Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 577–581. Pergamon Press. Printed in Great Britain

STEROL METABOLISM—XXXVIII. OXIDATION OF 4-CHOLSTEN-3β-OL BY SOYBEAN LIPOXYGENASE

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(*Received* 4 *August* 1975)

SUMMARY

4-Cholesten-3β-ol was oxidized by soybean lipoxygenase in reaction coupled to the oxidation of ethyl linoleate to give 4-cholesten-3-one as initial product which in turn was oxidized to a mixture of epimeric 3-oxo-4-cholestene-6-hydroperoxides. The 6-hydroperoxides were interconverted readily and thermally decomposed to the corresponding epimeric 6-alcohol and 6-ketone derivatives. The data implicate nonspecific free radical processes but not participation of electronically excited (singlet) molecular oxygen.

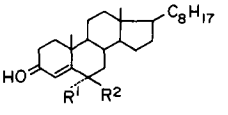
INTRODUCTION

Despite experimental evidence to the contrary [1–4] and retraction [5] of a claim initially made [6] of participation of electronically excited (singlet) molecular oxygen in the action of soybean lipoxygenase on polyunsaturated fatty acids [6–11], speculation that singlet molecular oxygen be formed in soybean lipoxygenase reactions continues [12]. Our previously reported approach via product analysis to the question of participation of singlet molecular oxygen in soybean lipoxygenase incubations utilized cholesterol (5-cholesten-3β-ol) which was peroxidized by the enzyme [1]. In that the sterol hydroperoxide products formed were interconvertible in the enzyme system a further test using another sterol was sought.

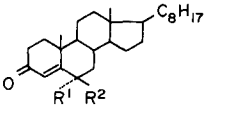
Radiation-induced free radical oxidation of 4-cholesten-3β-ol (Ia) yielded 4-cholesten-3-one (IIa) as major product with small amounts of 3β-hydroxy-4-cholestene-6α-hydroperoxide (Ib) and 3-oxo-4-cholestene-6β-hydroperoxide (IIc) [13], whereas oxidation of Ia by singlet molecular oxygen yielded 4α,5α-epoxy-5α-cholestan-3-one (III) and variable amounts of the ketone IIa [14–18]. Since the ketone IIa formed under both free radical and singlet oxygen conditions, formation of IIa from Ia under other conditions would not prove the reaction process involved. However, formation of the 4α,5α-epoxide III from Ia uniquely infers attack of singlet oxygen on Ia whereas formation of the 6-hydroperoxides Ib and IIc infer free radical processes. We report herein our results of the oxidation of Ia by soybean lipoxygenase in test of this matter.

EXPERIMENTAL

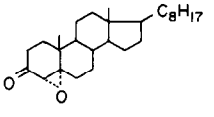
General procedures. Soybean lipoxygenase (EC 1.13.11.12, formerly also EC 1.13.1.13 and EC 1.99.2.1) purchased from Sigma Chemical Co., St. Louis, Mo. shown to be a mixture of isozymes (three major and six minor protein components) by polyacrylamide gel electrophoresis was used as received. Ethyl linoleate was purchased from Sigma Chemical Co. and used as received. Samples of the sterol Ia, 4-cholestene-3, 6-dione (IV), and the epimeric 3-oxo-4-cholestene-6α- and 6β-hydroperoxides IIb and IIc were purchased from Steraloids Inc., Pawling, N.Y. Each sample was purified by repeated thin-layer chromatography to homogeneity by thin-layer and gas chromatography. The epimeric 4-cholestene-3β, 6-diols Id and Ie were prepared by sodium borohydride reduction of the parent 6-hydroperoxides IIb and IIc. Samples of 5α-cholestane-3,6-dione (V) were prepared by pyrolysis of the 6-alcohols IId and IIe and purified to homogeneity by preparative gas and t.l.c.



