

Color Reaction of Cholesterol with Benzoyl Peroxide in Concentrated Trichloroacetic Acid

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Cholest-2,4,6-triene (I), 3,3'-bicholesta-2,4,6,5'-tetraene (II) and benzoic acid were isolated in the reaction of cholesterol with BPO in carbon tetrachloride, and the substances I and II were observed to color blue on shaking with conc. TCA. It was assumed that I formed initially was further subject to the radical reaction to yield II. It was also presumed that a series of reactions forming the substances I and II should proceed via the addition reaction of benzoxy radical caused by the thermolysis of BPO to cholesterol, resulting the radical dehydration and the radical dehydrogenation. The products I and II should be colored blue by the formation of corresponding cation or carbonium ion. It was found that this color reaction was specific for the steroids with the structure of 5-ene-3-ol or its ester, so that it may be useful for the detection and the determination of them.

It was clarified by the authors that the coloration of cholesterol in concentrated trichloroacetic acid (conc. TCA) and its mixture with hydrochloric acid was mainly due to the formation of 3,5-cholestadiene and 3,3'-bis(3,5-cholestadiene).²⁾ In continuation of this work, attempts were made to investigate the coloration of cholesterol with benzoyl peroxide (BPO) in conc. TCA. Regarding this type of coloration, it has been reported by Rosenheim³⁾ that the white product was obtained from the chloroform solution by heating cholesterol with BPO in chloroform, and that the product gave a blue color with reagents such as Brønsted and Lewis acids. He, however, did not describe any structure of the product and the mechanisms of this coloration in his report. Hereupon, the structure of the product was examined at first. For the conduct of the coloration, conc. TCA was used for the reaction solvent and carbon tetrachloride for the auxiliary solvent in order to avoid the possible side reaction.⁴⁾

A solution of cholesterol and BPO in carbon tetrachloride was refluxed on a boiling water bath, and then washed with 3% sodium carbonate solution. From the washings benzoic acid was obtained in 17.9% yield. The residue obtained after the evaporation of the organic layer was colored blue by shaking with conc. TCA. The absorption spectra of the colored solution are shown in Fig. 1, and the absorption maxima are observed at 585 and 656 nm. On the other hand, the reaction product was dissolved in a small amount of hexane and submitted to a column chromatography on silica gel. Two substances, I and II, were isolated both giving rise to a blue coloration on shaking with conc. TCA.

The substance I was colorless needles (mp 63-64°). The absorption spectra of the color developed by I with conc. TCA are shown in Fig. 2. The absorption maxima are observed at 585 and 656 nm. I was proved to be a steroidal monomer, possessing three double bonds from the data of elementary analysis and mass spectrum (MS). Moreover, according to the finding of Fieser⁵⁾ the double bonds were found to be conjugated from the data of the ultraviolet (UV) absorption spectrum shown in Fig. 3, which showed the absorption maxima at 296, 306

1) Location: *Shirokane, Minatoku, Tokyo, 108, Japan.*

2) Y. Kurasawa, A. Takada, and T. Ueda, *Chem. Pharm. Bul.* (Tokyo), **24**, (1976).

3) O. Rosenheim, *Biochem. J.*, **21**, 386 (1927).

4) R. Hiatt, "Organic Peroxides," Vol. II, ed. by D. Swern, John Wiley and Sons, Inc., New York, N. Y., 1971, pp. 822-826.

5) L.F. Fieser and M. Fieser (ed.), "Steroids," Chapter 2, Reinhold Publishing Corporation, New York, N. Y., 1959.

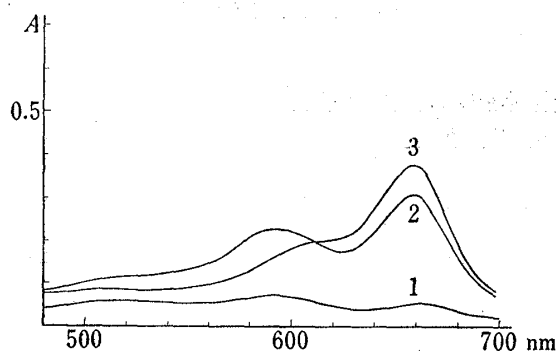


Fig. 1. Absorption Spectra of Cholesterol with BPO in Conc. TCA

A solution of 28.57 mg of cholesterol and 13.74 mg of BPO in 5 ml of carbon tetrachloride was refluxed for 12 minutes and then evaporated. The residue was dissolved in 3 ml of carbon tetrachloride, and 5 ml of conc. TCA was added. The solution was allowed to stand for a few periods at a room temperature. Spectrum 1 was recorded immediately, spectrum 2 at 18 min, and spectrum 3 at 80 min.

