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Color Reaction of Cholesterol in Concentrated Trichloroacetic Acid Media

Yoshihisa Kurasawa, Atsushi Takada, and Takeo Ueda

School of Pharmaceutical Sciences, Kitasato University[1]

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Reaction product of the coloration of cholesterol in 90% liquefied trichloroacetic acid (conc. TCA) and its mixture with hydrochloric acid were investigated in order to find the relationship between the coloration and the reaction products. It was found that 3,5-cholestadiene was the main product in all of TCA-containing media and 3,3′-bis(3,5-cholestadiene) was the minor product in conc. TCA–HCl (10: 1) medium, while any of bis-steroid was not found in the reaction with conc. TCA–HCl (1: 1) medium. The colored solution of these compounds showed the similar absorption spectrum to that of cholesterol in the respective medium. Cholesteryl chloride, which developed a red color gradually on dissolving in conc. TCA–HCl (10: 1) and (1: 1) media, was also obtained from the colored solution of cholesterol in all of HCl–containing media, and 3,5-cholestadiene was isolated from the colored solution of cholesteryl chloride. These results suggested that cholesteryl chloride might not be a direct coloring substance but 3,5-cholestadiene or a coloring intermediate derived from cholesteryl chloride in these media might contribute to the coloration.

It has been known to date that cholesterol showed specific coloration in various Brønsted or Lewis acid media. Some of these colorations are used for the identification of cholesterol in many of pharmacopoeias.

The reaction products associated with those colorations seem to vary with kinds of acids in media. 3,5-Cholestadiene (I), 3,3′-bis(2,4-cholestadiene) (II) and 3,3′-bis(3,5-cholestadiene) (III) were found by Watanabe with sulfuric acid-acetic anhydride (Liebermann-Burchard reaction),[2] I and II by Watanabe with zinc chloride-acetyl chloride (Tschugaeff reaction),[3] II and III by Dulou, *et al.* and Chopin with sulfuric acid (Salkowski reaction),[4] and III by Momose, *et al.* with ferric chloride-sulfuric acid.[5] Hirschsohn reported that cholesterol produced a red color with a weak fluorescence on warming in 90% liquefied trichloroacetic acid (conc. TCA), which changed to a blue color on standing at a room temperature for 12 hr, and that cholesterol developed a yellow color on standing in a mixture of conc. TCA and concentrated hydrochloric acid (HCl) (10: 1) at a room temperature for 5 min, which changed to a purplish red color.[6] He, however, did not describe the relationship between the coloration and the resulting products. On the reexamination of this coloration, we found that cholesterol afforded a purplish red color very soon by using a mixture of conc. TCA and HCl (1: 1), and recognized that the employment of this mixture as the reaction medium was of interest for the identification of cholesterol in the ease of the coloration. Attempts were made to clarify, in details, the reaction of cholesterol with conc. TCA and its mixture to HCl and particularly to find the relationship between the coloration and the reaction products.

At first, the reaction of cholesterol with conc. TCA was examined. A cholesterol solution in chloroform was mixed with conc. TCA, the mixture was heated in a boiling water bath for 6 min, and kept at a room temperature. During this period an intense red color was developed

1) Location: *Shirokane, Minato-ku, Tokyo, 108, Japan.*
2) T. Watanabe, *Eisei Shikenjo Hokoku,* **77**, 87 (1959).
3) T. Watanabe, *Yakugaku Zasshi,* **80**, 442 (1960); *idem, ibid.*, **80**, 445 (1960).
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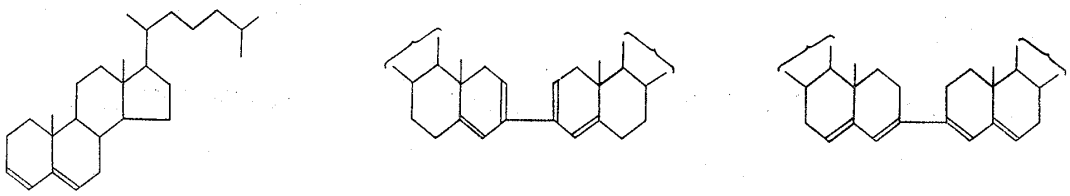