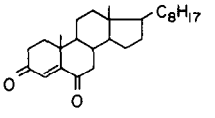
Ia $R^1 = R^2 = H$
Ib $R^1 = OOH, R^2 = H$
Ic $R^1 = H, R^2 = OOH$
Id $R^1 = OH, R^2 = H$
Ie $R^1 = H, R^2 = OH$



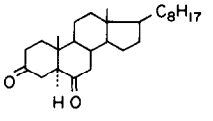
IIa $R^1 = R^2 = H$
IIb $R^1 = OOH, R^2 = H$
IIc $R^1 = H, R^2 = OOH$
IId $R^1 = OH, R^2 = H$
IIe $R^1 = H, R^2 = OH$



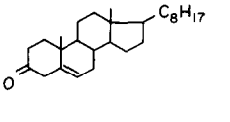
III



IV



V



VI

Melting points were obtained on a Kofler block under microscopic magnification. Infrared absorption spectra were recorded on pressed potassium bromide disks (0.5 mm dia.) incorporating the sample over the

Table 1. Chromatographic data for 4-cholestene derivatives

Steroid	Thin-layer chromatographic mobility, R_C^a Hexane–ethyl acetate (4:1 v/v)	Hexane–ethyl acetate (6:1 v/v)	Diethyl ether	Gas chromatographic retention time on 3% SP-2401 t_R^b
Ia	1.10	1.08	1.05	0.39, 0.44[c]
Id	0.15	0	0.32	0.48, 0.82, 1.90[d]
Ie	0.15	0	0.27	0.48, 0.82, 1.90[d]
IIa	1.40	1.64	1.14	3.12
IIb	0.58	0.50	1.12	6.70, 7.08[e]
IIc	0.77	0.64	1.12	6.70, 7.08[e]
IId	0.34	0.16	0.77	6.70[f]
IIe	0.44	0.26	0.85	6.70[f]
IV	1.27	1.25	1.17	7.08
V	1.16	1.05	1.13	6.70

[a] Triple ascending irrigation. Cholesterol as unit mobility.
[b] Retention times (t_R) with cholesterol as unit retention time. Absolute retention time, 6 min.
[c] Dehydration products 2,4-cholestadiene and 3,5-cholestadiene.
[d] 2,4,6-Cholestatriene, unidentified sterol, and the parent 3β,6-diol.
[e] Corresponding to V and IV respectively.
[f] Corresponding to V.

range 400–4000 cm.$^{-1}$ using a Perkin–Elmer Model 337 spectrophotometer equipped with a beam condenser [19]. Ultraviolet light absorption spectra were recorded over the range 200–270 nm on 95% ethanol solutions using a Cary Model 14 spectrophotometer. Thin-layer chromatography was conducted by methods previously described in detail [20] using 0.25 mm thick 20 × 20 cm. chromatoplates of Silica Gel HF_{254} (E. Merck, GmbH., Darmstadt) triply irrigated in ascending fashion. Steroids were detected sequentially by their U.V. light absorption properties under 254 nm light, by N,N-dimethyl-*p*-phenylenediamine specific for hydroperoxides [21], and by 50% aq. sulfuric acid for detection of all steroids [20]. Gas chromatography was conducted at 230° using 1.83 m long silanized glass U-tubes packed with 2% or 3% SP-2401 on 100–120 mesh Supelcoport (Supelco Inc., Bellafonte, Pa.) by means previously described in detail [22]. Mobility data are presented in Table 1.

Preparation of epimeric 6-hydroperoxides IIb and IIc. Because of extensive decomposition of commercially available samples of IIb and IIc it was necessary to synthesize both 6-hydroperoxides in our laboratory. Pure samples of the epimeric 6-hydroperoxides IIb and IIc were prepared from 5-cholesten-3-one (VI) by the method of Cox[23] as follows. A solution of 5 g VI in 50 ml cyclohexane was refluxed with 40 mg benzoyl peroxide for 36 h during which time oxygen was bubbled through the solution. The reaction mixture was evaporated under vacuum, dissolved in chloroform–methanol (2:1, v/v) and a portion chromatographed on thin-layer chromatoplates using hexane–ethyl acetate (4:1, v/v) and hexane–benzene–ethyl acetate (3:3:1, by vol.), yielding 9.8 mg of the crystalline 6α-hydroperoxide IIb, m.p. 141–142° from diethyl ether–methanol (lit. m.p. 150–151° [23]); λ_{max}242 nm (lit. λ_{max}241 nm [23]; $\tilde{\nu}_{max}^{KBr}$ 3350, 1660 cm.$^{-1}$; together with 49.1 mg of crystalline 6β-hydroperoxide IIc (from diethyl ether–methanol), m.p. 149–149.5°), 149.5–152.0° (capillary tube) (lit. m.p. 180° [24], 177° and 181° [25], 180–181° [23], and 177–180° [26]); λ_{max} 237.5 nm (ε14,500) (lit. λ_{max} 236 nm (ε16,850) [24], 235 nm [23], 237 nm [26]); $\tilde{\nu}_{max}^{KBr}$ 3325, 1675 cm.$^{-1}$. Repeated t.l.c. with the same solvent systems of commercial samples of IIb and IIc afforded homogeneous, crystalline preparations of IIb, m.p. 141–142°, and IIc, m.p. 148–150.5°, identical in chromatographic and spectral properties with samples prepared from VI.

