DETECTION OF STEROL HYDROPEROXIDES ON THIN-LAYER CHROMATOPLATES BY MEANS OF THE WURSTER DYES

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SUMMARY

A specific and sensitive means of detection of sterol hydroperoxides on irrigated thin-layer chromatoplates using N,N-dimethyl-*p*-phenylenediamine dihydrochloride or N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride has been developed. Using the color test the presence of specific sterol hydroperoxides in aged samples of cholesterol, stigmasterol, β-sitosterol, lanosterol, 24,25-dihydrolanosterol, 3α, 5-cyclo-5α-cholestan-6β-ol, 5α-cholest-7-en-3β-ol, and 5α-cholest-8(14)-en-3β-ol and the absence of sterol hydroperoxides in similarly stored samples of 5α-cholestan-3β-ol and 5β-cholestan-3β-ol are demonstrated. Autoxidation of cholesterol in the solid state, in the irradiated solid state, in colloidal dispersion, and in aerated solution is shown to proceed via initial formation of sterol hydroperoxides.

INTRODUCTION

We have recently established by isolation methods that specific sterol hydroperoxides are formed from cholesterol by aging in air[1]. Prior thin-layer chromatographic (TLC) studies suggested that hydroperoxides were formed from cholesterol under a variety of conditions, including mere heating in air and storage[2-5]. For lack of a really reliable color test for sterol hydroperoxides on thin-layer chromatograms no thorough study of the formation of specific sterol hydroperoxides in such reactions has been made. The present paper deals with an improved means of detection of sterol hydroperoxides on irrigated thin-layer chromatoplates using N,N-dimethyl- or N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride. Application of the procedure to studies of cholesterol autoxidation establishes that sterol hydroperoxides are formed from cholesterol as initial products.

EXPERIMENTAL

Reagents. N,N-Dimethyl-*p*-phenylenediamine dihydrochloride and N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride were products of the Eastman Kodak Co., Rochester, N.Y., used as received in their original bottles (catalog specifications 98 % pure by titration). Preparations of Wurster's red and Wurster's blue were made by bromine oxidation of these salts using published directions[6].

A 1% (w/v) solution of either salt in 50% aqueous methanol containing 1 ml of glacial acetic acid per 100 ml of reagent[5] was used for visualization procedures and for spectral studies. The 1% solution of N,N-dimethyl-p-phenylenediamine dihydrochloride slowly becomes colored a darker red on standing, but the darker solution retains its usefulness for detection of sterol hydroperoxides on thin-layer chromatograms for weeks. The darker color may be cleared by adding a small amount of zinc dust to the reagent, but this decolorization does not improve the reagent for hydroperoxide detection. The 1% solution of N,N,N',N'-tetramethyl-p-phenyl-enediamine dihydrochloride likewise turns rapidly to a dark violet color on standing. For both spectral and chromatographic visualization purposes 1% (w/v) solutions of crystalline Wurster's red and Wurster's blue in the same acidified 50% aqueous methanol were prepared.

Thin-layer chromatography. All TLC studies were conducted using 20 × 20 cm chromatoplates 0.25 mm thick of Silica Gel HF_{254} (E. Merck GmbH, Darmstadt) in the manner previously described in detail[2]. Reference sterol hydroperoxides[1] were spotted as 1 mg/ml solutions in diethyl ether, using disposable glass micro-pipettes of 1-μl volume.

Visualization procedures. Chromatographically irrigated thin-layer chromato-plates were dried in air and viewed routinely under both 254 and 366 nm ultraviolet light sources for absorbing and fluorescing components prior to visualization by spray techniques.

