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Study on the Correspondence of Color Change with Polyenyl Cation Formation of Cholesterol in Strong Acids

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A method was developed to study the correspondence of color change with structural change of the polyenyl cation in the color reaction of cholesterol in strong acid. Five unsaturated steroids having conjugated systems consisting of 3,5-diene (I), 3,5,7-triene (II), 4,6,8(14)-triene (III), 3α,5-cyclo-6,8(14)-diene (IV) and 2,4,6-triene (V), and four bisteroids having 3,3′-linkages and conjugated systems consisting of 3,3′,5-triene (VI), 3,3′,5,5′-tetraene (VII), 3,3′,5,5′,7-pentaene (VIII) and 3,3′,5,5′,7,7′-hexaene (IX) were prepared and their electronic spectra were measured in trifluoroacetic acid (TFA), in a $CHCl_3$ solution of TFA, in H_2SO_4 and in 80% H_2SO_4. The absorption maxima observed immediately after the preparation of the solution and those observed after standing for various times at room temperature were correlated with the chemical species presumed to be produced in strong acids. The following chemical conversions were found to take place: ready conversion of I to VI and VII, and of VI to VII; very rapid conversions of both II and IV to III; ready conversion of V to a dimeric molecule; conversion of III to a dimeric molecule. The color change of cholesterol in strong acid was found to be mainly due to the formation of VI and VII and to their chemical conversions.

Keywords——color reaction mechanism; cholesterol; polyenyl cation; trifluoroacetic acid; H_2SO_4; bisteroid; UV spectrum

Storong acids, especially conc. H_2SO_4, are generally used as color developing reagents in color reactions of steroids having no specific functional group suitable for color development by reaction with organic reagents. The Liebermann-Burchard, the Salkowsky and the Zak-Henly reactions of cholesterol, for example, are color reactions of this type. In every one of these reactions the reaction mixture changes color on standing.

In the Liebermann-Burchard reaction of cholesterol, Watanabe[2] reported the isolation of cholesta-3,5-diene (I), (3,3′-bicholesta)-3,3′,5-triene (VI),[3] and 3,3′-bi(cholesta-3,5-diene) (VII) as coloring compounds. In the Salkowsky reaction of cholesterol, the isolation of VI and VII was reported by Dulou *et al.*,[4a] and in the Zak-Henly reaction of cholesterol, VI was isolated by one of the authors.[5] These compounds are also known to be produced in color reactions of cholesterol using other kinds of color developing reagents. Namely, the color

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2) T. Watanabe, *Eisei Shikensho Hokoku*, **77**, 87 (1959).
3) This compound was erroneously reported to be 3,3′-bi(cholesta-2,4-diene) in several references.[2,5,7b] Chopin showed that this compound has a conjugated three double bond system, and he considered its structure to be (3,3′-bicholesta)-2′,3,5-triene from the results of ozonolysis, though this structure cannot account for the results of oxidation with N-bromo-succinimide.[4b] We found that this compound is easily derivable from I by dissolution of the latter compound in a $CHCl_3$ solution of trifluoroacetic acid, as mentioned in the text. The structure given by Chopin is inconsistent with this observation. We consider that the compound probably has the structure VI, which can explain not only our experimental result but also Chopin's result on oxidation with N-bromo-succinimide. Rearrangement of the conjugated system may account for Chopin's ozonolysis results.
4) *a*) R. Dulou, J. Chopin, and Y. Raoul, *Bull. Soc. Chim. Fr.*, **1951**, 616; *b*) J. Chopin, *ibid.*, **1956**, 258.
5) T. Momose, Y. Ueda, M. Kageura, and T. Masumura, *Chem. Pharm. Bull.*, **12**, 1500 (1964).

reactions of cholesterol with a mixture of perchoric acid, phosphoric acid and ferric chloride,[6] with a mixture of trichloroacetic acid and HCl or with the same mixture containing hydrogen peroxide,[7a] and with antimony trichloride and acetyl chloride[7b] were reported to produce I and VI, I and VII, and I and VI, respectively.