different from those of I, and observed at a wave length longer than those of I, indicating the presence of an alkyl substituent at some position in which the double bonds existed. According to those findings, it was assumed that the substance II might be the derivative of the substance I having the steroidal substituent in A or B ring. Thus five structures are possible as shown in Chart 1. However, the observation of a singlet signal for an olefinic proton at

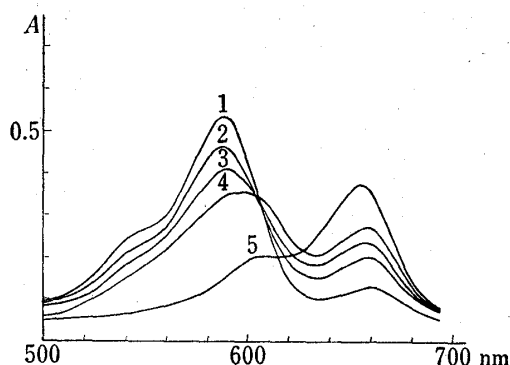
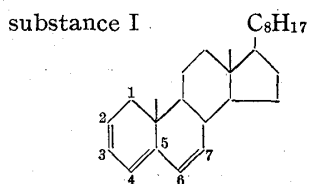


Fig. 2. Absorption Spectra of Cholest-2,4,6-triene in Conc. TCA

To a solution of 0.05 mg of cholest-2,4,6-triene in 1 ml of chloroform was added 20 ml of conc. TCA. The solution was allowed to stand for several periods at a room temperature.

Spectrum 1 was recorded at 4 min, spectrum 2 at 16 min, spectrum 3 at 40 min, spectrum 4 at 97 min, and spectrum 5 at 16 hr.



and 320 nm. Five olefinic protons were observed at 5.42 to 5.90 ppm in the nuclear magnetic resonance (NMR) spectrum, indicating the structure of cholest-2,4,6-triene for I as shown in Chart 1. The UV and infrared (IR) absorption spectra were identical with those of the authentic sample prepared by the method of Eckhardt.⁶⁾

The substance II was colorless needles (mp 164–165°), and gave a blue coloration on shaking with conc. TCA. The absorption spectra are shown in Fig. 4, and the absorption maxima are observed at 585 and 656 nm. II was suggested to be a dimeric steroid having four double bonds from the data of elementary analysis and MS. The UV absorption maxima were observed at 302, 315 and 330 nm as shown in Fig. 3. The UV absorption maxima were

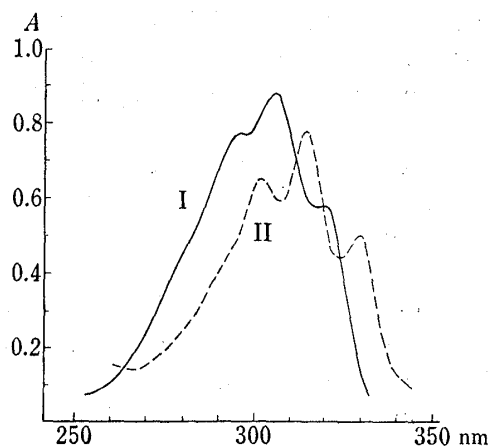
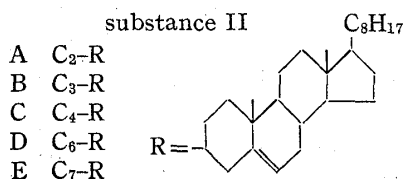


Fig. 3. UV Spectra of I and II

I (—), 0.0970 mg of I was dissolved in 25 ml of isopropyl alcohol.

II (---), 0.182 mg of II was dissolved in 25 ml of isopropyl alcohol.



B may be the most possible structure for the substance II.

Chart 1

6) H.J. Eckhardt, *Ber.*, **71B**, 461 (1938).