The prepared chromatograms were lightly sprayed with the 1% solution of either the N,N-dimethyl- or the N,N,N',N'-tetramethyl-p-phenylenediamine rea-gents. After drying in air for a few minutes reddish pink colored zones of Wurster's red appeared against a lighter pink background when the N,N-dimethyl-p-phenyl-enediamine reagent was used. Contrast between colored spots and background was improved by warming on a hot plate. A similar behavior was obtained for the N,N,N',N'-tetramethyl-p-phenylenediamine reagent, violet spots of Wurster's blue being formed against a lighter violet background, and for which contrast was improved by brief warming on a hot plate. The visualized chromatoplate colors were stable for several days, during which time, however, the background became increasingly colored with either the Wurster's red or Wurster's blue as the case might be.

After the sterol hydroperoxide zones were suitably marked the chromatoplate could be sprayed with 50% aqueous sulfuric acid and heated to develop the character-istic colors of the individual sterols in the usual manner[2].

Visualization of sterol hydroperoxides with starch–potassium iodide and ammo-nium thiocyanate–ferrous sulfate reagents was made using standard color test con-ditions[7].

Visible absorption spectra. Visible absorption spectra over the range 400–700 nm were recorded on solutions of either substituted p-phenylenediamine salt and of their semiquindiimine oxidation products Wurster's red and Wurster's blue in acidified 50% aqueous methanol using a Cary Model 14 spectrophotometer. Spectra were recorded *versus* acidified 50% aqueous methanol as blank.

The Wurster dyes obtained on thin-layer chromatograms of the sterol hydro-peroxides after spraying with the N,N-dimethyl-p-phenylenediamine or N,N,N',N'-tetramethyl-p-phenylenediamine reagents were eluted with acidified 50% aqueous

methanol after scraping the silica gel containing the colored zones from the chromatoplate. The aqueous methanolic elute was scanned against a blank prepared by a similar elution of a comparable area (1–4 cm²) from the same chromatoplate in which no visible dye color had developed.

Spectra were also recorded on solutions of either reagent salt in acidified aqueous methanol to which benzoyl peroxide or hydrogen peroxide were added. Both peroxides oxidized the reagent solutions to their respective Wurster dyes, as evinced by visible absorption spectra.

Purified N,N-dimethyl-p-phenylenediamine dihydrochloride gave little absorption at 518 and 555 nm characteristic of Wurster's red[8], and purified N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride gave little adsorption at 564 and 604 nm characteristic of Wurster's blue[8]. Exposure of N,N-dimethyl-p-phenylenediamine or of N,N,N′,N′-tetramethyl-p-phenylenediamine in solution to air invariably resulted in an increase in adsorption at the characteristic band positions such that these air-oxidized solutions could not be used conveniently as reagent blanks for visible spectra studies. Consequently, solvent blanks not containing the substituted p-phenylenediamine salts were used for the work herein reported.

The visible absorption bands characteristic of Wurster's red obtained from oxidation by air, by bromine, by hydrogen peroxide, by benzoyl peroxide, or by sterol hydroperoxides were at 518 and 555 nm, with an inflection at 482 nm and minima at 425 and 535 nm (Fig. 1). Bands for Wurster's blue obtained by similar oxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride were at 564 and 604 nm, with an inflection at 524 nm and minima at 420 and 592 nm.

Autoxidation experiments. Preparations of Lifschütz's "oxycholesterol" (benzoyl peroxide in acetic acid) and of autoxidized cholesterol in aqueous dispersion in sodium stearate solutions (at 85°) were made as previously described[2]. Autoxidation of cholesterol dissolved in toluene (25 mg/ml) was achieved by bubbling air through the refluxing solution for 144 h. Photosensitized autoxidation of cholesterol in pyridine solution was achieved by published means[9-11]. Autoxidation of solid cholesterol was achieved by heating in air at 60° or at 100° in an electric oven. Films of solid cholesterol on glass plates were prepared by evaporation of a diethyl ether solution of cholesterol, and these films were exposed in air to light (254 and 366 nm ultraviolet and visible light). Crystalline cholesterol samples were exposed to ⁶⁰Co gamma radiation in a Gammacell 200 (Atomic Energy of Canada, Ltd., Ottawa) for periods of up to 64 h at radiation levels of 2.7×10^5 Rad/h.