Brieskorn *et al.*[8] and Burke *et al.*[9] questioned the formation of dimeric molecules in the course of the Liebermann-Burchard and the Zak-Henly reactions of cholesterol, and claimed that intramolecular prolongation of the conjugated double bond system was a more probable mechanism of the color change observable in these reactions. However, their conclusion lacks experimental support, though the isolation of cholestapolyenes from the reaction mixture might be difficult.

In this work, the color reaction mechanism of cholesterol in strong acids was studied by synthesizing steroidal polyenes thought to be responsible for the coloration, and measuring their electronic spectra in strong acid not having oxidizing character. Measurements of the electronic spectra of synthesized polyenes having a variety of conjugated double bond systems in dilute solutions should aid in clarifying the correlation between the color and the structure of the chemical species involved in the color reactions of steroids.

Results and Discussion

Nine compounds, I—IX, were prepared and their electronic spectra were measured in strong acids at room temperature. Among them, II, III, IV, V, VIII and IX have the side chain(s) of ergosterol, but the double bond in this side chain was considered not to exert any significant influence on the color change. Compound I is considered to be produced in the initial step of the color reaction of cholesterol in strong acid and to be the starting substance in the color change. Ergosta-3,5,7,22-tetraene (II), ergosta-4,6,8 (14) ,22-tetraene (III) and ergosta-2,4,6,22-tetraene (V) are compounds having structures thought to correspond to the first step of extension of a conjugated system such as that of I. 3α,5-Cyclo-5α-ergosta-6,8 (14), 22-triene (IV) is the compound whose cation is assumed to be formed in the color reaction of ergosterol. Compounds VI, VII, (3,3′-biergosta)-24′-nor-22′-dihydro-3,3′,5,5′,7,22-hexaene (VIII) and 3,3′-bi(ergosta-3,5,7,22-tetraene) (IX) have structures thought to correspond to the extension of a conjugated system such as that of I by dimerization.

Trifluoroacetic acid (TFA) and H_2SO_4 were used as strong acids. TFA is a stronger acid than trichloroacetic acid and it dissolved all of the compounds fairly well. However, in order to carry out the determination of spectra as quickly as possible after the dissolution of compounds in TFA, the spectra of all the compounds were also determined in a $CHCl_3$ solution of TFA (2 to 1 v/v unless otherwise mentioned; hereafter this solution is written as $CHCl_3$–TFA). In practice, TFA was added to a solution of a compound in $CHCl_3$. H_2SO_4 was used in order to observed the color change in a strong acid having oxidizing character. Eighty per cent H_2SO_4 was also used in order to observed the color change in more dilute H_2SO_4 solution. The results are described below in the order of numbering of the compounds.

I——A TFA solution showed two rather broad absorption bands, one with a maximum at 424 nm and the other with a maximum at 487 nm accompanied by a shoulder at about 470 nm, immediately after the preparation of the solution. On standing, the former maximum shifted to 417 nm and then disappeared comparatively quickly, and the latter band became increasingly intense and showed a distinct maximum at 491 nm (Fig. 1-a).

6) T. Momose, Y. Ueda, M. Kageura, T. Masumura, and K. Ohta, *Chem. Pharm. Bull.*, **12**, 1415 (1964).
7) *a*) Y. Kurasawa, A. Takada, and T. Ueda, *Chem. Pharm. Bull.*, **24**, 859 (1976); *b*) *Idem, ibid.*, **24**, 487 (1976).
8) C.H. Brieskorn and H. Hofmann, *Arch. Pharm. Ber. Dtsch. Pharm. Ges.*, **297**, 577 (1964).
9) R.W. Burke, B.I. Diamondstone, R.A. Velepoldi, and O. Menis, *Clin. Chem.* **20**, 794 (1974).