Incubations with soybean lipoxygenase. To 1 ml of 50 mM Tris–HCl buffer (pH 6.6 or pH 9.0) in a 25 ml flask was added 5 mg of Ia in 0.2 ml acetone containing 10% Tween 80 and 50 μmol ethyl linoleate in 50 μl 95% ethanol. Following evaporation of the organic solvents under vacuum the substrates were suspended in 9 ml of buffer and 5 mg of soybean lipoxygenase was added. Incubations were conducted at 30° and 37° on a rotary shaker bath for varying time periods and were terminated by cooling in ice water, acidification to pH 2.0 with 2N HCl, and extraction four times with equal vol. of ethyl acetate. The pooled extracts were dried over anhydrous sodium sulfate and evaporated under vacuum without heat. Incubations with the 3-ketone IIa as substrate were conducted in the same manner. The progress of incubations was monitored by withdrawal of 0.2 ml aliquots for t.l.c. analysis.

In the same manner, control incubations using active soybean lipoxygenase and soybean lipoxygenase previously inactivated by boiling in buffer were conducted at 37° for 2 h at both pH 6.6 and pH 9.0, using the 6β-hydroperoxide IIc as substrate.

The reaction products mixtures were subjected to preparative t.l.c. and individual components were eluted with acetone. Repeated t.l.c. led to isolation of pure, homogeneous preparations of each product, identified by comparisons of chromatographic and spectral properties with those of authentic reference samples. Each identified component was estimated

Table 2. Oxidation of steroids by soybean lipoxygenase at 37° (4 h)

Substrate	pH	Enzyme	Products, %[a] IIa	IIb	IIc	IId + IIe	IV	III
4-Cholesten-3β-ol (Ia)	6.6	Active	10	0	0	0	0	0
		Boiled	1	0	0	0	0	0
	9.0	Active	3.8	0	0	0	0	0
		Boiled	0.53	0	0	0	0	0
4-Cholesten-3-one (IIa)	6.6	Active	[b]	0.36	0.52	0.036	<0.008	0
		Boiled	[b]	0	0	0	0	0
	9.0	Active	[b]	0.64	1.2	0.20	0.056	0
		Boiled	[b]	0	0	0	0.008	0

[a] Products for which 0% is given were not detected.
[b] Substrate IIa present but not measured.

quantitatively from the area under its peak on gas chromatogram elution curves in comparison with known amounts of reference steroids.

RESULTS

Chromatographic examination of the products of the action of soybean lipoxygenase on ethyl linoleate and the sterol Ia as substrates revealed the presence of one major product identified as the ketone IIa and five minor products identified as the epimeric 6-hydroperoxides IIb and IIc, the epimeric 6-alcohols IId and IIe, and the 3,6-diketone IV. By analysis at different times it was established that the initial and sole reaction of Ia was its oxidation to the ketone IIa and that the minor products IIb, IIc, IId, IIe, and IV derived only after the ketone IIa had formed. Soybean lipoxygenase incubations of the ketone IIa as substrate supported this formulation in detail, the ketone IIa being transformed into IIb, IIc, IId, IIe, and IV. Control incubations of IIc with boiled soybean lipoxygenase established that IIc was transformed nonenzymatically into a mixture of IId, IIe, and IV. Data of Table 2 for incubations conducted at 37° for 4 h establish these points quantitatively. The 4α,5α-epoxy-3-ketone III was not detected among the products despite careful examination.

