-ene-3β,20α,21-triol and cholest-5-ene-3β,20α,22R-triol (*1–3*) established a means of stereospecific enzymic hydroxylation of steroids not involving molecular oxygen or NADPH and suggested that cholesterol 20α-hydroperoxide or a formal equivalent be an intermediate in the biosynthesis of 3β-hydroxypregn-5-en-20-one from cholesterol. However, enzymic formation of the requisite 20α-hydroperoxide infers oxidation of cholesterol by a dioxygenase rather than by a monooxygenase (mixed function oxidase). In order to support the concept of dioxygenase action on sterols we have examined several tissues and enzyme systems for their capacity to form hydroperoxides from unsaturated sterols.

We report herein experimental work demonstrating the actions of soybean lipoxygenase, horseradish peroxidase, and the NADPH-dependent microsomal lipid peroxi-

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[2] Systematic nomenclature for sterols given trivial names includes: 3β-hydroxycholest-5-ene-20α-hydroperoxide, cholesterol 20α-hydroperoxide; 3β-hydroxycholest-5-ene-7α-hydroperoxide, cholesterol 7α-hydroperoxide; 3β-hydroxycholest-5-ene-7β-hydroperoxide, cholesterol 7β-hydroperoxide. Systematic nomenclature for the all-*cis* polyunsaturated fatty acid esters given trivial names include: ethyl octadeca-Z-9, Z-12-dienoate, ethyl linoleate; ethyl octadeca-Z-9, Z-12, Z-15-trienoate, ethyl linolenate; ethyl eicosa-Z-11, Z-14-dienoate, ethyl eicosadienoate; ethyl eicosa-Z-11, Z-14, Z-17-trienoate, ethyl eicosatrienoate; ethyl eicosa-Z-5, Z-8, Z-11, Z-14-tetraenoate, ethyl arachidonate.

dation system of rat liver on cholesterol to form the epimeric cholesterol 7-hydroperoxides Ia and Ic as initial and chief products, together with several secondary products derived by further alteration of the initially formed 7-hydroperoxides. These results formally establish dioxygenase action on sterols, a matter demonstrated unequivocally for the first time. Preliminary accounts of some of these points have appeared (*4*, *5*).

EXPERIMENTAL

All organic solvents were redistilled shortly before use. General details of thin-layer (*6*) and gas (*7*) chromatography have been described elsewhere. Thin-layer chromatography was conducted on 0.25 mm thick 20 × 20 cm chromatoplates of Silica Gel HF_{254} (E. Merck GmbH., Darmstadt) with triple ascending solvent irrigations in all cases using specified solvent mixtures. Gas chromatography was conducted using 1.83 m long silanized glass U-tubes packed with 2 or 3% OV-210 and SP-2401 on 100–120 mesh Supelcoport (Supelco, Inc., Bellafonte, Pa.) and Hewlett–Packard F and M Model 400 and Model 402 gas chromatographs.

[1,2-^{3}H]Cholesterol (53.2 Ci/mmole) and [4-^{14}C]cholesterol (56 Ci/mole) were purchased from New England Nuclear, Boston, Mass. The [4-^{14}C]5α-hydroperoxide IIa (8.2×10^6 dpm/mmole) was prepared by photosensitized oxygenation of [4-^{14}C]-cholesterol (*8*); the [4-^{14}C]7α-hydroperoxide Ia (7.5×10^6 dpm/mmole) was prepared by allylic rearrangement of [4-^{14}C] IIa in chloroform solution (*9*); and the [4-^{14}C]7β-hydroperoxide Ic (8.5×10^6 dpm/mmole) was prepared by irradiation of [4-^{14}C]-cholesterol in air with ^{60}Co gamma radiation for 8 hr (*10*). Sodium borohydride reduction of the parent [4-^{14}C]hydroperoxides in methanol solution yielded the corresponding [4-^{14}C]alcohol derivatives, thus, [4-^{14}C] IIb (4.4×10^6 dpm/mmole) from [4-^{14}C] IIa; [4-^{14}C] Ib (6.7×10^6 dpm/mmole) from [4-^{14}C] Ia; [4-^{14}C] Id (7.05×10^6 dpm/mmole) from [4-^{14}C] Ic. All radioactive sterol preparations were free from other detectable components and were radiochemically pure as judged by thin-layer chromatography. Each radioactive sterol was purified by thin-layer chromatography prior to use, using chloroform-acetone (24:1, v/v) followed by benzene–ethyl acetate (18:7, v/v). Polyunsaturated fatty acid ethyl esters[2] including ethyl linoleate, ethyl linolenate, ethyl eicosadienoate, ethyl eicosatrienoate, and ethyl arachidonate were purchased from Sigma Chemical Co., St. Louis, Mo. Ethyl linoleate hydroperoxides were prepared by incubations of ethyl linoleate with soybean lipoxygenase using 50 m*M* Tris–HCl buffer at pH 6.6 and 9.0 at 30°C for 2 hr. Thin-layer chromatography of the ester hydroperoxides using isooctane-diethyl ether-acetic acid (40:20:1, v/v/v), with elution from the chromatoplate with acetone and evaporation under vacuum afforded the purified mixed ethyl linoleate hydroperoxides. Hydroperoxides prepared at pH 6.6 and 9.0 were used in subsequent experiments conducted at pH 6.6 and 9.0, respectively.

Soybean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12, formerly also EC 1.13.1.13 and EC 1.99.2.1) was purchased from four different sources (Sigma Chemical Co., Pabst Laboratories Biochemical Co., Milwaukee, Wis., Nutritional Biochemical Corp., Cleveland, Ohio, and Worthington Biochemical Corp., Freehold,

N.J.). Polyacrylamide gel electrophoresis of the commercial soybean lipoxygenase preparation was conducted using 250 μg samples on a 7.5% acrylamide gel for 5 hr and 25 V and 24 mA for six sample tubes. Proteins were stained with amido black. Each commercial enzyme was a mixture of three major protein components and up to six minor components, very much like previously reported soybean lipoxygenase analyses (*11*, *12*). Except for preliminary comparison data the soybean lipoxygenase of Sigma Chemical Co. was used.

Incubation procedures with lipoxygenase for preparative purposes involved addition of 220 μmole of [1,2-^{3}H]cholesterol in 0.1 ml acetone containing 10% Tween-80 to 650 μmoles of ethyl linoleate in 0.65 ml 95% ethanol in a 800 ml flask. After evaporation of solvent under nitrogen 245 ml of 50 m*M* Tris HCl buffer (at pH 6.6 and 9.0) previously oxygenated for 15 min, was added slowly to the flask to suspend the substrates. A solution of 30 mg of soybean lipoxygenase in 5 ml of buffer was then added, and incubation at 30°C for 2 hr in a rotary shaker water bath followed. Incubations for analytical purposes were conducted in the same manner using 50 ml flasks containing 11 μmoles of [1,2-^{3}H]cholesterol, 25 μmole ethyl linoleate, and 2 mg lipoxygenase in 10 ml of oxygenated 50 m*M* Tris-HCl buffer (pH 6.6 and 9.0). Incubations were terminated by cooling in ice water, acidifying to pH 2.0 with 2 *N* HCl, and extracting four times with equal volumes of ethyl acetate. The extracts were pooled, dried over anhydrous sodium sulfate, and evaporated under vacuum without heating. Recovery of radioactivity was generally 98% or more of that added.