A $CHCl_3$–TFA solution showed two weak and rather broad absorption bands with maxima at 428 and 496 nm immediately after the preparation of the solution. Thirty min later the band at the shorter wavelength region had become intense, with a maximum at 420 nm and the other band had become slightly more intense, with a maximum at 500 nm accompanied by a shoulder at about 560 nm. Four hr later, the former band became weak and the latter band became very intense (Fig. 1-b). In order to find the reason for this spectral change we tried to identify the structures of compounds produced in the solution on standing at room temperature for a definite time. Namely, a part of the $CHCl_3$–TFA solution was neutralized at the elapsed time when the maximum at 420 nm was most intense. Another part of the same solution was neutralized at the elapsed time when the maximum at 500 nm was most intense. The ultraviolet spectrum of the $CHCl_3$ layer of the former reaction mixture had two bands, one composed of three maxima at 271, 281 and 292 nm and the other composed of three maxima at 299, 313 and 323 nm, with an intensity ratio of the two bands of about 3 to 1. The former and the latter bands clearly correspond to the absorption bands of VI and VII in $CHCl_3$, respectively. This indicates the conversion of I to VI and VII in $CHCl_3$–TFA. The infrared spectrum of the crystals obtained from the latter reaction mixture was identical with that of VII.

The structure of the protonated species of I is considered likely to be either X or XI. However, it is obvious that the length of the conjugated system of either of these species is not long enough to accout for the absorption bands of I mentioned above.[10] This means that the conjugated system of I must be extended by the action of strong acid. The detection of VI is convincing evidence that the absorption band of I having a maximum at 420 nm in $CHCl_3$–TFA is the absorption band of the protonated form of VI, because VI was found to show an absorption maximum at the same wavelength in $CHCl_3$–TFA. The isolatoin of VII similarly indicated that the absorption band having a maximum at 500 nm (or at 491 nm in TFA) is the absorption band of the protonated form of VII, because VII was found to show an absorption maximum at 500 nm in $CHCl_3$–TFA (or at 491 nm in TFA). The existence

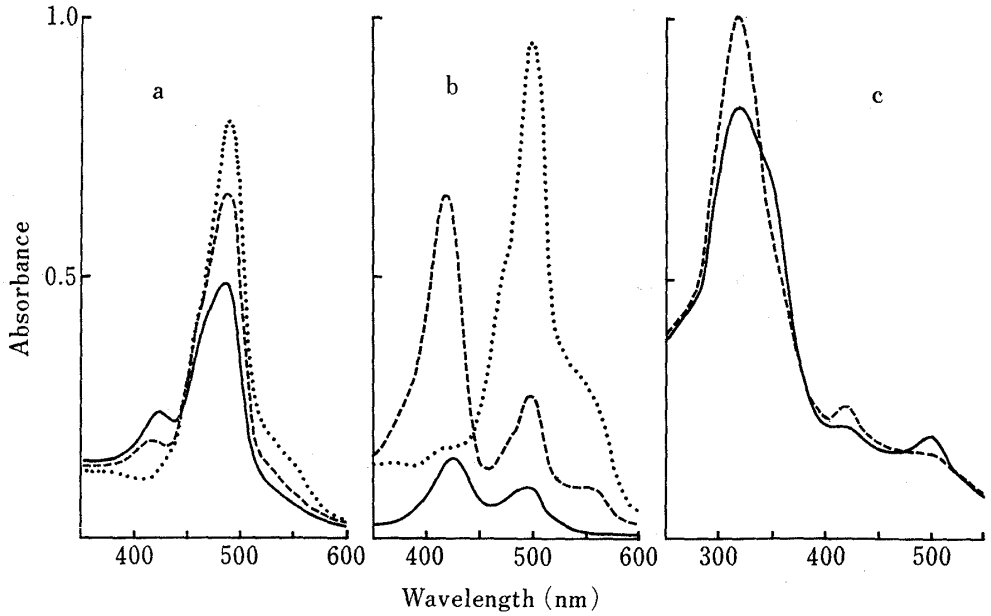

Fig. 1. Spectral Changes of I in Strong Acid

(a) 3.8×10^{-5} M (TFA), ——: 1 min, – – –: 20 min, ……: 3 hr.
(b) 8.7×10^{-5} M ($CHCl_3$–TFA), ——: 1 min, – – –: 30 min, ……: 4 hr.
(c) 3.8×10^{-4} M (H_2SO_4), ——: 3 min, – – –: 5 hr.