Incubations at pH 6.6 and at pH 9.0 corresponding to the pH optimum of soybean lipoxygenase isozymes L-2 and L-1 respectively established that oxidation of Ia to IIa was more effective at pH 6.6 but that oxidation of IIa was more effective at pH 9.0. These results may reflect the differential effects of the soybean lipoxygenase isozymes or merely different amounts or activities of the isozymes in the commercial enzyme mixture.

Recovery of the several reaction products II and IV from the incubations was attended by alteration of the major product hydroperoxides IIb and IIc, thus necessitating a careful study of the nature of the transformations in order to avoid misunderstanding of the processes at work in the enzyme oxidations. The data of Table 2 were obtained using the utmost care and attention to conditions such that factitious alterations of the products were minimized.

It was readily demonstrated that the thermal stability of the 6-hydroperoxides IIb and IIc was poor under a variety of circumstances, including storage as a solid at room temperature, warming in solution, etc. Decomposition of IIb and of IIc occurred by three processes: (i) interconversion of IIb and IIc, (ii) dehydration of both IIb and IIc to the 3,6-diketone IV, and (iii) reduction to the corresponding 6-alcohols (IIb to IId, IIc to IIe). These stability characteristics of the epimeric 6-hydroperoxides IIb and IIc thereby provide a basis for the presence of IId, IIe, and IV among the products of the enzyme transformations, being secondary products derived from the 6-hydroperoxides initially formed from IIa.

In distinction to the case of the analogous 3β-hydroxy-4-cholestene-6-hydroperoxides Ib and Ic where epimerizations were not observed [27], the interconversion of the 6-hydroperoxides IIb and IIc occurred on storage of the solid samples and in organic solvent solutions, with epimerization of the quasiaxial 6β-hydroperoxide IIc to the quasiequatorial 6α-hydroperoxide IIb being favored over the reverse epimerization which also occurred, but to a lesser extent. Our results now clearly establish the facile epimerization of the 6β-hydroperoxide IIc, a matter given prior consideration but no experimental study [23, 26]. The interconversions of IIb and IIc accordingly account for the presence of both eprimeric 6-hydroperoxides IIb and IIc and 6-alcohols IId and IIe among the products of soybean lipoxygenase attack on substrate IIa.

The epimerization of the quasiequatorial 6α-hydroperoxide IIb, though observed, was a minor transformation of the steroid, for dehydration to the 3,6-diketone IV was the predominant reaction of IIb. Ethanol solutions (1 mg/ml) of the 6α-hydroperoxide IIa showing maximum absorption at 242 nm exhibited a slow shift in absorption such that after 14 days the 242 nm band had disappeared and a new band at 250 nm attributed to the 3,6-diketone IV (absorption maximum at 251.5–252 nm [23, 28]) has appeared. In contrast, similar solutions of the 6β-hydroperoxide IIc with absorption maxima at 238 nm retained that band after 14 days but had gained additional weak absorption near 255 nm as a shoulder on the main absorption band which was also attributed to formation of the 3,6-diketone IV from IIc. The concomitant in-

terconversions of IIb and IIc or their reductions to the corresponding 6-alcohols IId and IIe would not influence these observed absorption changes, for both 6α-derivatives IIb and IId absorb near 240 nm (IIb at 241 nm [23] or 242 nm as given herein; IId at 240 nm [29] or 241.5 nm [23]) and both 6α-derivatives IIc and IIe absorb near 237 nm (IIc at 236 nm [24], 237 nm [26], or 237.5 nm as given herein; IIe at 237 nm [29] or 237.5 nm [23]). Thin-layer chromatographic observations of these events confirmed the dehydration as a major reaction pathway of the 6α-hydroperoxide IIb and as a less prominent pathway of the 6β-hydroperoxide IIc whose major transformation was epimerization to IIb. These data suggest that the 6β-hydroperoxide IIc would be ultimately transformed, via IIb, to the 3,6-diketone IV and account for the presence of IV as a major product among the products of soybean lipoxygenase incubations of IIa and amoung products of reactions in which the 6-hydroperoxides IIb and IIc are involved.