Soybean lipoxygenase incubations in which [1,2-^{3}H] Ia, Ic, or IIc were substrates instead of [1,2-^{3}H] cholesterol were conducted in the same fashion, using 11 μmole of sterol hydroperoxide, 25 μmoles of ethyl linoleate, and 2 mg lipoxygenase in 10 ml 50 m*M* Tris-HCl buffer (pH 6.6 and 9.0) at 30°C for 2 hr.

The progress of the oxygenation of ethyl linoleate by soybean lipoxygenase was followed by withdrawing a 1 ml aliquot of incubation medium, mixing with 0.5 ml of 95% ethanol, and adding 50 μl of the diluted solution to 3 ml of 95% ethanol, and measuring the absorbance of the solution at 234 nm versus a 95% ethanol blank with a Cary Model 14 ultraviolet-visible spectrophotometer. A molar absorbance of 28 000 for the product *cis,trans* conjugated diene was used in calculations.

Horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) and beef liver catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) were purchased from Sigma Chemical Co. Incubations were conducted for 2 hr at 37°C using 6.2 μmole [4-^{14}C]-cholesterol (1.6×10^6 dpm) or 2.0 μmole [1,2-^{3}H]cholesterol (10^7 dpm) in 100 μl of acetone containing 10% Tween-80 added to 10 ml of 0.1 *M* sodium acetate buffer (pH 5.5) containing 180 μmole H_2O_2, 8.0 μmole $MnCl_2$, and 0.02 μmole horseradish peroxidase (RZ 1.6). After cooling the incubation mixture in ice water, sterols were extracted four times with equal volumes of ethyl acetate. The pooled extracts were dried over anhydrous sodium sulfate and evaporated under vacuum without heating. Recovery of radioactivity was generally greater than 98% of the amount added.

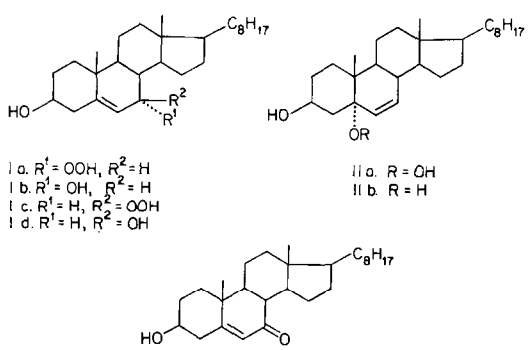
For experiments with rat liver, male albino rats of the inbred ACI/Tex and outbred Tex:(SD) strains obtained from the Texas Inbred Mice Co., Houston, Texas were used. Rats were maintained on Purina animal chow fed *ad libitum* pending experimental use. Only Tex:(SD) strain rats weighing 500–600 g were used for quantitative data. Several rats (250–300 g) were pretreated with phenobarbital as previously des-

cribed (*13*). Animals were anesthetized with diethyl ether and killed by cutting the spinal cord. Livers were perfused immediately *in situ* with ice cold 0.25 *M* sucrose to remove blood completely. Livers were excised, chopped in a blender in a ratio of 1 g to 2 ml of 0.15 *M* Tris–HCl (pH 6.8) at 4°C for 10 sec, and the disrupted tissue was homogenized in a glass homogenizer and diluted appropriately with buffer to give a preparation containing 1 g (wet weight) of liver in 4 ml of buffer. The homogenate was fractionated by centrifugation at 4°C using a Beckman Model L ultracentrifuge, yielding mitochondria after 10 min at 9000 *g*, microsomes after 60 min at 105000 *g*, and a soluble fraction.

Incubations with rat liver subcellular fractions were conducted in 50 ml flasks containing 5 ml of 0.15 *M* Tris–HCl buffer (pH 6.8) to which 10 μmoles [1,2-^{3}H]-cholesterol (6×10^6 dpm) in 250 μl acetone (containing 20% Tween-80) had been added. After evaporation of the acetone under nitrogen, 6.5 μmole NADPH, 33 μmoles glucose 6-phosphate, and 1 unit glucose 6-phosphate dehydrogenase in 1 ml of buffer was added. The reaction was started upon addition of the enzyme preparation (4 ml equivalent to 1 g of wet tissue) and was carried out under oxygen at 30°C for 2 hr. The reaction was terminated by addition of 2 ml of 2 *N* HCl and extracted four times with equal volumes of ethyl acetate. The pooled extracts were dried over anhydrous sodium sulfate and evaporated under vacuum for analysis.

Control experiments for soybean lipoxygenase and horseradish peroxidase involved boiled enzymes. The control for the rat liver experiments of Table 7 involved no enzyme.

Products of incubations were analyzed qualitatively for product identification and quantitatively for the amounts of radioactivity from labeled substrate cholesterol incorporated into each product. Product identification required resolution of the seven possible primary (Ia, Ic, IIa) and secondary (Ib, Id, IIb, and III) products, whereas quantitative analysis required this plus rigorous exclusion of radioactive substrate cholesterol from the products.



I a. R^1 = OOH, R^2 = H
I b. R^1 = OH, R^2 = H
I c. R^1 = H, R^2 = OOH
I d. R^1 = H, R^2 = OH

II a. R = OH
II b. R = H

III.

Thin-layer chromatography readily resolves the diols Ib, Id, and IIb from one another and from the 7-ketone III and the sterol hydroperoxides Ia, Ic, and IIa, and resolution of the 7β-hydroperoxide Ic from Ia and IIa can be achieved. Resolution of

Ia and IIa cannot be achieved directly, and resort must be had to initial resolution of the sterol hydroperoxides as a group, followed by sodium borohydride reduction and subsequent resolution of the product alcohols (Ib from Ia, Id from Ic, IIb from IIa). Rigorous exclusion of the 7-ketone III from the hydroperoxide mixture must be emphasized to avoid generation of Id and Ib from III in the reduction step.

Qualitative analyses of the sterol product mixture and of the borohydride reduced product mixture was routinely conducted using benzene-ethyl acetate (18:7, v/v). Mobility data relative to cholesterol as unity: cholesterol, 1.00; Ic, 0.69; Ia and IIa, 0.61; III, 0.53; IIb, 0.41; Id, 0.33; Ib, 0.25. Chromatoplates were examined under 254 nm light to detect III, sprayed with *N,N*-dimethyl-*p*-phenylenediamine (*14*) to detect sterol hydroperoxides, and then sprayed with 50% aqueous sulfuric acid and warmed gently for full color display to detect all sterols. Although these procedures sufficed for product identification, the identity of each resolved component was confirmed by gas chromatography. Inspection of the characteristic pyrolysis patterns obtained (*15*, *16*) allowed independent identification of the sterols I, II, and III. Additional evidence of identity and purity was adduced following borohydride reduction of the hydroperoxides Ia, Ic, and IIa to the diols [1,2-^{3}H] Ib, [1,2-^{3}H] Id, and [1,2-^{3}H] IIb by adding [4-^{14}C] Ib, [4-^{14}C] Id, and [4-^{14}C] IIb and purification by successive thin-layer chromatography to constant ^{3}H:^{14}C isotope ratios.