10) N.C. Deno, "Carbonium Ions," Vol. 2, ed. by G.A. Olah and P. von R. Schleyer, Wiley-Interscience, New York, 1970, p. 738.

of a shoulder at about 560 nm in the spectrum of a $CHCl_3$–TFA solution kept for 4 hr supports this view, because VII was found to show an absorption maximum at 560 nm in $CHCl_3$–TFA on standing. The above findings provide conclusive evidence that the main reason for the color change of a TFA or a $CHCl_3$–TFA solution of I is the formation of the dimeric molecules VI and VII.

An H_2SO_4 solution showed a broad absorption band having a maximum at 320 nm and a shoulder at about 340 nm. On standing, the band became somewhat stronger and lost its shoulder, while a new weak absorption band having a maximum at 420 nm appeared (Fig. 1-c). The chemical species absorbing at 320 nm is presumably either X or XI.

II and III——Compounds II and III showed identical spectral changes in all kinds of strong acid employed, so only the spectral change of II is described.

A TFA solution showed an intense absorption band having a maximum at 453 nm immediately after the preparation of the solution. On standing, this band became gradually weaker and a broad absorption band appeared having a maximum at 498 nm accompanied by a shoulder at about 520 nm (Fig. 2-a).

A $CHCl_3$–TFA solution showed a similar spectral change. Namely, the spectrum determined immediately after the preparation of the solution had an intense absorption maximum at 458 nm. Ten min later this band was lost and a rather weak and broad absorption band having a maximum at 511 nm was observed (Fig. 2-b). A $CHCl_3$–TFA solution was neutralized immediately after the preparation of the solution . The ultraviolet spectrum of the $CHCl_3$ layer of the resulting reaction mixture was identical with that of a $CHCl_3$ solution of III. This means that II was converted to III very quickly in $CHCl_3$–TFA, and also that the chemical species absorbing at 458 nm in $CHCl_3$–TFA (or at 453 nm in TFA) is the polyenylic cation (XII) derivable from both II and III. A $CHCl_3$–TFA solution was also neutralized at the elapsed time when the solution showed only one absorption band having a maximum at 511 nm. The ultraviolet absorption spectrum of the $CHCl_3$ layer of the resulting reaction mixture had an intense absorption maximum at 290 nm and a rather weak absorption maximum at 347 nm, and the ratio of their absorbances was about 3 to 2. The wavelength and the shape of the former band coincide with those of the absorption spectrum of a $CHCl_3$ solution of III. Another absorption maximum at 347 nm must be due to compound(s) having a conjugated system longer than that of III, as predicted by the Woodward or the Fieser and Fieser rule. The mass spectrum of the residue left on concentration of the above-mentioned $CHCl_3$ layer had a peak (*m/e* 756) assignable to a dimeric molecule ($C_{56}H_{84}$), but it was very weak. The high-speed liquid chromatogram (HLC) of the same residue had four peaks, and the most abundant one was found to correspond to the peak of III. These results mean that the compounds responsible for the absorption maximum at 511 nm in $CHCl_3$–TFA are mainly monomeric molecule including III as the major component. If the conjugated system of XII is presumed to shift to form XIII by the action of strong acid, these results can be understood on the basis of the results described below for IV.

An H_2SO_4 solution showed an intense absorption maximum at 502 nm accompanied by a shoulder at about 470 nm immediately after the preparation of the solution. On standing, this band gradually became weak (Fig. 2-c).