4.48 ppm in the NMR spectrum excluded the structure A, C and D. Taking into consideration of the steric hindrance in the dimerizing reaction as shown in Chart 2b, E was also thought to be eliminated. Therefore, B may be the most plausible structure for II. A double bond in cholesteryl group, R in Chart 1, was postulated to be at 5'-position from the data of NMR, which showed a doublet signal ($J=3$ Hz) at 5.20 ppm indicating the coupling between the olefinic proton and the methylenic protons, and by considering the reaction mechanism in Chart 2b. Furthermore, the NMR spectrum of this product showed a double doublet signals ($J=9$ Hz, $J=3$ Hz) for 7-olefinic proton at 5.48 ppm and a doublet signal ($J=9$ Hz) for 6-olefinic proton at 5.88 ppm, and 2-olefinic proton was postulated to exist between 5.60 ppm and 5.40 ppm from the integral curve of the NMR spectrum. These data supported the structure of 3,3'-bicholesta-2,4,6,5'-tetraene.

It has been described by Sosnovsky and Rawlinson⁷⁾ that the thermal decomposition of BPO gives benzoxy radical, and resulting benzoxy radical causes the addition reactions to various kinds of olefins. In the present investigation, it was anticipated that benzoxy radical yielded by the thermolysis should add to the double bond of cholesterol molecule existing in B ring. In order to confirm whether the reaction was brought about radically, the electron spin resonance (ESR) measurement was attempted. A free radical (g value, 2.002) was

detected in the ESR absorption spectrum as shown in Fig. 5 from the reaction mixture in carbon tetrachloride. Any hyperfine structure was not observed in the ESR absorption spectrum. Therefore, the reaction was postulated to proceed radically. On the other hand, it has been reported by Kochi⁸⁾ that benzoxy radicals react with olefins by addition to yield benzoxyalkyl adduct radicals, whereas hydrogen abstraction from the allylic position of olefins by benzoxy radicals is minor. Based on these findings, it may be assumed that the

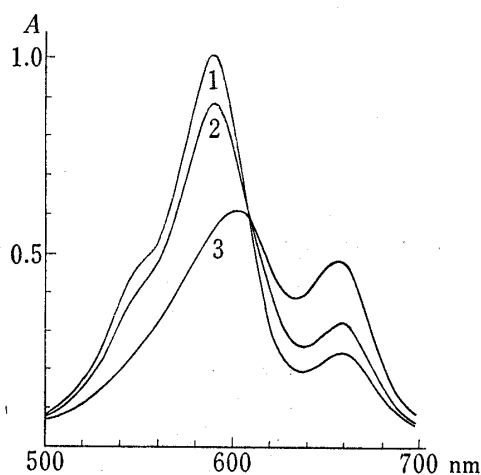


Fig. 4. Absorption Spectra of 3,3'-Bicholesta-2,4,6,5'-tetraene in Conc. TCA

To a solution of 0.02 mg of 3,3'-bicholesta-2,4,6,5'-tetraene in 1 ml of chloroform was added 13 ml of conc. TCA. The solution was allowed to stand for a few periods at a room temperature.

Spectrum 1 was recorded immediately, spectrum 2 at 93 min, and spectrum 3 at 17.5 hr.

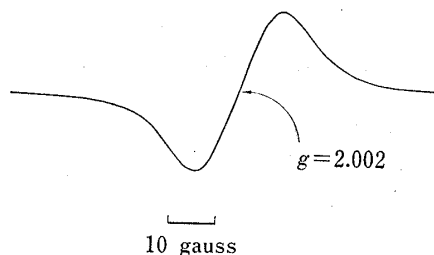


Fig. 5. ESR Spectrum of Cholesterol with BPO in Carbon Tetrachloride

reaction mechanism was proposed as shown in Chart 2. It was also inferred that benzoxy radical caused by the thermolysis of BPO should add to the double bond of cholesterol and then the dehydration and the dehydrogenation should take place through the formation of the transient three membered ring.

It has been presumed by many investigators⁹⁾ that the colorations of the steroids possessing conjugated double bonds with Brønsted and Lewis acids were attributed to the formation

7) G. Sosnovsky and D.J. Rawlinson, "Organic Peroxides," Vol. I ed. by D. Swern, John Wiley and Sons, Inc., New York, N. Y., 1971, pp. 564—584.