For measurement of radioactivity incorporation the product residues obtained from experimental incubations were immediately redissolved in a small volume (200 μl) of acetone. In some cases 50 μg of 3β-hydroxycholest-5-en-7-one was previously dissolved in the acetone to act as marker on subsequent thin-layer chromatography. The sample was applied to a 0.25-mm thick 20 × 20 cm chromatoplate and converged to a fine line by brief chromatographic development with acetone. The chromatoplate was then developed by triple ascending irrigation to full height using benzene-ethyl acetate (18:7, v/v). The sterol hydroperoxides were found within a 1.2 cm wide zone just above that of 3β-hydroxycholest-5-en-7-one whose location was determined by viewing under 254 nm light. Reference sterol hydroperoxides applied to one edge of the chromatoplate detected by spraying with *N,N*-dimethyl-*p*-phenylenediamine further guided location of the product hydroperoxides. The excised chromatographic zone was eluted with acetone and the solvent removed under nitrogen in a small tube. Sodium borohydride (1–2 mg) was added to the tube, and the reduction was initiated by addition of 0.5 ml methanol. After 1 hr the reduced solution was chromatographed directly on a chromatoplate using benzene-ethyl acetate (18:7, v/v). Reference samples of the 3β,7-diols Ib and Id and the 3β,5α-diol IIb spotted along one edge of the chromatoplate were revealed by spraying carefully with 50% aqueous sulfuric acid. Exposure of the chromatoplate to an iodine atmosphere for a few minutes also facilitated location of the product zones.

After dissipation of the iodine vapors the appropriate zones were individually excised from the chromatoplate, and the silica gel containing the radioactive sterol was added to 10 ml of scintillation fluid (0.5% PPO and 0.025% POPOP in toluene containing 5% methanol). Radioactivity was measured to a 5% precision using a Beckman Model LS-200 liquid scintillation counter, with 35% efficiency for ^{3}H and 67% for ^{14}C. Chromatogram regions between sterol zones of interest also eluted assayed for radioactivity at background levels only. Results are expressed as percent

radioactivity (average of two or three independent experiments) incorporated into each sterol from substrate.

RESULTS

Incubations of radioactive cholesterol with soybean lipoxygenase, horseradish peroxidase, or the NADPH-dependent microsomal lipid peroxidation system of rat liver yielded uniformly the epimeric cholesterol 7-hydroperoxides Ia and Ic as initial and chief products, the quasi-equatorial 7β-hydroperoxide Ic (*17*) predominating. Both Ia and Ic could be detected within the first 15–30 min of incubation, with the secondary products Ib, Id, IIa, IIb, and III appearing later. In the rat liver case the amounts of secondary products equaled or exceeded levels of the primary products Ia and Ic. The soybean lipoxygenase, horseradish peroxidase, and NADPH-dependent microsomal lipid peroxidation system were characterized by 7α:7β product ratios of 2:3 to 1:3 (range 2:3 to 1:20 with yield 0.4–2.8%), 1:1 to 1:2 (range 2:1 to 1:2 with yield 0.04–0.63%), and 1:2 to 1:3 (range 2:3 to 1:7 with yield 0.21–4.8%) respectively. In no instance was the 5α-hydroperoxide IIa detected as an initial product. Rather, where observed, the 5α-hydroperoxide IIa was formed at later times after initial formation of the epimeric 7-hydroperoxides Ia and Ic. These product ratios and yields are quite comparable to those obtained in the free radical oxidation of crystalline cholesterol induced by ^{60}Co gamma radiation characterized by 7α:7β product ratio of ca. 1:10 and yields of 5.8–7.4% (*10*).

Results with Soybean Lipoxygenase

General incubation conditions. A study of buffer composition and strength established that at pH 7.4 both Tris–HCl and phosphate buffers were optimally effective at 50–100 m*M* concentration with combined yields of Ia and Ic of 2.25% for Tris-HCl and 2.0% for phosphate buffers respectively. A companion study of pH effects established that 50 m*M* Tris–HCl buffer was equally effective over the pH range 6.6–9.0, whereas 50 m*M* phosphate buffer was optimally effective at pH 8.0, with combined yields of Ia and Ic being 2.5 and 3.25%, respectively. However, phosphate buffer promoted isomerization of Ia and Ic to the 5α-hydroperoxide IIa over the pH range 5.0–9.0 (yield 0.3%) and therefore, was not used for further studies. Incubation temperatures varying from 10–30°C did not influence the nature or extent of peroxidation of cholesterol, with yields of Ia of 0.65–0.8% and of Ic of 1.34–1.38%. At 38°C yields were increased to 1.03% for Ia and 1.74% for Ic, but increased dehydration of Ia and Ic to the 7-ketone III was observed. The previously demonstrated temperature optimum of 30°C (*18*) was accordingly used in all studies.

The initial rates of peroxidation of ethyl linoleate and of cholesterol yielding the corresponding product hydroperoxides were essentially linear over the first 2 hr of incubation (Fig. 1), with a constant relationship (9.4–9.8%) up to 4 hr between the amount of cholesterol and ethyl linoleate oxidized. The peroxidation rates decreased beyond 2 hr although the yields of peroxidized products increased such that after 12 hr the combined yield of Ia and Ic was 7.5% and of ethyl linoleate hydroperoxide 62.5%. A standard incubation time of 2 hr was chosen.

Different commercial samples of soybean lipoxygenase recognized as protein mixtures containing isozymes L-1, L-2, and L-3 (*19–21*) were equally effective in oxidizing cholesterol at pH 6.6 (0.22–0.28% Ia, 1.43–1.53% Ic), but at pH 9.0 the Sigma Chemical Co. enzyme was more effective (0.12% Ia, 1.24% Ic), than others (0.03–0.08% Ia, 0.59–0.78% Ic) and was used accordingly.

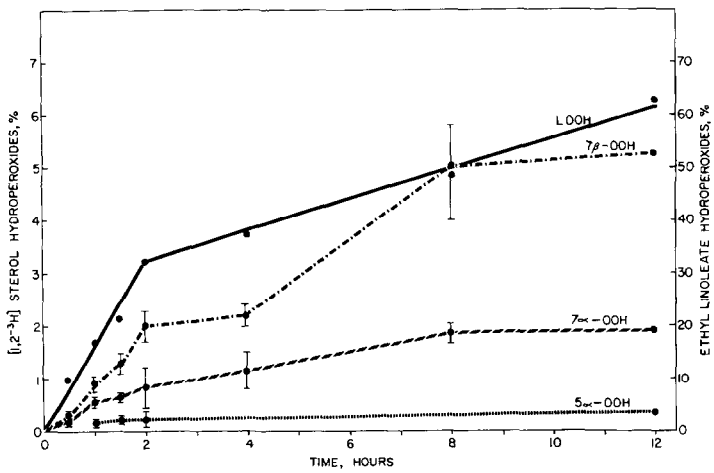


FIG. 1. Formation of ethyl linoleate hydroperoxides (—, LOOH) and of Ia (---), Ic (-·-), and IIa (· · ·) in soybean lipoxygenase incubations of 11 μmole [1,2-^{3}H]cholesterol, 25 μmole ethyl linoleate, and 2 mg soybean lipoxygenase in 10 ml 50 m*M* Tris–HCl (pH 9.0) buffer at 30°. Data are averages of three separate experiments (standard deviations indicated) adjusted for boiled enzyme control values.