An 80% H_2SO_4 solution first showed a broad absorption band having a maximum at 454 nm accompanied by a weak shoulder at about 500 nm. On standing, the absorption maximum was lost and the shoulder became an intense absorption band having a maximum at 502 nm accompanied by a shoulder at about 470 nm (Table I). The maxinum at 454 nm is considered to be due to the chemical species XII. An aliquot of the solution was neutralized at the elapsed time when the absorption maxima at 454 and 502 nm showed almost the same intensity, and another aliquot of the same solution was neutralized at the elapsed time when the solution showed only the absorption maximum at 502 nm. The mass spectrum of the $CHCl_3$ extract of the former reaction mixture did not have any peak assignable to a dimeric molecule.

However, the mass spectrum of the $CHCl_3$ extract of the latter reaction mixture had a very weak peak (*m/e* 756) assignable to a dimeric molecule ($C_{56}H_{84}$). Therefore, the absorption maximum at 502 nm is considered to be due mainly to protonated species of monomeric molecules. A hexane solution of II was shaken vigorously with H_2SO_4 for 1 min, then the mixture was neutralized. The hexane layer showed an absorption band consisting of three maxima at 332, 351 and 370 nm. This spectrum resembles fairly well both the spectrum of an Et_2O solution of cholesta-4,6,8,11-tetraen-3β-ol[11] and that of an EtOH solution of ergosta-3,5,7,9 (11), 22-pentaen-3β-yl acetate.[12] This suggests that the chemical species responsible for the absorption maximum at 502 nm in both H_2SO_4 and 80% H_2SO_4 are species such as XIV and XIV'.

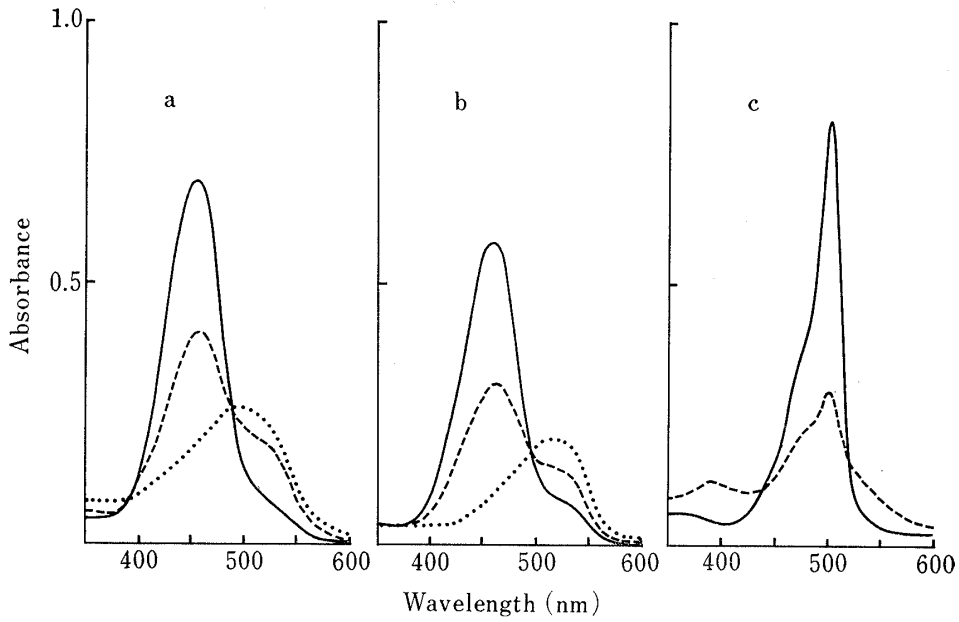

Fig. 2. Spectral Changes of II in Strong Acid

(a) 3.1×10^{-5} M (TFA), ——: 1 min, ----: 1 hr, ·····: 6 hr.
(b) 4.3×10^{-4} M ($CHCl_3$–TFA), ——: 30 sec, ----: 3 min, ·····: 10 min.
(c) 3.1×10^{-5} M (H_2SO_4), ——: 1 min, ----: 12 hr.