8) J.K. Kochi, *J. Am. Chem. Soc.*, **84**, 1572 (1962).

9) T. Watanabe, *Eisei Shikenjo Hokoku*, **77**, 87 (1959); R.W. Burke, B.I. Diamondstone, R.A. Velapoldi, and O. Menis, *Clin. Chem.*, **20**, 794 (1974).

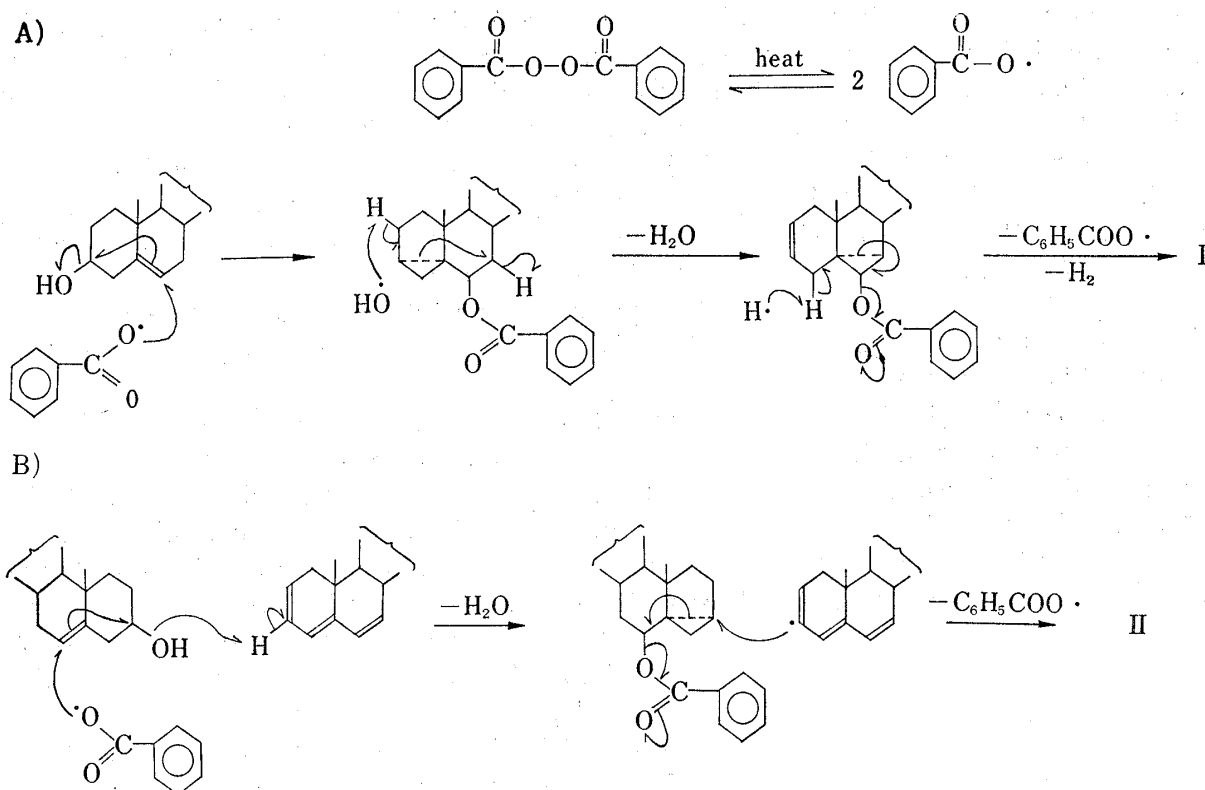


Chart 2

TABLE I. Coloration of the Various Steroids with BPO in Conc. TCA

Compound	Coloration	$\lambda_{\max}$ nm (log ϵ)
Cholesteryl chloride	blue	605 (1.41)
Cholesteryl acetate	blue	659 (1.59)
Dehydro-iso-androsterone	blue	620 (1.97) ^{a)}
Ergosterol	brown	660 (2.21)
7-Dehydrocholesterol	brownish green	610 (1.02) ^{a)}
3,5-Cholestadiene	blue	650 (1.29)
Testosterone	colorless	—
Estrone	colorless	—
Cholic acid	colorless	—
Cholanic acid	colorless	—
3,3'-Bis(3,5-cholestadiene)	red ^{b)}	—
3,3'-Bis(2,4-cholestadiene)	red ^{b)}	—