RESULTS

The color responses of over fifty steroids to N,N-dimethyl-and N,N,N′,N′-tetramethyl-p-phenylenediamine have been determined, and it is clearly established that these reagents may be used reliably for detection of sterol hydroperoxides on thin-layer chromatograms. Both of these reagents enjoy wide use in a variety of radical reactions, and the N,N-dimethyl-reagent has been used in prior chromatographic detection of sterol hydroperoxides[5] and in other chromatographic analytical work[12].

With the N,N,N′,N′-tetramethyl-reagent a positive response was the formation of the characteristic violet color of Wurster's blue, but with the N,N-dimethyl-reagent

TABLE I

COLOR RESPONSES OF STEROIDS TO N,N-DIMETHYL-p-PHENYLENEDIAMINE

1. *Formation of Wurster's red*

   Cholesterol 7α-hydroperoxide, cholesterol 20α-hydroperoxide, cholesterol 24-hydroperoxide, cholesterol 25-hydroperoxide, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide.

2. *Yellow, beige, or tan colors* (Δ^4-and Δ^5-3-ketosteroids)

   Cholest-4-en-3-one, cholesta-4,6-dien-3-one, progesterone, androst-4-ene-3,17-dione, testosterone, 2β,17α,21-trihydroxypregn-4-ene-3,20-dione, 9α-fluoro-11β,16α,17α,20β,21-pentahydroxypregna-1,4-dien-3-one, 2α,17β-dihydroxyandrost-4-en-3-one, 6β,17α,21-trihydroxypregn-4-ene-3,20-dione, 17α-ethinyltestosterone, 10β,17β-dihydroxyestr-4-en-3-one, cholest-5-en-3-one.

3. *Orange to orange-pink colors* (Δ^4-3-ketosteroid α-ketols)

   Hydrocortisone, cortisone, 9α-fluorohydrocortisone, prednisolone, corticosterone, triamcinolone, 17α,21-dihydroxypregn-4-ene-3,20-dione,   11α,17α,21-trihydroxypregn-4-en-3,20-dione,   21-hydroxypregna-4,16-diene-3,20-dione, 20-hydroxy-3-oxopregna-4,17(20)-dien-21-al.

4. *Slowly developing blue colors*

   Cholest-5-ene-3β,7α-diol, cholest-5-ene-3β,7β-diol.

5. *Negative (no color) responses*

   Cholesterol, 5α-cholestan-3β-ol, 5β-cholestan-3β-ol, 3α,5-cyclo-5α-cholestan-6β-ol (i-cholesterol), stigmasterol, β-sitosterol, 5α-cholest-7-en-3β-ol, 5α-cholest-8(14)-en-3β-ol, lanosterol, 24,25-dihydrolanosterol, cholest-5-ene-3β,4β-diol, cholest-5-ene-3β,20α-diol, cholest-5-ene-3β,25-diol, cholest-5-ene-3β,20α,22R-triol, 3β,5-dihydroxy-5α-cholestan-6-one, 5α-cholestane-3β,5,6β-triol, 3β-hydroxycholest-5-en-7-one, cholesta-3,5-dien-7-one, cholesterol 5α,6α-oxide, cholic acid, 3β-hydroxypregn-5-en-20-one,   3β-hydroxyandrost-5-en-17-one,   androst-5-ene-3β,17β-diol, estradiol, estriol, equilin, 17α-ethinylestradiol, 16-ketoestradiol.

positive color responses ranged over four distinct color categories, as tabulated in Table I. Only sterol hydroperoxides gave the characteristic reddish pink color of Wurster's red, however. Some Δ^4-3-ketosteroid α-ketols gave yellow colors instead of the orange or orange-pink colors characteristic of the group. Cyclic α-ketols (16-ketoestradiol, 3β,5-dihydroxy-5α-cholestan-6-one) gave negative responses. Those Δ^4-3-ketosteroids giving yellow, beige, or tan colors and those Δ^4-3-ketosteroids α-ketols giving orange or orange-pink colors with N,N-dimethyl-p-phenylenediamine gave diminished, weak (but positive) colors of Wurster's blue with N,N,N',N'-tetramethyl-p-phenylenediamine.