I II III

Chart 1

and the intensity of this coloration increased gradually with the elapse of time. The absorption maxima of the colored solution were observed at 500, 534 and 555 nm as shown in Fig. 1. The reaction mixture was poured into ice-water, and the resulting mixture was extracted with chloroform. The crude product obtained from the chloroform extract was found to be consisted of three components by thin-layer chromatography (TLC) (silica gel). Among them, the spot at *Rf* 0.24 (chloroform) coincided in position with that of cholesterol, and two other products (*Rf* 0.88 and 0.27, hexane) were purified by column chromatography on silica gel eluting with hexane. The former (*Rf* 0.88) was colorless prisms of mp 78—79° and identified as I by the inspection of its spectral data and by the mixing melting point test with the authentic sample prepared by the method of Mauthner.[7] The latter (*Rf* 0.27) was colorless prisms of mp 148—149° and showed a positive Beilstein test. The infrared (IR) spectrum of this compound showed the absorption of $\nu_{C=O}$ at 1760 cm^{-1}. The latter prisms (*Rf* 0.27) were treated with ethanolic potassium hydroxide solution to obtain cholesterol. On the basis of the above findings, the latter compound was identified as cholesteryl trichloroacetate (IV). By shaking with conc. TCA, I showed an intense red color immediately and the absorption maxima were observed at 500, 534 and 555 nm as shown in Fig. 1, while IV did not show any coloration, suggesting that I was the main product associated with the coloration.

When cholesterol was mixed with conc. TCA without dissolving in chloroform, the similar coloration to that described above was also observed. The colored solution was poured into ice-water and the resulting mixture was extracted with chloroform. Four components, cholesterol, I, IV and a trace amount of a yellow syrup, were obtained from the chloroform extract by the chromatographic fractionation on silica gel. The yellow syrup showed the molecular ion peak at *m/e* 736 in a mass spectrum (MS), suggesting that this compound might be a dimer of steroid having three double bonds, but this dimer could not be further characterized in details, on account of its very poor yield.

The colored solution of cholesterol in conc. TCA–HCl (10: 1) medium at a room temperature exhibited three absorption maxima at 420, 500 and 560 nm as shown in Fig. 2. The maxima at 420 and 500 nm were gradually shifted to 406 and 505 nm, and their intensity increased with the passage of time. The colored solution was treated according to the similar method to that described above. The resulting crude product was chromatographed and five components were obtained. Their *Rf* values on TLC (silica gel) were 0.88, 0.78, 0.58, 0.27 (hexane) and 0.24 (chloroform). Among them, three were found to be identical with cholesterol (*Rf* 0.24), I (*Rf* 0.88) and IV (*Rf* 0.27) by comparing the melting point and the spectral data. The component of *Rf* 0.78 was almost colorless needles of mp 245—247° and showed a molecular ion peak at *m/e* 734 in MS. This compound was identified as III by comparing the melting point, IR and UV spectra with those of the authentic sample prepared by the Squire's method.[8] The component of *Rf* 0.58 was white needles of mp 95—96° and showed a positive Beilstein test. This compound was identified as cholesteryl chloride (V) by comparing the melting point and the spectral data with those of the authentic sample.

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8) E.N. Squire, *J. Am. Chem. Soc.*, 73, 2586 (1951).

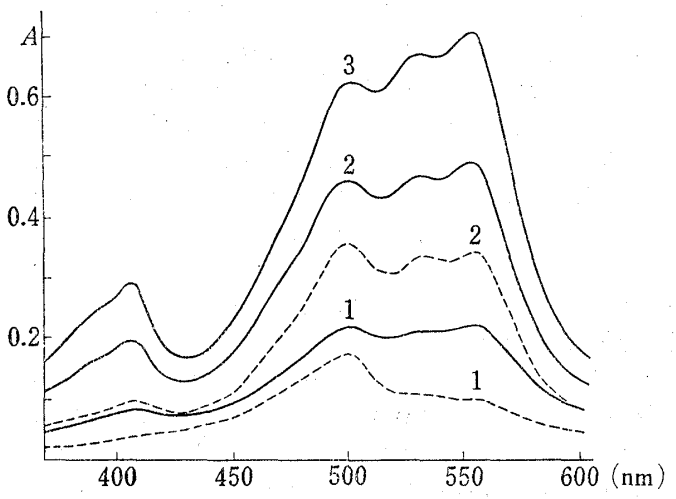

Fig. 1. Absorption Spectra of Cholesterol and I in conc. TCA

——— : cholesterol
To a solution of 0.6691 g of cholesterol in 2 ml of $CHCl_3$, 10 ml of conc. TCA was added, the mixture was heated for 2 min in a boiling water bath and kept for several periods (1—3) at a room temperature. 1, immediately; 2, 3 min; 3, 7 min
--------: I
To a solution of 2.27 mg of I in 2 ml of $CHCl_3$, 10 ml of conc. TCA was added with shaking and the mixture was kept for several periods (1—2) at a room temperature. 1, immediately; 2, 6 min