Effects of polyunsaturated substrates. Data of Table 1 demonstrate that both active enzyme and polyunsaturated fatty acid derivative are required for the peroxidation of cholesterol. Although yields varied with pH and different C_{18}- and C_{20}-substrates it is obvious that either ω3 or ω6 polyunsaturated fatty acid esters supported the oxidation of cholesterol.

These data also established that it was the polyunsaturated fatty acid ester serving as principal substrate and not product hydroperoxides formed from the esters that supported the oxidation of cholesterol. The characteristic cholesterol peroxidation pattern obtained with ethyl linoleate as principal substrate with 7α:7β ratio of 2:3 was substantially altered in favor of the 7β-hydroperoxide Ic in incubations in which ethyl linoleate and ethyl linoleate hydroperoxides served as cosubstrates, and the amounts of hydroperoxide products were diminished. Incubations in which ethyl linoleate hydroperoxides alone were added as substrates yielded little or no peroxidation of cholesterol, thereby establishing the essentiality of polyunsaturated fatty acid esters as principal substrates in the lipoxygenase reaction.

Inhibition of lipoxygenase reactions. The well-known inhibition of soybean lipoxygenase oxidation of linoleate by propyl gallate and by α-tocopherol (*18, 22–25*) was also obtained under our incubation conditions. During the initial period of linear rates 0.15 m*M* propyl gallate inhibited the oxygenation of ethyl linoleate much more

TABLE 1

EFFECTS OF DIFFERENT FATTY ACID ESTER SUBSTRATES

		Amount of product formed (%)[a]										
		Enzyme	Control		Substrate[b]					Hydroperoxide effect		
pH	Product	None	Boiled	Active[c]	18:2	18:3	20:2	20:3	20:4	18:2	18:2 + LOOH[d]	LOOH[d]
6.6	Ia	0.05	0.04 ± 0.03	0.06 ± 0.04	0.96 ± 0.18	1.20 ± 0.79	0.44 ± 0.09	0.52 ± 0.06	0.39 ± 0.10	0.90 ± 0.09	0.30 ± 0.09	0.00
	Ic	0.08	0.06 ± 0.04	0.10 ± 0.04	1.36 ± 0.67	1.57 ± 0.38	0.63 ± 0.32	0.72 ± 0.58	0.39 ± 0.11	1.36 ± 0.33	0.90 ± 0.02	0.00
9.0	Ia	0.05	0.07 ± 0.02	0.16 ± 0.05	0.58 ± 0.04	0.88 ± 0.13	0.20 ± 0.00	0.67 ± 0.17	0.56 ± 0.07	0.38 ± 0.08	0.00 ± 0.00	0.00
	Ic	0.07	0.12 ± 0.07	0.31 ± 0.10	1.02 ± 0.03	1.22 ± 0.36	0.34 ± 0.01	1.13 ± 0.22	0.88 ± 0.16	0.64 ± 0.20	0.34 ± 0.04	0.10 ± 0.14

[a] Amount of hydroperoxide formed as per cent of substrate [1,2-^{3}H]cholesterol, expressed as an average of three independent experiments ± standard deviation of the mean. Incubations conducted at 30° for 2 hr using 11 μmole [1,2-^{3}]cholesterol, 25 μmole fatty acid ethyl ester, and 2 mg soybean lipoxygenase in 10 ml of oxygenated 50 m*M* Tris–HCl buffer (pH 6.6 or 9.0). Nonhydroperoxide products Ib, Id, and III not measured.

[b] Substrates abbreviated: 18:2, ethyl linoleate; 18:3, ethyl linolenate; 20:2, ethyl eicosadienoate; 20:3, ethyl eicosatrienoate; 20:4, ethyl arachidonate.

[c] Active enzyme but no fatty acid ester.

[d] Ethyl linoleate hydroperoxides (abbreviated LOOH) prepared by separate lipoxygenase incubations of ethyl linoleate at pH 6.6 or 9.0 and isolated by thin-layer chromatography were added in 50-μmole amounts.

effectively (61 %) than did 1.0 m*M* *dl*-α-tocopherol (9 %). Propyl gallate was also much more inhibitory in the coupled oxygenation of cholesterol (Table 2), producing 83–85 % inhibitions of cholesterol 7-hydroperoxide formation at concentration as low as 0.05 m*M* and 90–94 % inhibitions at 0.15–3.0 m*M* concentrations. The effects of *dl*-α-tocopherol on the coupled oxidation were more complicated, ranging from no

TABLE 2

INHIBITION OF THE LIPOXYGENASE REACTION

Inhibitor	Concentration (m*M*)	Products formed (%)[a]	
		Ia	Ic
n-Propyl gallate	0.05	0.12	0.20
	0.15	0.07	0.09
	0.50	0.05	0.06
	1.00	0.04	0.06
	3.00	0.04	0.06
dl-α-Tocopherol	0.20	0.63	1.17
	0.40	0.63	1.25
	0.80	0.73	1.46
	1.20	0.75	1.64
	1.60	0.60	1.09
	3.00	0.58	0.96
None	—	0.70	1.37

[a] Average of two independent incubations at 30° for 2 hr using 11 μmole [1,2-^{3}H]cholesterol, 25 μmole ethyl linoleate, and 2 mg soybean lipoxygenase in 10 ml of oxygenated 50 m*M* Tris-HCl buffer (pH 9.0).

inhibition at 0.80–1.20 m*M*, 10–15 % inhibitions at 0.20–0.40 m*M*, and 15–20 % inhibitions at 1.60–3.0 m*M* concentrations. The 7α:7β product ratio (Ia:Ic) of 1:2 was unaffected by *dl*-α-tocopherol or by the lowest level (0.05 m*M*) of propyl gallate used, the product ratio being more nearly 1:1 for the fully inhibited reactions.