TABLE I. Spectral Changes of II, IV and V in 80% H_2SO_4

Compound	Concentration (M)	Standing time	λ_{max} nm (A)
II	3.1×10^{-5}	3 min	454 (0.25), 500 (0.10)
		7 hr	470 (0.24),[a] 502 (0.65)
IV	4.1×10^{-4}	7 min	— 501 (0.15), 577 (0.10)
		5 hr	390 (0.35), 501 (0.10), 577 (0.25)
V	9.5×10^{-5}	1 min	470 (0.15),[a] 502 (0.25)
		3 hr	470 (0.40),[a] 502 (0.80)

a) Shoulder.

IV——A TFA solution showed an absorption band having a maximum at 504 nm accompanied by a shoulder at about 520 nm. This band resenbles that of a TFA solution of II determined after standing. On standing, the intensity of the absorption band decreased gradually and its maximum shifted to 495 nm (Fig. 3-a).

11) A. Windaus, U. Riemann, and G. Zuhlsdorff, *Ann.*, **552**, 135 (1942).
12) I.M. Heilbron, T. Kennedy, F.S. Spring, and G. Swain, *J. Chem. Soc.*, **1938**, 869.

A $CHCl_3$–TFA solution showed an intense absorption band having a maximum at 513 nm, which is fairly close to that of a $CHCl_3$–TFA solution of II determined after standing. On standing, the solution became colorless rather quickly (Fig. 3-b). A $CHCl_3$–TFA solution was neutralized immediately after the preparation of the solution. The $CHCl_3$ layer of the resulting reaction mixture showed an absorption band having a maximum at 290 nm, and the wavelength and shape of this band coincided with those of a $CHCl_3$ solution of III. This means that IV was converted to III very quickly by the action of strong acid. This conversion is considered to take place through a protonated species of IV. This species must not only be responsible for the absorption maximum at 513 nm in $CHCl_3$–TFA (or at 504 nm in TFA), but must also be deprotonated easily to give III. Therefore, its structure is thought to be XIII.

An H_2SO_4 solution showed an intense absorption maximum at 502 nm accompanied by a shoulder at about 470 nm. On standing, the intensity of this band decreased gradually (Fig. 3-c). The spectrum and its change resemble those of the H_2SO_4 solution of II.

An 80% H_2SO_4 solution did not have any appreciable color immediately after its preparation, but on standing three rather weak absorption bands appeared having maxima at 390, 501 and 577 nm (Table I). This result was quite different from that observed in the case of an 80% H_2SO_4 solution of II. The mass spectrum of the $CHCl_3$ extract of an 80% H_2SO_4 solution neutralized at the elapsed time when the maximum at 577 nm was most intense had a major peak at *m/e* 756, which corresponds to the parent peak of a dimeric molecule ($C_{56}H_{84}$).