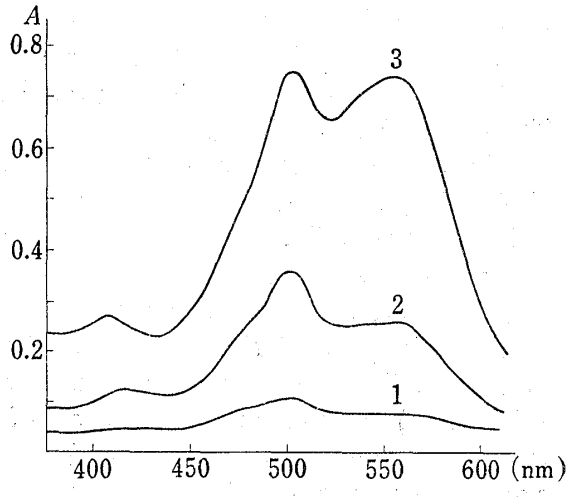

Fig. 2. Absorption Spectra of Cholesterol in conc. TCA–HCl (10: 1) Medium

To a solution of 2.05 mg of cholesterol in 1 ml of $CHCl_3$, 10 ml of conc. TCA–HCl (10: 1) medium was added and the mixture was kept for several periods (1—3) at a room temperature. 1, 14 min; 2, 28 min; 3, 55 min

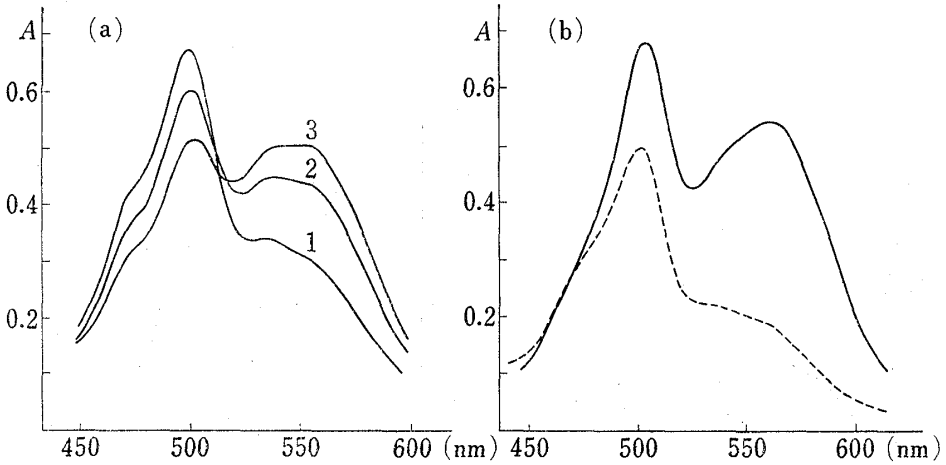

Fig. 3. Absorption Spectra of I (a), and III and V (b) in conc. TCA–HCl (10: 1) Medium

a : To a solution of 11.1 μg of I in 1 ml of $CHCl_3$, 10 ml of conc. TCA–HCl (10: 1) medium was added and the mixture was kept for several periods (1—3) at a room temperature. 1, 10 min; 2, 13 min; 3, 16 min
b : ——— III, -------- V
To a solutions of 1.11 mg of III in 5 ml of $CHCl_3$ and 21.4 mg of V in 1 ml of $CHCl_3$, 10 ml of conc. TCA–HCl (10: 1) medium was added respectively and the mixtures were kept for 15 min at a room temperature.

By shaking with conc. TCA–HCl (10: 1) medium, I and III showed an intense red color immediately, and the absorption spectra are shown in Fig. 3. On the other hand, V showed a weak coloration immediately, increasing its intensity gradually with the elapse of time, while IV did not show any coloration in conc. TCA–HCl (10: 1) medium.