Lipoxygenase effects on sterol hydroperoxides. The disposition of sterol hydroperoxides Ia, Ic, and IIa in soybean lipoxygenase incubations disclosed by data of Table 3 included reactions of allylic rearrangement, epimerization, reduction, and dehydration. The 7α-hydroperoxide Ia was transformed chiefly by epimerization to the 7β-hydroperoxide Ic, both Ia and Ic then being dehydrated to the 7-ketone III and reduced to the corresponding alcohols Ib and Id. However, significant levels of the 5α-hydroperoxide IIa were detected, which establish that allylic rearrangement of the 7α-hydroperoxide Ia to the 5α-hydroperoxide IIa occurs in aqueous protein dispersions. The matter is in distinction to behaviour of the 7α-hydroperoxide Ia in other systems (*15–17*). The 7β-hydroperoxide Ic was likewise epimerized to the 7α-hydroperoxide *Ia* as a major transformation, with the usual secondary products Ib, Id, and III also formed. The 5α-hydroperoxide IIa (formed from 7α-hydroperoxide *Ia*) and the 3β,5α-diol IIb were also detected. The 5α-hydroperoxide IIa was the least

TABLE 3

METABOLISM OF STEROL HYDROPEROXIDES BY SOYBEAN LIPOXYGENASE

Substrate	pH	Enzyme	Products found (%)[a]							
			Ia	Ib	Ic	Id	IIa	IIb	III	Others[b]
Ia	6.6	Active	71.0 ± 3.34	1.71 ± 0.94	14.4 ± 3.10	0.85 ± 0.17	0.80 ± 0.25	1.45 ± 0.50	5.84 ± 3.22	3.94 ± 3.20
		Boiled	74.1 ± 3.16	1.00 ± 0.32	17.4 ± 2.94	0.72 ± 0.19	1.16 ± 0.21	1.32 ± 0.32	3.16 ± 0.39	1.18 ± 0.18
	9.0	Active	57.9 ± 5.64	1.84 ± 0.02	11.9 ± 2.29	0.77 ± 0.06	0.54 ± 0.12	2.12 ± 0.41	20.7 ± 1.64	4.28 ± 0.85
		Boiled	68.5 ± 2.08	1.68 ± 0.90	16.3 ± 3.86	1.05 ± 0.25	1.03 ± 0.05	1.73 ± 0.34	5.09 ± 0.54	4.62 ± 1.69
Ic	6.6	Active	7.12 ± 0.15	0.32 ± 0.06	77.4 ± 3.14	1.12 ± 0.37	0.34 ± 0.08	0.39 ± 0.36	1.25 ± 0.26	12.0 ± 2.16
		Boiled	4.88 ± 2.73	0.50 ± 0.13	82.0 ± 2.51	0.85 ± 0.16	0.35 ± 0.03	0.48 ± 0.35	2.76 ± 0.45	8.16 ± 0.15
	9.0	Active	8.95 ± 2.51	1.21 ± 0.26	81.1 ± 5.35	1.73 ± 0.55	0.44 ± 0.05	0.73 ± 0.06	2.96 ± 0.15	2.86 ± 1.11
		Boiled	7.19 ± 4.72	0.43 ± 0.12	84.7 ± 3.95	1.13 ± 0.08	0.43 ± 0.11	0.59 ± 0.02	1.97 ± 0.90	3.61 ± 0.58
IIa	6.6	Active	37.0 ± 1.11	3.85 ± 0.80	2.79 ± 0.16	0.67 ± 0.08	38.8 ± 0.84	1.21 ± 0.13	8.38 ± 2.79	7.30 ± 1.21
		Boiled	35.1 ± 1.88	0.48 ± 0.15	3.57 ± 0.75	0.35 ± 0.05	49.3 ± 2.04	1.05 ± 0.07	0.57 ± 0.02	9.55 ± 1.37
	9.0	Active	41.1 ± 1.65	9.80 ± 4.30	7.90 ± 1.04	1.78 ± 0.19	11.3 ± 1.97	2.60 ± 0.55	13.5 ± 3.16	12.0 ± 7.42
		Boiled	35.3 ± 5.90	1.10 ± 0.26	10.5 ± 0.61	0.44 ± 0.18	44.3 ± 3.31	1.21 ± 0.25	2.67 ± 0.40	4.49 ± 0.02

[a] Amount of product formed as percentage of substrate hydroperoxide, expressed as an average of three independent experiments ± standard deviation of the mean. Incubations conducted at 30° for 2 hr using 11 μmole [4-^{14}C]sterol hydroperoxide, 25 μmole ethyl linoleate, and 2 mg soybean lipoxygenase in 50 m*M* Tris–HCl buffer.

[b] Radioactivity of unidentified components from regions of the chromatograms not occupied by I, II, or III.

stable of the three hydroperoxides and was extensively rearranged to the 7α-hydroperoxide Ia (from which were derived Ib, Ic, Id, and III) and reduced to the 3β,5α-diol IIb.

Thus, each sterol hydroperoxide Ia, Ic, and IIa yielded a common mixture of seven sterols I, II, and III distinguished by their proportions. Considering only the product hydroperoxides approximate Ia: Ic: IIa ratios of 80:20:1 were obtained from Ia and 20:250:1 from Ic, with a more variable proportion ranging from 13:1:14 to 10:2:3 from IIa. Although these product ratios were obtained using high substrate

levels of sterol hydroperoxides, the relative stabilities indicated probably hold for the same hydroperoxides when formed from cholesterol in active soybean lipoxygenase incubations. These data suggest that the product hydroperoxides are sufficiently stable to survive secondary transformations and be detected by our methods in incubations using cholesterol as substrate.

By the usual consideration of differences between active and boiled enzyme experiments, only the dehydration at pH 9.0 of the 7α-hydroperoxide Ia to the 7-ketone III could be enzyme catalyzed. Although levels of the 3β,7α-diol Ib derived from the 5α-hydroperoxide IIa in active enzyme experiments were eight- to ninefold those in boiled enzyme controls, the 3β,7α-diol Ib cannot form from IIa directly but must derive by the two-step process IIa to Ia to Ib, neither phase of which is clearly enzyme catalyzed. Therefore, Ib formed from IIa is probably not of enzyme origin. Similar arguments for other cases of Table 3 suggest these transformations result from an erratic ionic catalysis of protein or buffer but not of enzyme.

Results with Horseradish Peroxide

General incubation conditions. Most of the experimental parameters for horseradish peroxidase incubations were examined qualitatively by thin-layer chromatography only, without quantitative measurements. Incubations in which the enzyme was absent did not show sterol hydroperoxide formation, and heat inactivation of the enzyme markedly reduced the levels of sterol peroxidation (Table 4). Peroxidation occurred only if preoxygenated buffer was employed or H_2O_2 added as cosubstrate, but preoxygenated buffer resulted in an increased level of the 7-ketone III. Examination of the

TABLE 4

PEROXIDATION OF CHOLESTEROL BY HORSERADISH PEROXIDASE

Product	Amounts of product (%)[a]	
	Active enzyme	Boiled enzyme
Ia	0.085	0.020
Ic	0.159	0.030
IIa	0.017	0.005
Ib	0.042	0.020
Id	0.095	0.020
IIb	0.00	0.00
III	0.061	0.040

[a] Amounts of product formed as percent of substrate, expressed as an average of two independent experiments. Incubations conducted at 37° for 2 hr using 0.62 m*M* [4-^{14}C]cholesterol (1.6×10^6 dpm), 18 m*M* H_2O_2, 0.8 m*M* $MnCl_2$, and 2 μ*M* horseradish peroxidase in 10 ml of 0.1 *M* sodium acetate (pH 5.5) buffer.

effects of pH using 0.1 *M* sodium acetate buffer (pH 4.5 and 5.5), 0.1 *M* phosphate buffer (pH 6.5 and 7.4), and 0.1 *M* Tris–HCl buffer (pH 8.2 and 9.5) indicated that maximal peroxidation occurred at pH 5.5. Different batches of horseradish peroxidase with Reinheitzahl (RZ) number 1.4, 1.6, 1.9, 3.2, and 3.4 were examined qualitatively, and an apparent inverse relationship between RZ number and extent of cholesterol peroxidation resulted. Accordingly, batches of enzyme with RZ 1.6 number were used for quantitative studies.