   The epimeric 7-hydroxycholesterols cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol gave negative responses to N,N-dimethyl- and N,N,N',N'-tetramethyl-p-phenylenediamine immediately after spraying. However, with the N,N-dimethyl-reagent a blue color developed slowly, (more rapidly on warming the sprayed chromatoplate). Acidified aqueous methanol alone did not give the blue color; therefore, it is unlikely that this color response to the N,N-dimethyl-reagent is the same chemical reaction giving the rapid, intense blue colors of the epimeric 7-hydroxycholesterols with strong acid reagents[2].

   Solutions of preformed Wurster's red or blue in acidified aqueous methanol gave no color responses when used as a spray for detection of the sterol hydroperoxides.

   The visible absorption spectrum of the red color formed on thin-layer chromatograms of cholesterol 25-hydroperoxide sprayed with N,N-dimethyl-p-phenylenediamine (Fig. 1) possesses features which are identical with those of authentic

Wurster's red prepared by bromide oxidation of N,N-dimethyl-p-phenylenediamine[8] or by hydrogen peroxide oxidation.

Attempted recovery of the Wurster dyes from thin-layer chromatograms for quantitative measurement of the sterol hydroperoxides led to some difficulties, relating mainly to dilution effects and non-adherence to the Beer–Lambert law.
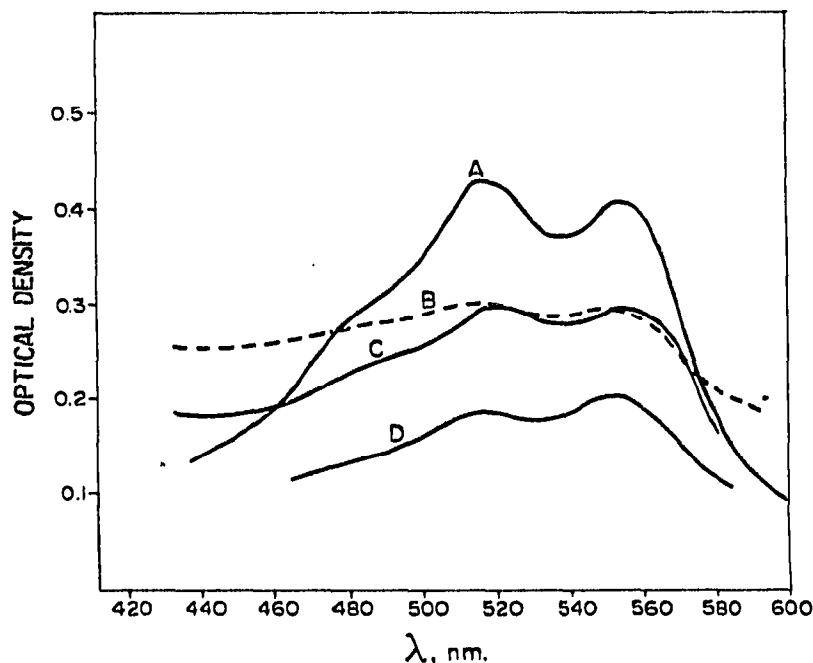

Fig. 1. Visible spectra of oxidation products from N,N-dimethyl-p-phenylenediamine, in reaction with: (A) 30 % hydrogen peroxide, 16.7 nl/ml; (B) cholesterol 25-hydroperoxide, 400 μg, chromatographed, sprayed, and eluted into 3 ml; final concentration 133 μg/ml; (C) cholesterol 25-hydroperoxide standard, 133 μg/ml; (D) 5α-cholestane-3β,5,6β-triol, containing peroxidic components, 1 mg, chromatographed, sprayed, and eluted into 3 ml; final sterol concentration, 333 μg/ml.