A solution of respective steroids and BPO in carbon tetrachloride was refluxed for 20 minutes and then evaporated. The spectra were measured at 7 minutes after the addition of conc. TCA to the residue.

a) shoulder

b) This coloration may be caused by these steroids in conc. TCA.

of the corresponding cation or carbonium ion. It was postulated that cholest-2,4,6-triene and 3,3'-bicholesta-2,4,6,5'-tetraene were colored blue with conc. TCA in the same way as above, since none of free radical was observed in the colored solutions.

It appeared of interest to investigate further the scope of this coloration using various steroids, because this coloration showed a specific blue color. The experimental results are shown in Table I.

As shown in Table I, it was found that this color reaction was specific for the steroids with the structure of 5-ene-3-ol or its ester. Therefore, this color reaction may be useful for the detection and the determination of the steroids having the structure of 5-ene-3-ol in biological fluids, since the coloration shows a characteristic blue color, so that the influence of impurities in the test solutions may be eliminated.

Experimental¹⁰⁾

Reaction of Cholesterol with Benzoyl Peroxide—A solution of 10 g of cholesterol and 10 g of benzoyl peroxide in 100 ml of carbon tetrachloride was refluxed on a water bath for 2 hr. During this period, the solution was colored yellow gradually.

After cooling the solution, the reaction mixture was washed with 3% Na_2CO_3 solution and the washings was acidified with 10% HCl to give 1.79 g of benzoic acid. Recrystallization from water gave colorless needles, mp 120–121°.

The above organic layer was dried over Na_2SO_4 and evaporated *in vacuo*. An oily residue obtained was dissolved in a small amount of hexane and submitted to column chromatography on silica gel with hexane. The first fraction was collected and evaporated to give a faint yellow oily product (I). This oily product was further chromatographed on alumina with hexane to give colorless crystals. Recrystallization from acetone by dissolving the colorless crystals at 20–25° and then cooling below 0° gave analytically pure cholest-2,4,6-triene as colorless needles, mp 63–64°. Yield, 339 mg (3.6%). Mass Spectrum m/e : 366 (M^+). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 2960, 2880, 1466 (CH_2), 1380, 1369 (CH_3). UV $\lambda_{\text{max}}^{\text{iso-PrOH}}$ nm (log ϵ): 296 (4.86), 306 (4.92), 320 (4.72). *Anal.* Calcd. for $\text{C}_{27}\text{H}_{42}$: C, 88.45; H, 11.55. Found: C, 88.26; H, 11.47.

The original silica gel column was eluted with hexane successively, and the second fraction was collected and evaporated to give another oily product (II). Further purification of II by column chromatography on alumina with hexane yielded colorless crystals. Recrystallization from acetone–hexane gave analytically pure 3,3'-bicholesta-2,4,6,5'-tetraene as colorless needles, mp 164–165°. Yield, 98 mg (1.03%). Mass Spectrum m/e : 734 (M^+). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 2960, 2880, 1468 (CH_2), 1380, 1368 (CH_3). NMR ppm (CCl_4): 5.88 (1H, d, 6-olefinic, $J=9$ Hz), 5.48 (1H, dd, 7-olefinic, $J=9$ Hz, $J=3$ Hz), 5.48 (1H, s, 4-olefinic), 5.20 (1H, d, 6'-olefinic, $J=3$ Hz). UV $\lambda_{\text{max}}^{\text{iso-PrOH}}$ nm (log ϵ): 302 (4.82), 315 (4.90), 330 (4.70). *Anal.* Calcd. for $\text{C}_{54}\text{H}_{86}$: C, 88.21; H, 11.79. Found: C, 88.36; H, 11.51.

10) Absorption spectra were measured by Hitachi Recording Spectrophotometer Type EPS-3 in a cell of 10 mm optical length, IR spectra by JASCO IRA-1 Spectrophotometer, NMR spectra by JEOL JNM-PS-100 Spectrometer at 100 MHz with tetramethylsilane as internal standard, MS by JEOL JMS-01S Mass Spectrometer, and ESR spectra by JEOL JES-ME-1X Spectrometer with manganese monoxide as external standard.