When the cholesterol solution in chloroform was treated with conc. TCA–HCl (1: 1) medium at a room temperature, an intense red color was found to develop immediately. The absorption maxima were observed at 420, 499 and 535 nm in the early stage and the maxima of 420 and 499 nm gradually shifted to 410 and 500 nm respectively, as shown in

Fig. 4, while the maximum near 560 nm which had been observed in the coloration with conc. TCA–HCl (10: 1) medium was not observed. The crude product was obtained by the procedure as described above, and submitting the crude product to column chromatography on silica gel afforded cholesterol, I, IV and V, but III or another dimeric substance derived from cholesterol was not found to be produced in this color reaction. By dissolving I in conc. TCA–HCl (1: 1) medium, an intense red color was developed immediately, and the absorption maxima were observed at 410 and 500 nm as shown in Fig. 5. The solution of V in conc. TCA–HCl (1: 1) medium showed a weak orange color immediately and changed to an intense red color gradually. The absorption maxima were observed at 420 and 500 nm. On the other hand, IV did not show any coloration in this medium.

As described above, it was found that the reaction products associated with the coloration varied with hydrochloric acid concentration in TCA medium. From the colored solution in all of TCA media, I was obtained as the main product, III as the minor product from conc. TCA–HCl (10: 1) medium and V from all of HCl-containing TCA media. The coloration of V required a while to develop complete coloration in conc. TCA–HCl medium. In order to clarify this mechanism, the colored solution of V in conc. TCA–HCl (1: 1) medium was treated as described above and a crude product was obtained. From the crude product, I was obtained in 5.6% yield. It, therefore, was assumed from the results that V might not be a direct coloring substance, but V might be converted to I or a coloring intermediate produced from I in conc. TCA–HCl medium.

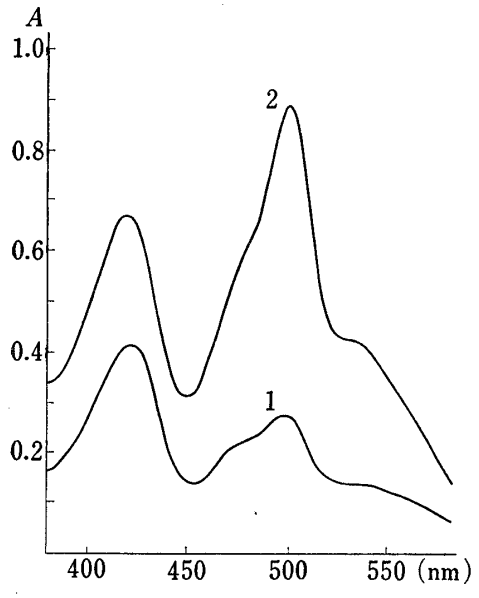

Fig. 4. Absorption Spectra of Cholesterol in conc. TCA–HCl (1: 1) Medium

To a solution of 1.575 mg of cholesterol in 1 ml of $CHCl_3$, 10 ml of conc. TCA–HCl (1: 1) medium was added and the mixture was kept for several periods (1—2) at a room temperature. 1, 12 min; 2, 19 min

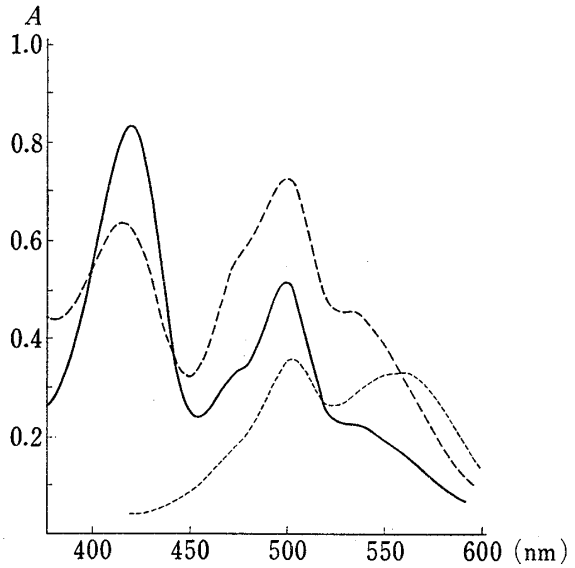

Fig. 5. Absorption Spectra of I, III and V in conc. TCA–HCl (1: 1) Medium

--------: I, ——— : V, ----------: III

To a solutions of 1.16 mg of I in 1 ml of $CHCl_3$, 0.52 mg of III in 1 ml of $CHCl_3$ and 19.52 mg of V in 1 ml of $CHCl_3$, 10 ml of conc. TCA–HCl (1: 1) medium was added respectively, and the mixtures were kept at a room temperature. I, 8 min; III, 16 min; V, 11 min

It is also of interest that any of dimeric substance was not obtained in conc. TCA–HCl (1: 1) medium. Since the colored solution of cholesterol in this medium did not show the absorption maximum at 560 nm and III was not obtained from the reaction mixture, it was assumed that the maximum at 560 nm might be caused by III. To confirm this assumption, the absorption spectrum of III in conc. TCA–HCl medium was investigated as follows: The colored solution of III in conc. TCA (1: 1) medium showed the maxima at 500 and 560 nm, and, between these maxima, the maximum at 560 nm was rather intensive, as shown in Fig. 5.