This problem may derive through the polymerization of Wurster's red semiquindiimine, which is known to occur under a variety of temperature and concentration conditions[6,13]. Wurster's blue is thought not to polymerize, however[6], and our preliminary studies do not exhaust the possibilities for development of a spectral assay for sterol hydroperoxides. Indeed, N,N-dimethyl-p-phenylenediamine salts have been reported to be of use in a colorimetric analysis for trace peroxides in other work[14].

The sterol products of reaction with N,N-dimethyl-p-phenylenediamine were also examined. After visualization of cholesterol 25-hydroperoxide and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide with N,N-dimethyl-p-phenylenediamine sterols were eluted and rechromatographed. Other than some unaltered sterol hydroperoxide only a single product sterol was detected in either case, and the product had the chromatographic mobility and color test behavior of the alcohols cholest-5-ene-3β, 25-diol and 5α-cholest-6-ene-3β, 5-diol, respectively.

The sensitivity of the procedure for the detection of sterol hydroperoxides on irrigated thin-layer chromatoplates is as good or better than that obtained with the use of 50 % aqueous sulfuric acid for the general detection of unsaturated sterols[2]. With the N,N-dimethyl-p-phenylenediamine reagent it was possible to detect reliably 0.5 μg

of cholesterol 25-hydroperoxide and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide on irrigated chromatoplates. Quantities less than 0.5 μg could not be detected reliably. With the N,N,N′,N′-tetramethyl-p-phenylenediamine reagent an improved sensitivity obtained, it being possible to detect with confidence as little as 0.05 μg of cholesterol 25-hydroperoxide on irrigated thin-layer chromatoplates. This increased sensitivity results in part from a greater contrast between the Wurster's blue spot and the background which retains a faint violet tinge. The added expense of the N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (over ten times as costly in the United States) limits use to special situations, however.

Sensitivity of these reagents is, in our hands, superior to that of either the standard starch–potassium iodide or ammonium thiocyanate–ferrous sulfate reagents[7] used in our prior TLC studies of sterol hydroperoxides[1,2]. Although these reagents have been reported as being sensitive to 1 μg of organic peroxides[15], we have not been able to use either reagent with confidence at this reported sensitivity. An additional difficulty with these standard reagents is the diffusion of the color response outside of the bounds nominally occupied by the sterol hydroperoxide on the irrigated chromatoplate, such that accurate positioning of the sterol hydroperoxide in the chromatograms of mixtures is difficult.

Hydroperoxides could be detected with either reagent in common sterol samples which had been stored at room temperature in the bottles in which they had been obtained several years ago. Thus, cholesterol, stigmasterol, β-sitosterol, 5α-cholest-7-en-3β-ol, 5α-cholest-8(14)-en-3β-ol, lanosterol, and 24,25-dihydrolanosterol, as examples of Δ^5-, Δ^7-, $\Delta^{8(9)}$-, and $\Delta^{8(14)}$-3β-hydroxysteroids, and 3α,5-cyclo-5α-cholestan-
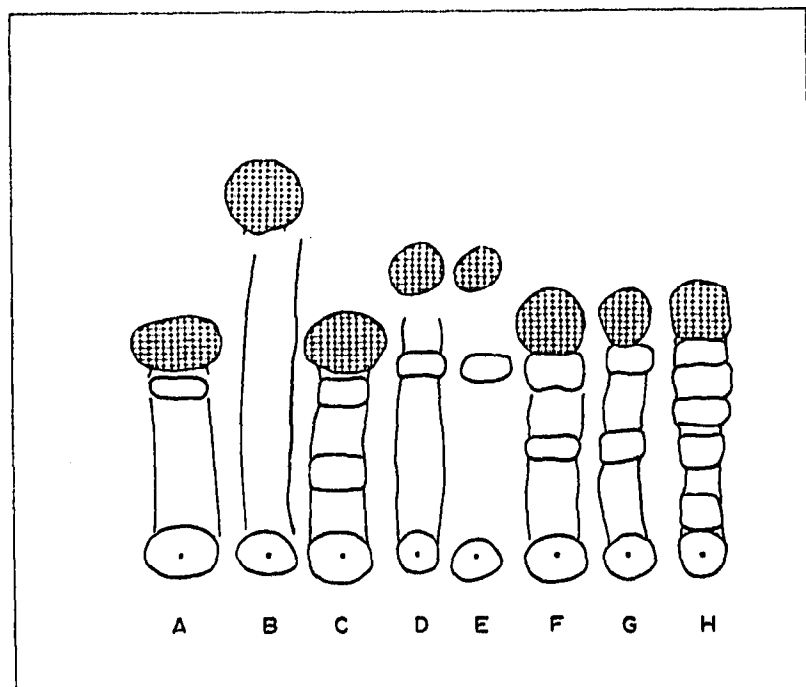