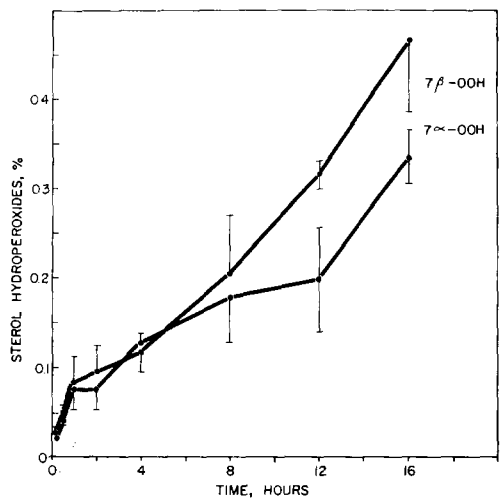


FIG. 2. Formation of Ia (- - -) and Ic (—) in horseradish peroxidase incubations of 88 μmole [1,2-^{3}H]-cholesterol (10^7 dpm), 1.8 mmole H_2O_2, and 16 mg horseradish peroxidase in 80 ml 0.1 *M* sodium acetate (pH 5.5) buffer at 37°. Data are averages of three separate experiments (standard deviations indicated) adjusted for boiled enzyme control values.

The effect of time on the progress of cholesterol peroxidation by horseradish peroxidase is shown in Fig. 2. In distinction to the progress of the soybean lipoxygenase reaction (Fig. 1), levels of the 7-hydroperoxides Ia and Ic were the same at early times.

TABLE 5

METABOLISM OF STEROL HYDROPEROXIDES BY HORSERADISH PEROXIDASE

Substrate	Enzyme	Products Found, %[a]							
		Ia	Ib	Ic	Id	IIa	IIb	I	Others[b]
Ia	Active	36.94	4.30	3.14	2.04	0.83	2.32	30.50	19.93
	Boiled	29.30	6.96	2.67	2.59	0.68	3.40	43.34	11.06
Ic	Active	4.87	2.97	57.26	4.83	0.08	1.57	6.59	21.85
	Boiled	4.78	2.70	58.74	3.91	0.05	1.46	8.07	20.29
IIa	Active	53.88	4.67	3.67	1.43	11.00	2.69	9.22	13.42
	Boiled	44.51	4.52	1.37	1.54	9.08	2.51	10.82	25.59

[a] Amounts of products in percent of substrate, expressed as an average of two independent experiments. Incubations conducted at 37° for 2 hr using 0.4 m*M* [4-^{14}C]Ia (5×10^3 dpm), [4-^{14}C]Ic (3.6×10^3 dpm), or [4-^{14}C]IIa (10^4 dpm) with 18 m*M* H_2O_2, 0.8 m*M* $MnCl_2$, and 2 μ*M* horseradish peroxidase in 10 ml of 0.1 *M* sodium acetate (pH 5.5) buffer.

Only after prolonged incubations did the 7β-hydroperoxide Ic level exceed that of the 7α-hydroperoxide Ia. A standard incubation time of 2 hr was selected for data of Tables 1 and 5.

Effects of inhibitors of horseradish peroxidase reaction. The effects of propyl gallate and catalase on the peroxidation of cholesterol catalyzed by horseradish peroxidase acting on H_2O_2 were measured in one experiment. A total yield of 0.205% of combined sterol hydroperoxides Ia and Ic obtained for the uninhibited reaction was reduced to 0.075% by 2 μM catalase and was completely reduced to unmeasurable levels (0.00%) by 1 mM propyl gallate.

Peroxidase effects on sterol hydroperoxides. The metabolism of the hydroperoxides Ia, Ic, and IIa by horseradish peroxidase summarized in Table 5 established that each was transformed into a mixture of sterols I, II, and III. Considering only the hydroperoxide products, ratios of Ia:Ic:IIa of 45:12:1 were obtained from Ia, 7:84:1 from Ic, and 15:1:3 from IIa, thus assuring hydroperoxide stabilities sufficient for our detection of even the least stable IIa were it formed in incubations of cholesterol as substrate.

Results with Rat Liver

Effects of age and strain of rats. Qualitative analysis by thin-layer chromatography of the products of the NADPH-dependent oxidation of cholesterol by microsomal, mitochondrial, and soluble fractions of liver homogenate established that although the epimeric 3β,7-diols Ib and Id and the ketone III were present throughout, the presence of the parent 7-hydroperoxides Ia and Ic varied greatly with the age and strain of rats used, as is illustrated by the qualitative data of Table 6. Product sterol hydroperoxides

TABLE 6

CHOLESTEROL PEROXIDATION IN DIFFERENT STRAINS OF RATS

Rat strain	Weight (g)	Number of rats	Products detected[a]				
			Ia	Ib	Ic	Id	III
ACI/Tex	120–140	3	+	+	++	+	++
	220–240	4	+	+	++	+	++
	250	1	+	+	++	+	++
Tex: (SD)	200–210	3	—	+	—	+	+
	240–300	4	—	+	—	+	+
	500–550	2	+	++	++	++	+++
	600	2	++	++	+++	++	+++

[a] Amounts of products estimated from intensity of blue color developed on thin-layer chromatograms following reduction *in situ* with methanolic $NaBH_4$ and spraying with 50% aqueous sulfuric acid (no warming).

Ia and Ic were readily demonstrated in incubations of liver microsomes from inbred male albino rats of the ACI/Tex strain weighing more than 120 g, but ready detection of

TABLE 7

OXIDATION OF [1,2-^{3}H]CHOLESTEROL BY RAT LIVER SUBCELLULAR FRACTIONS

			Microsomal fraction[a]			Soluble fraction[a]			Microsomal and soluble fractions[a]			
Product	No enzyme	Mitochondrial fraction[a]	As is	With linoleate[b]	With linoleate hydroperoxides[b]	As is	With linoleate[b]	With linoleate hydroperoxides[b]	As is	Without NADPH	With EDTA[c]	With propyl gallate[c]
Ia	0.05	0.06	0.14	0.21	0.88	0.05	0.12	0.59	0.10	0.04	0.06	0.04
Ic	0.05	0.08	0.17	0.98	4.02	0.06	0.21	1.36	0.12	0.08	0.08	0.04
Ib	0.07	0.26	0.50	1.13	1.13	0.47	1.62	0.78	0.40	0.19	0.32	0.31
Id	0.16	0.52	1.35	3.05	2.14	1.28	1.55	1.14	1.16	1.42	0.42	0.45
III	0.27	1.10	2.85	7.19	9.21	2.62	2.79	2.92	3.67	1.25	0.60	1.25
Totals	0.60	2.02	5.01	12.56	17.38	4.48	6.29	6.79	5.45	2.98	1.48	2.09

[a] Amount of products formed as percent of substrate, expressed as an average of two experiments. Incubations conducted with rat liver subcellular fractions from 1 g wet liver at 30° for 2 hr with 10 μmole [1,2-^{3}H]cholesterol (6×10^6 dpm), 6.5 μmoles NADPH, 33 μmoles glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase in 6 ml of 0.15 *M* Tris-HCl (pH 6.8).