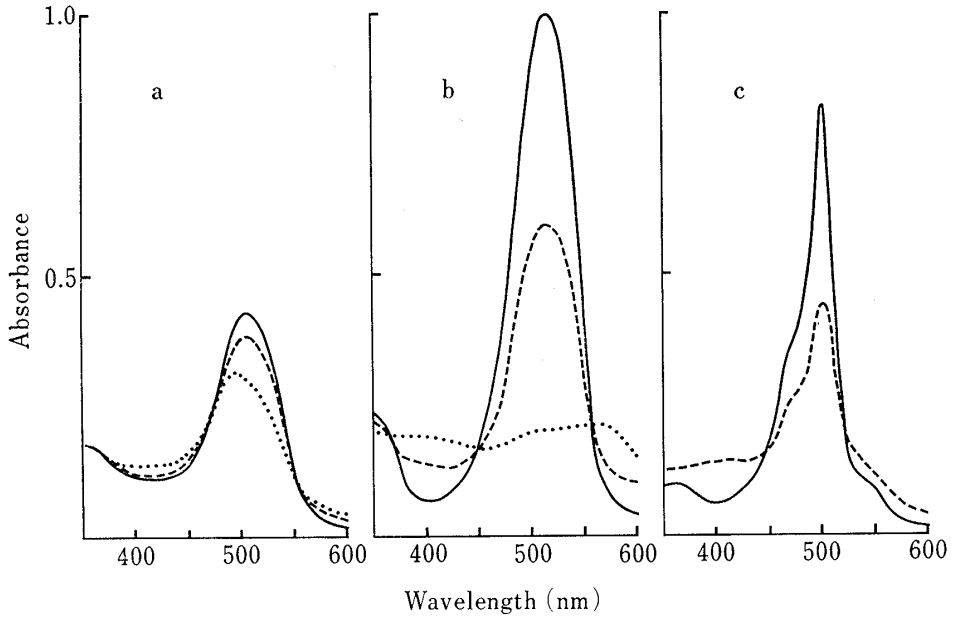

Fig. 3. Spectral Changes of IV in Strong Acid

(a) 4.1×10^{-5} M (TFA), ——: 1 min, ----: 4 hr, ·····: 22 hr.
(b) 6.6×10^{-5} M ($CHCl_3$–TFA), ——: 1 min, ----: 10 min, ·····: 3 hr.
(c) 2.1×10^{-5} M (H_2SO_4), ——: 1 min, ----: 13 hr.

V——A TFA solution showed a broad absorption band having three maxima at 532, 576 and 645 nm accompanied by a shoulder at about 500 nm immediately after the preparation of the solution. On standing, the absorption maximum at 645 nm became gradually more intense, the absorption maxima at 532 and 577 nm and the shoulder at about 500 nm became gradually weaker, and a new absorption band having a maximum at 745 nm appeared (Fig. 4-a).

A $CHCl_3$–TFA solution showed a rather intense absorption band having a maximum at 588 nm accompanied by a shoulder at about 540 nm and a rather weak absorption band hav-

ing a maximum at 658 nm immediately after the preparation of the solution. On standing, the band at 658 nm increased in intensity fairly quickly, the band at 588 nm decreased in intensity rather slowly and the shoulder changed to an absorption band having a maximum at 540 nm and then it was gradually lost (Fig. 4-b).

A solution of V in a mixture of $CHCl_3$ and TFA (6: 1, v/v), which showed two absorption maxima at 589 and 659 nm immediately after the preparation of the solution ,was neutralized at the elapsed time when the maximum at 659 nm became most intense. The crystals obtained by repeated recrystallization of the residue left on concentration of the $CHCl_3$ layer of the reaction mixture had the elemental composition $C_{56}H_{84}$, corresponding to a dimeric molecule. A *n*-heptane solution of these crystals showed two absorption maxima at 352 and 369 nm. This result indicates that the dimeric molecule must have a conjugated five-double-bond system. These crystals showed an absorption maximum at 589 nm in a $CHCl_3$ solution of TFA (6: 1, v/v) immediately after the preparation of the solution . Therefore, the absorption maximum of V at 588 nm in $CHCl_3$–TFA or at 589 nm in a $CHCl_3$ solution of TFA (6: 1, v/v) appeared to be due to the protonated species of dimeric molecule mentioned above. Despite the experimental fact that the solution of V was neutralized at the elapsed time when the absorption maximum at 659 nm was most intense, as mentioned before, no compound(s) showing the absorption maximum at 659 nm in a $CHCl_3$ solution of TFA (6: 1, v/v) could be isolated as crystals.

An H_2SO_4 solution showed an intense absorption band having a maximum at 501 nm accompanied by a shoulder at about 470 nm. The wavelength and the shape of the band are very similar to those of the spectra of H_2SO_4 solutions of II, III and IV. Accordingly, it appears that all four compounds may produce the same chemical species on dissolution in H_2SO_4. On standing, both bands became weak (Fig. 4-c).

In contrast to the cases of II and III, an 80% H_2SO_4 solution did not show any band at 454 nm but showed a weak absorption maximum at 502 nm accompanied by a shoulder at about 470 nm immediately after the preparation of the solution (Table I). On standing, this absorption band became intense.