Fig. 2. Thin-layer chromatogram of peroxide-containing sterol samples (80 μg each), irrigated with benzene–ethyl acetate (2:1), detection with N,N-dimethyl-p-phenylenediamine reagent. The named parent sterols, not detected by N,N-dimethyl-p-phenylenediamine, are drawn as cross-hatched spots, detected with 50% sulfuric acid. A = 5α-Cholest-8(14)-en-3β-ol; B = 3α,5-cyclo-5α-cholestan-6β-ol (i-cholesterol); C = 5α-cholest-7-en-3β-ol; D = 24,25-dihydrolanosterol; E = lanosterol; F = β-sitosterol; G = stigmasterol; H = cholesterol.

6β-ol, as an example of a related reactive system, all form characteristic sterol hydroperoxides (Fig. 2). None of these hydroperoxides except for those of cholesterol[1] have been identified, although several specific lanosterol derivative hydroperoxides have been described[16-18].

Examination of a cholesterol monohydrate sample prepared in 1937 via the dibromide which had not autoxidized on storage[19] established that this sample did not contain sterol hydroperoxide or other autoxidation impurities.

Positive Wurster red color responses were obtained with stored samples of 5α-cholestane-3β,5,6β-triol and 4-hydroxycholest-4-en-3-one, the red color in both cases being superimposed in the sterol spot on thin-layer chromatograms developed in several solvent systems. However, the peroxides responsible for the positive test could be removed from the sterol samples by recrystallization. This result with 5α-cholestane-3β,5,6β-triol suggests that peroxide formation may occur in certain saturated sterols devoid of unsaturated functional groups in the molecule. However, the saturated stanols 5α-cholestan-3β-ol and 5β-cholestan-3β-ol did not have detectable amounts of sterol hydroperoxide impurities on extended storage in air.

Sterol hydroperoxides were detected with either reagent in cholesterol samples subjected to standard autoxidation procedures. The times necessary for initial detection of a sterol hydroperoxide (either the 5α-or the 7α-hydroperoxide or both) and for the development of the complete spate of autoxidation products are given in Table II for a variety of conditions. In each case cholesterol 20α-and 25-hydroperoxides could be detected after the initial 5α and/or 7α-hydroperoxides had formed but before the full elaboration of degradation products occurred.