[b] Before addition of the enzyme 25 μmole ethyl linoleate or 50 μmole ethyl linoleate hydroperoxides in 25 μl of 95% ethanol was added.

[c] Added as 1 m*M* solutions in buffer.

the hydroperoxides where liver microsomes from outbred male Tex: (SD) strain rats were used was achieved only when the rats weighed more than 500 g. Consistently good results were obtained using livers from the aged Tex: (SD) rats, and they were the strain used for quantitative studies. The 5α-hydroperoxide IIa was not encountered in any rat liver incubation.

Failure to detect hydroperoxides Ia and Ic in incubations of liver microsomes from young rats (less than 120 g for the ACI/Tex strain, less than 500 g for the Tex: (SD) strain) cannot be a consequence of the hydroperoxides not being formed, witness the ubiquitous presence of the secondary products Ib, Id, and III. The extent of sterol peroxidation may be moderated by such items as diminished or undeveloped microsomal peroxidation systems in the young or declining peroxidase activities in the aged rats, by the varying presence of endogenous inhibitors, by limitation of NADPH supply, failure or diversion of the microsomal electron transport system, or yet other factors. However the matter is quite complicated, there being data suggesting diminution of NADPH-dependent liver microsomal lipid peroxidation (*26*, *27*) and of declining hepatic 7α-hydroxylation and side-chain oxidations of cholesterol (*28*) in aging rats.

The ease of detection of the 7-hydroperoxides Ia and Ic also depended on incubation media pH, greater stability of the sterol hydroperoxides being attained in acid incubation media rather than at pH 7.4 used routinely. The extent of perfusion of liver with 0.25 *M* sucrose also influenced the matter, any hemoglobin not removed by the perfusion tending to catalyze decomposition of the sterol hydroperoxides to the secondary products Ib, Id, and III. In experiments to which ADP and $FeCl_3$ (final concentrations 3 m*M* each) were added four-fold increases in levels of secondary products Ib, Id, and III were observed, with no detectable formation of the parent 7-hydroperoxides Ia and Ic.

Effects of Subcellular Fractionation

Data of Table 7 establish that cholesterol peroxidation occurred in all subcellular fractions of rat liver but was maximal in the microsomal fraction. Ready detection of the product hydroperoxides Ia and Ic was achieved only in incubations of microsomes, and data of Table 8 demonstrate the radiochemical purity of the products Ia and Ic formed by microsomal peroxidation. The secondary products Ib, Id, and III derived from the initial products Ia and Ic were also formed in all subcellular fractions.

Effects of Added Components

The stimulatory nature of added NADPH on the peroxidation of cholesterol is demonstrated by data of Table 8 for the microsomal fraction and in Table 7 for the combined microsomal and cytosol fractions. Without added NADPH the levels of 7-hydroperoxides Ia and Ic formed were at control levels. Although these data suggest that hydroperoxide formation occurred at diminished levels in those incubations to which NADPH was not added, an absolute dependence on added NADPH was not demonstrated.

A more marked stimulation was observed with added ethyl linoleate or with added ethyl linoleate hydroperoxides. Added linoleate more than doubled the amount of

TABLE 8

EFFECTS OF NADPH ON STEROL PEROXIDATION BY LIVER MICROSOMES

Recrystallization	Product	Specific activity (dpm/mg)[a] With NADPH	Specific activity (dpm/mg)[a] Without NADPH
Benzene-hexane (1:1)	Ib	1338	40
	Id	2310	112
Ethyl acetate-benzene (1:5)	Ib	1350	41
	Id	2300	85

[a] Incubation of liver microsomes equivalent to 1 g wet liver from 620 g Tex: (SD) rat with [1,2-^{3}H]cholesterol (6×10^6 dpm) to which 5 mg carrier cholesterol was added prior to incubation. Chromatographically recovered products Ia and Ic were reduced with $NaBH_4$ in methanol, 5 mg each of inactive carrier Ib and Id was added, and the products Ib and Id recovered by recrystallization to constant specific activity from binary solvent mixtures specified.

cholesterol oxidation; added linoleate hydroperoxides more than tripled the amount. A similar but less extensive stimulation of cholesterol oxidation occurred on addition of linoleate or linoleate hydroperoxides to incubations involving the cytosol fraction as enzyme.

Qualitative chromatographic data indicated that diminished cholesterol oxidation occurred in incubations of liver microsomes derived from liver homogenate to which trypsin (1 mg/g homogenate) had been added. Cholesterol oxidation was greatly diminished using boiled microsomal enzyme preparations, and no oxidation products were formed in incubations containing 1 m*M* EDTA, 1 m*M* propyl gallate, dithionite, or dithionite plus carbon monoxide. Carbon monoxide treatment alone was without effect on the peroxidation reactions. Quantitative data for the inhibitory effects of added EDTA and propyl gallate on incubations conducted using combined microsomal and cytosol fractions as enzyme are presented in Table 7. Both agents reduced 7-hydroperoxides Ia and Ic formation as well as that of the secondary products Ib, Id, and III.

Phenobarbital Pretreatment

Liver microsomes prepared from rats of both the Tex: (SD) and ACI/Tex strains which had been pretreated with phenobarbital were incubated in comparison experiments with microsomes from untreated rats. Qualitative chromatographic data established the common presence of the secondary products Ib, Id, and III in all cases, with the hydroperoxides Ia and Ic present erratically. No quantitative measurements were attempted, but it was clear that phenobarbital pretreatment of either strain did not alter the nature or extent of cholesterol peroxidation in standard incubations.

DISCUSSION

Although there are established cases of plant and mammalian tissue lipoxygenases acting on polyunsaturated fatty acids (*29*) together with suggestions of dioxygenase actions on carotene (*30, 31*) and steroids (*32*), little else broadly adumbrating dioxygenase metabolism of lipids has been reported (*33–35*). Our present results establish that dioxygenases of higher plants and mammalian tissue may oxidize cholesterol by introduction of both atoms of molecular oxygen into the substrate, yielding the epimeric 7-hydroperoxides Ia and Ic. As is typical of dioxygenases, sequential transformations of the primary enzyme products Ia and Ic to secondary products Ib, Id, and III confounded ready demonstration of the true dioxygenase character of these reactions. The formal enzymic nature of the peroxidation is assured by the sharply diminished degree of oxidation in the absence of active enzyme, but we have not attempted to characterize the mechanisms by which these dioxygenases act on cholesterol other than to consider three points: (i) formation of an enzyme-substrate complex, (ii) participation of electronically excited (singlet) molecular oxygen, and (iii) participation of nonspecific free radial processes.