The pyrolysis of the 6β-hydroperoxide IIc to yield the 3,6-diketone IV and the 5α-3,6-diketone V (formed by pyrolysis of the 6β-alcohol IIe initially formed from IIc) has previously been demonstrated [13], and we have now completed study of the pyrolysis of the epimeric 6α-hydroperoxide IIb which yields the same pair of 3,6-diketones IV and V. In each case, dehydration of the 6-hydroperoxide yielded the 3,6-diketone IV and initial reduction of the 6-hydroperoxides to the corresponding 6-alcohols IId and IIe followed by pyrolysis of the 6-alcohols yielded the saturated 3,6-diketone V.

The thermal reduction of the 6β-hydroperoxide IIc to the 6β-alcohol IIe was observed by t.l.c. of samples subjected to warming on a Kofler block (as if to take the melting point) to 30°, 50°, 100°, 150°, and 175°. Warming between 30–100° yielded the 6β-alcohol IIe as a sole minor product, with IIc manily unaltered. Heating at 150–175° completely decomposed IIc to a mixture of approximately equal parts of IId, IIe, IV, and unidentified polar components in which the 3,6-diketone IV predominated. These experiments provide a basis for rationalization of our observed melting point of 149–149.5° for the 6β-hydroperoxide IIc whereas others have reported melting points in the range 177–181° [23–26]. In that the 6β-hydroperoxide IIc does not survive warming beyond 150°, it is possible that the previously reported melting points involved mixtures of IId, IIe, and IV and other products whose melting behavior fortuitously appeared to be that of a pure compound.

DISCUSSION

Our results establish that soybean lipoxygenase oxidizes the sterol Ia to the corresponding ketone IIa which is in turn oxidized to the epimeric 6-hydroperoxides IIb and IIc which are then thermally decomposed to the corresponding 6-alcohol and 6-ketone derivatives IId, IIe, and IV. The action of soybean lipoxygenase on Ia to yield steroid hydroperoxides is therefore less direct than is its action on the isomeric cholesterol (5-cholesten-3β-ol) where sterol hydroperoxides were obtained directly as initial products [1].

Moreover, enzymic oxidation of Ia followed a pathway different from those previously demonstrated for its oxidation by radiation-induced free radical [13] or singlet molecular oxygen [14–18] mechanisms. Whereas the ketone IIa initially formed from Ia by the enzyme gave rise to 6-hydroperoxides IIb and IIc via the sequence Ia to IIa to IIb and IIc, the ketone IIa was stable to oxidation induced by ^{60}Co gamma radiation and the 6-hydroperoxides Ib and IIc were formed in that instance *via* the sequences Ia to Ib and Ia to Ic to IIc [13], neither involving the ketone IIa. The epimeric 3β-hydroxy-4-cholestene-6-hydroperoxides Ib and Ic were not detected in soybean lipoxygenase incubations of Ia.

The differential behavior of the ketone IIa in these two systems may be rationalized on realization that enolization of IIa (to 3,5-cholestadien-3-ol) is a more likely event in the aqueous buffered enzyme system than in the solid state, with subsequent oxidation of the putative enol to the epimeric 6-hydroperoxides IIb and IIc in analogy to the recorded facile autoxidation of the ethyl enol ether of IIa to IIb and IIc [30].

Careful examination of the products was necessary in order to determine whether the 4α,5α-epoxide III (or putative metabolites) whose presence infers participation of singlet molecular oxygen was formed. However, the minor product 6-alcohols IId and IIe were clearly derived from the corresponding major product 6-hydroperoxides IIb and IIc by thermal degradation, as was the 3,6-diketone IV. No 4α,5α-epoxide III nor unidentified steroid was detected among the products.

Moreover, the ketone IIa formed in both free radical [13] and photosensitized oxidations [14–18] of Ia though once regarded as a product of singlet molecular oxygen attack [17, 18] is properly to be regarded as a product of free radical processes [13, 16, 31]. Accordingly, we conclude that participation of electronically excited molecular oxygen in the action of soybean lipoxygenase on Ia is not supported. The formal similarity between the enzyme products IIa, IIb, and IIc and free radical oxidation products Ib, IIa, and IIc coupled with the recognized participation of free radical processes in the enzyme action on polyunsaturated fatty acid derivatives [12, 32–34] suggest that the oxidation of the sterol Ia occurs as consequence of nonspecific free radical processes.

Acknowledgements—The financial support of these studies by the Robert A. Welch Foundation, Houston, Texas and the U.S. Public Service (grant ES-00944) is gratefully acknowledged.

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