On the other hand, the solution of cholesterol in conc. TCA–HCl (10: 1) medium showed the maximum at 560 nm with a considerable intensity as shown in Fig. 2 and III was obtained from the reaction mixture, which showed the maxima at 500 and 560 nm in conc. TCA–HCl (10: 1) medium as shown in Fig. 3(b). The colored solution of I in conc. TCA–HCl (10: 1) medium showed the maximum at 500 nm, but the maximum at 560 nm was scarecely observed immediately. On standing the mixture at a room temperature, the maximum at 560 nm was found to increase as shown in Fig. 3(a), suggesting that III might be produced from I in this condition. The solution of I in conc. TCA–HCl (1: 1) medium, on the contrary, did not show the maximum at 560 nm even if the mixture was allowed to stand for a long time, indicating that bis-steroid was not produced in this reaction condition.

From these findings, it was found that the main product associated with the coloration was I, and III might be produced from I in conc. TCA–HCl (10: 1) medium. Watanabe reported that an adduct of dimeric dehydrated cholesterol with sulfuric acid possessing the formula of $(C_{27}H_{43})_2 \cdot 4H_2SO_4$ was isolated in Liebermann-Burchard reaction, and that the coloration might be ascribed to this cation.[2] In the coloration of cholesterol in conc. TCA medium, it may also be assumed that I or III should be converted to corresponding cation by their protonation with the strong acid and these cations should contribute to the coloration.

Experimental[9]

Media of the Color Reaction——A solution of 9 g of trichloroacetic acid liquefied by adding 1 ml of water was used as conc. TCA, a mixture of 10 ml of conc. TCA and 1 ml of hydrochloric acid ($d=1.18$) as conc. TCA–HCl (10: 1) and a mixture of 10 ml of conc. TCA and 10 ml of hydrochloric acid as conc. TCA–HCl (1: 1).

Isolation of the Reaction Products of Cholesterol in Conc. TCA——To a solution of 20 g of cholesterol in 50 ml of $CHCl_3$ was added 400 ml of conc. TCA, and the mixture was heated in a boiling water bath for 6 min with stirring. The colored reaction mixture was poured into ice water, and the resulting mixture was extracted with $CHCl_3$. The extract was washed with water, aqueous saturated solution of $NaHCO_3$ and water successively, dried over Na_2SO_4, and $CHCl_3$ was removed by evaporation. The residue was dissolved in a small amount of hexane, adsorbed to the column packed with silica gel, and eluted with hexane to separate two fractions.

From the first fraction, 1.52 g of crude 3,5-cholestadiene was obtained, and purified by rechromatography on silica gel with hexane. Recrystallization from acetone–EtOH gave colorless prisms of mp 78—79°, undepressed on admixture with the sample prepared by the method of Mauthner. *Anal.* Calcd. for $C_{27}H_{44}$: C, 87.97; H, 12.03. Found: C, 87.71; H, 12.05. UV λ_{max}^{hexane} nm (log ε): 230 (4.38), 236 (4.42), 244 (4.24).

From the second fraction, 5.4 g of cholesteryl trichloroacetate was obtained. Recrystallization from petr. ether gave colorless prisms of mp 148—149°. *Anal.* Calcd. for $C_{29}H_{45}O_2Cl_3$: C, 65.47; H, 8.53. Found: C, 65.21; H, 8.31. IR ν_{max}^{KBr} cm^{-1}: 1760 (C=O).

Hydrolysis of Cholesteryl Trichloroacetate——To a solution of 200 mg of cholesteryl trichloroacetate in 5 ml of $CHCl_3$, 10 ml of ethanolic KOH (5%) was added, and the mixture was stirred for 10 min at a room temperature. Emerging precipitate was filtered off, and the filtrate was evaporated *in vacuo*. The residue was recrystallized from EtOH to give 187 mg of white plates of mp 147—148°. IR spectrum of this plates was identical with authentic cholesterol, and the melting point was not depressed on admixture with authentic cholesterol. *Anal.* Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found: C, 83.91; H, 11.96.