TABLE II

DETECTION OF STEROL HYDROPEROXIDES IN CHOLESTEROL AUTOXIDATION

Condition[a]	Time required for development of	
	Initial hydroperoxides[b]	Full spate of autoxidation products[c]
Heating at 60°	9 days	48 days
Heating at 100°	17 h	2–3 days
254 nm radiation	16 h	18 h
366 nm radiation	11 days	29 days
60Co radiation	16 h	24 h
Colloidal dispersion	30 min	1 h
Refluxing toluene	4 h	27–67 h

a Details are given in EXPERIMENTAL.
b As detected with N,N-dimethyl-p-phenylenediamine on thin-layer chromatograms.
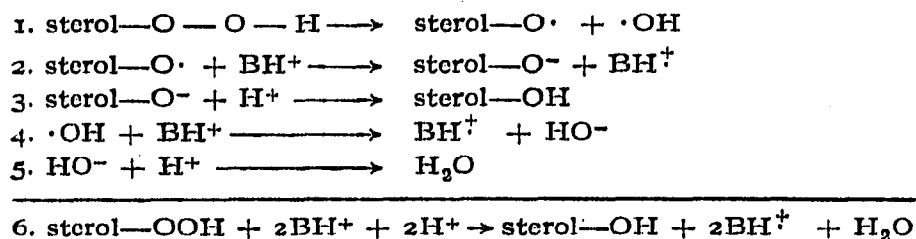c As detected with 50% aqueous sulfuric acid on thin-layer chromatograms.

The photosensitized autoxidation of cholesterol in pyridine solution used preparatively for 3β-hydroxy-5α-cholest-6-ene-5-hydroxyperoxide[9-11] was shown to give only the 5α-hydroperoxide as a product. In refluxing toluene the 5α-hydroperoxide was detected within 4 h, but after 27 h a new peroxidic component more mobile than cholesterol was also detected, along with cholesterol 7α-hydroperoxide and 3β-hydroxycholest-5-en-7-one. After 67 h the epimeric 7-hydroxycholesterols were

present. The new peroxidic component is derived from cholesterol, since toluene alone did not give rise to the component. The well-known autoxidation of toluene to benzaldehyde did occur in this experiment, as evinced by the characteristic odor of benzaldehyde, both in the reaction solution and also on the irrigated chromatoplate, by which means benzaldehyde was readily located in a region more mobile than cholesterol.

The complex "oxycholesterol" of Lifschütz, prepared by action of benzoyl peroxide on cholesterol, contained several peroxides, none of which was recognized as corresponding to previously identified cholesterol hydroperoxides. The major products of this reaction give strong blue colors with sulfuric acid but are not hydroperoxides.

DISCUSSION

The Wurster dyes[20-22] are formed by one electron oxidation of the phenylenediamine salts to give semiquindiimines whose structures are supported by potentiometric titration[8,13] and magnetic susceptibility[6] measurements. Based on our spectral and chromatographic evidence formation of the Wurster dyes from sterol hydroperoxides may be summarized as follows:

1. $sterol—O—O—H \longrightarrow sterol—O\cdot + \cdot OH$

2. $sterol—O\cdot + BH^+ \longrightarrow sterol—O^- + BH^{\overset{+}{\cdot}}$

3. $sterol—O^- + H^+ \longrightarrow sterol—OH$

4. $\cdot OH + BH^+ \longrightarrow BH^{\overset{+}{\cdot}} + HO^-$

5. $HO^- + H^+ \longrightarrow H_2O$

6. $sterol—OOH + 2BH^+ + 2H^+ \rightarrow sterol—OH + 2BH^{\overset{+}{\cdot}} + H_2O$

where BH^+ represents the protonated bases N,N-dimethyl- or N,N,N',N'-tetramethyl-p-phenylenediamine and $BH^{\overset{+}{\cdot}}$ represents the respective Wurster dye.

These studies accordingly offer an improved procedure for detection of sterol hydroperoxides on thin-layer chromatograms by means of which the chemistry is established. Application of the method to a variety of cholesterol autoxidation conditions demonstrates the utility of the procedure and also suggests that the initial product of cholesterol autoxidation which is detected is the 5α-hydroperoxide. Only after formation of the 5α-hydroperoxide are there detectable amounts of cholesterol 20α- and 25-hydroperoxides. Finally, after elaboration of the hydroperoxides the more commonly described autoxidation products are formed.

ACKNOWLEDGEMENT

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