The dioxygenase action of soybean lipoxygenase on polyunsaturated fatty acid derivatives appears to involve binding of the requisite *cis,cis*-1,4-diene moiety (*36*) of the substrate to the enzyme in a planar structure (*37, 38*) with rate-limiting abstraction of hydrogen from the $\omega 8$ methylene group and subsequent reaction with molecular oxygen proceeding from opposite sides of the plane. Dioxygenase action on steroids, heretofore unnoticed (*1, 39–42*), by analogy could proceed by binding of the reactive B-ring features of cholesterol (Δ^5-double bond and allylic C-7 methylene group) constrained by the nature of the steroid BCD ring system in a configuration similar to that of *cis,cis*-1,4-diene substrates. However, soybean lipoxygenase actions are quite complex, being characterized by complicated substrate and product specificities and pH optima as well as by the presence of associated hydroperoxide isomerase, β-carotene oxidase, and lipoxygenase isozyme (*19–21, 43*) activities, for which precise control of experimental conditions has been emphasized (*44, 45*). Whereas the coupled oxidation of β-carotene was preferentially associated with isozymes L-2 and L-3 of pH 6.6 optima and not with the Theorell isozymes L-1 of pH 9.0 optimum (*43, 46*), our data of Table 1 at pH 6.6 and 9.0 do not suggest differential isozyme actions on cholesterol. We have not examined the individual soybean lipoxygenase isozymes in this regard.

However, binding of enzyme and cholesterol does not adequately account for the observed requirement of polyunsaturated fatty acid derivative for cholesterol peroxidation, and the sterol product hydroperoxides *Ia* and *Ic* are not analogous to the conjugated *trans*-$\omega 7$,*cis*-$\omega 9$-diene-$\omega 6$-hydroperoxide or *cis*-$\omega 6$,*trans*-$\omega 8$-diene-$\omega 10$-hydroperoxide products from polyunsaturated substrates. Indeed, we have not demonstrated formation of lipoxygenase-cholesterol complexes, and product distribution and temperature effects suggest that direct interaction of cholesterol and soybean lipoxygenase may not occur.

Speculations implicating singlet molecular oxygen in soybean lipoxygenase catalyzed oxidation of linoleate set forth on trapping data (*47*) since retracted (*48*), mechanistic grounds (*49*), ultraweak chemiluminescence (*50, 51*), and deuterium oxide solvent effects (*52*) are not supported by other trapping experiments (*53*) or by our present

results. Were generation of singlet molecular oxygen an important aspect of enzyme action the 5α-hydroperoxide *IIa* recognized as the major product of singlet molecular oxygen attack on cholesterol (*54*) should have been detected prominently, as was the case of generation of singlet molecular oxygen in other aqueous buffered protein systems (*55*). Moreover, attribution of ultraweak chemiluminescence of the soybean lipoxygenase reaction directly to singlet molecular oxygen (*50, 51*) rather than to recombination of peroxy radicals as demonstrated by kinetics and other data (*56, 57*) is now disputed (*57*). Similarly, deuterium oxide solvent effects interpreted in support of participation of singlet molecular oxygen (*52*) have received opposing interpretations (*58*).

Thus, enzyme activation of neither cholesterol nor molecular oxygen appears to account for our observations. In that product ratios and yields of the enzymic peroxidation mimic in detail those of the free radical oxidation of cholesterol in the solid (*10*) and dispersed (*59*) states, we are compelled to a formal comparison of the two processes. Moreover, the well-established participation of free radicals in the peroxidation of polyunsaturated fatty acids by soybean lipoxygenase (*60–62*) prompts us to posit nonspecific free radical processes as the means by which cholesterol is oxidized in this system.

Horseradish peroxidase catalyzes the reduction of hydrogen peroxide and may utilize organic compounds as electron donors (*63*). The enzyme oxidizes phenolic steroids (*64, 65*), hydroxylates steroid β-diketones (*66*), and acts as a dioxygenase on a Δ^5-3-ketosteroid (*67*). Our present observations confirm the dioxygenase nature of horseradish peroxidase acting on cholesterol.

In similarity with soybean lipoxygenase the peroxidation of cholesterol by horseradish peroxidase required an active enzyme acting on its norminal substrate hydrogen peroxide. Formation of a peroxidase–cholesterol complex was not indicated, nor was speculation of participation of singlet molecular oxygen (*51, 68*) supported by our observations. The formal similarity of our results of soybean lipoxygenase and free radical oxidations of cholesterol with those of horseradish peroxidase is obvious, and generation of free radicals in the horseradish peroxidase reaction is manifest (*63*). These considerations suggest that soybean lipoxygenase and horseradish peroxidase may be alike in regard to the mechanism by which they both peroxidize cholesterol.

The oxidation of cholesterol by the NADPH-dependent microsomal lipid peroxidation system of rat liver is a much more complicated matter, for pure enzymes were not had, and the possibilities for competing and consecutive reactions were greatly increased. Nonetheless, demonstration of the formation of the epimeric 7-hydroperoxides Ia and Ic as initial and chief products was possible, but the aforementioned complexities led to extensive and rapid loss of the initially formed 7-hydroperoxides such that the secondary product 3β,7-diols Ib and Id and the 7-ketone III regularly predominated in the standard incubations. Previous investigations of cholesterol oxidation in this system had established the presence of the secondary products Ib, Id, and III (*41, 69–71*) among others, and although the participation of sterol hydroperoxides was suspected (*69, 70, 72*) the dioxygenase nature of the reaction was not demonstrated with steroids. The peroxidation of susceptible xenobiotic substrates such as tetralin and fluorene that bear readily oxidized benzylic methylene groups (*73, 74*) appear to be related examples of these dioxygenase processes.

The secondary product 3β,7-diols Ib and Id may best be considered as enzyme reduction products of the 7-hydroperoxides Ia and Ic by sterol (*75–77*) and other (*78*) hydroperoxide peroxidases of rat liver. Formation of the 3β,7α-diol Ib from the 7α-hydroperoxide Ia derived by dioxygenase action on cholesterol is distinct from formation of Ib directly from cholesterol by action of the microsomal 7α-hydroxylase implicated in hepatic bile acid biosynthesis (*41, 70, 71*). Whereas the sterol 7α-hydroxylase is a monoxygenase involving participation of cytochrome P-450 (*79*) inhibited by carbon monoxide (*41*) but not by EDTA or cysteamine (*70*), the lipid peroxidation system acts as a dioxygenase involving nonheme iron and is inhibited by EDTA or cysteamine (*70*) but not by carbon monoxide (*41*).

The secondary product 7-ketone III does not appear to derive by action of 7-hydroxysterol dehydrogenases on either 3β,7-diol Ib or Id (*70, 72*) and must derive by dehydration of the 7-hydroperoxides Ia and Ic. However, hydroperoxide dehydratases are undescribed enzymes, and we regard formation of III from Ia and Ic as probably nonenzymic but promoted by unrecognized components of the microsomal lipid peroxidation system.

In view of the complexity of the microsomal enzyme preparation we have not examined details of the peroxidation mechanism, including the matter of enzyme–substrate complex formation. The participation of singlet molecular oxygen in NADPH-dependent microsomal lipid peroxidations of rat liver has been variously suggested (*80–85*), but neither our present results nor prior studies of xenobiotic substance metabolism (*86*) support this possibility. Indeed, the general character of our results together with electron spin resonance data supporting the formation of carbon free radicals in aerated rat liver microsomal preparations (*87*) suggest that the microsomal enzyme system oxidize cholesterol as a dioxygenase by processes involving free radicals in manner like the plant dioxygenases soybean lipoxygenase and horseradish peroxidase.

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