Isolation of the Reaction Products of Cholesterol in Conc. TCA–HCl (10: 1) Medium——To a solution of 10 g of cholesterol in 50 ml of $CHCl_3$ was added 440 ml of conc. TCA–HCl (10: 1) medium with stirring for 90 min at a room temperature. The colored reaction mixture was treated by the similar way described above to obtain the crude product.

The crude product was dissolved in a small amount of $CHCl_3$ and submitted to column chromatography on silica gel eluted with $CHCl_3$ in order to remove unreacted cholesterol. The whole eluate was evaporated to dryness, and the residue was dissolved in a minimal volume of petr. ether with warming. After cooling in an ice bath, a yellow powder precipitated was collected by filtration, and 101 mg of crude 3,3′-bis(3,5-cholestadiene) was obtained. After purification by chromatography on alumina using benzene as an eluate, recrystallization from $CHCl_3$ then from benzene gave almost colorless needles of mp 245—247°. IR spectrum of thisneedles was identical with that of the authentic sample prepared by the Squire's method. *Anal.* Calcd. for

9) Absorption spectra were measured by Shimazu MPS-50L Spectrophotometer in a cell of 10 mm optical length, infrared (IR) spectra by JASCO IRA-1 Spectrophotometer and MS by JEOL JNS-01S Mass Spectrometer.

$C_{54}H_{86}$: C, 88.21; H, 11.79. Found: C, 87.99; H, 11.82. UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 298 (4.68), 312 (4.80), 327 (4.66). Mass Spectrum *m/e*: 734 (M^+).

The filtrate of 3,3′-bis(3,5-cholestadiene) was evaporated *in vacuo*. The residue was dissolved in a small amount of hexane and fractionated by column chromatography on silica gel with benzene. From the first fraction, 2.1 g of crude 3,5-cholestadiene was obtained and the purification was accomplished in the similar procedure described above, mp 78—79°. *Anal.* Calcd. for $C_{27}H_{44}$: C, 87.97; H, 12.03. Found: C, 87.91; H, 11.86.

From the second fraction, 416 mg of crude cholesteryl chloride was obtained as white crystals. After purification by column chromatography on alumina with hexane, recrystallization from benzene–EtOH gave white needles of mp 95—96°. IR spectrum of this needles was identical with that of the authentic sample. *Anal.* Calcd. for $C_{27}H_{45}Cl$: C, 80.05; H, 11.20. Found: C, 80.19; H, 11.17.

The third fraction gave cholesteryl trichloroacetate (403 mg), and purified by the similar way described above, mp 147—148°. *Anal.* Calcd. for $C_{29}H_{45}O_2Cl_3$: C, 65.47; H, 8.53. Found: C, 65.43; H, 8.38.

Isolation of the Reaction Products of Cholesterol in Conc. TCA–HCl (1 : 1) Medium——To a solution of 10 g of cholesterol in 50 ml of $CHCl_3$ was added 400 ml of conc. TCA–HCl (1 : 1) medium, and the mixture was stirred for 90 min at a room temperature. The colored solution was treated by the similar method to the reaction in conc. TCA, and three fractions were obtained.

From the first fraction, 1.1 g of crude 3,5-cholestadiene was obtained, and the purification was carried out by the similar procedure described above to afford colorless prisms of mp 78—79°. *Anal.* Calcd. for $C_{27}H_{44}$: C, 87.97; H, 12.03. Found: C, 87.78; H, 12.12.

From the second fraction, 190 mg of crude cholesteryl chloride was obtained. The purification was carried out by the similar procedure described above to afford white needles of mp 95—96°. *Anal.* Calcd for $C_{27}H_{45}Cl$: C, 80.05; H, 11.20. Found: C, 80.18; H, 11.17.

From the third fraction, 195 mg of crude cholesteryl trichloroacetate was obtained. Recrystallization from petr. ether afforded colorless prisms of mp 147—148°. *Anal.* Calcd. for $C_{29}H_{45}O_2Cl_3$: C, 65.47; H, 8.53. Found: C, 65.41; H, 8.42.

Reaction Product of Cholesteryl Chloride in Conc. TCA–HCl (1 : 1) Medium——To a solution of 5 g of cholesteryl chloride in 30 ml of $CHCl_3$ was added 100 ml of conc. TCA–HCl (1 : 1) medium with stirring for 3 hr at a room temperature. The reaction mixture was treated by the similar procedure described above, and the crude product was purified by a chromatographic method on alumina with hexane to obtain 3,5-cholestadiene. Recrystallization from EtOH–acetone gave colorless prisms of mp 78—79°, yield, 282 mg (5.6%). No depression in melting point was observed on admixture with the authentic sample.