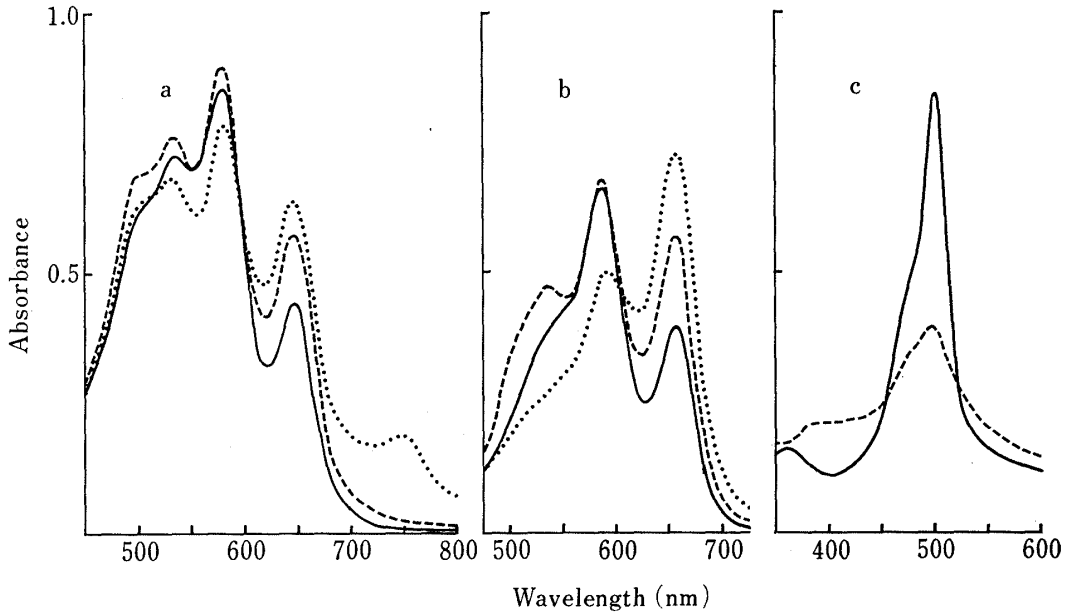

Fig. 4. Spectral Changes of V in Strong Acid

(a) 9.5×10^{-5} M (TFA), ——: 1 min, – – –: 30 min, ·····: 15 hr.
(b) 1.4×10^{-4} M ($CHCl_3$–TFA), ——: 1 min, – – –: 5 min, ·····: 2 hr.
(c) 5.7×10^{-5} M (H_2SO_4), ——: 1 min, – – –: 5 hr.

VI——A TFA solution showed an intense absorption band having a maximum at 413 nm and two very weak absorption bands having maxima at 464 and 491 nm at 15 min after the preparation of the solution. On standing, the band at 413 nm disappeared and the other two bands combined to form an intense absorption band having two maxima at 464 and 491 nm. The maximum at 464 nm was more intense than that at 491 nm. On prolonged standing, the maximum at 464 nm became rather weak and the maximum at 491 nm became more intense (Fig. 5-a).

A $CHCl_3$–TFA solution showed an intense absorption band having a maximum at 420 nm immediately after the preparation of the solution. On standing, this band gradually became weaker and disappeared by 20 hr, while an intense absorption band having a maximum at 500 nm accompanied by two shoulders at about 470 and 560 nm appeared (Fig. 5-b). The maximum at 420 nm can be considered to be due to the protonated species of VI, namely XV, for the reasons given in the case of I. The solution was neutralized when the maximum at 500 nm became most intense. The residue obtained by concentration of the $CHCl_3$ layer of the reaction mixture was found to contain a considerable amount of VII. Since VII showed an absorption maximum at 500 nm in $CHCl_3$–TFA (or at 491 nm in TFA), as mentioned later, the spectral change of VI from 420 to 500 nm in $CHCl_3$–TFA (or from 413 to 491 nm in TFA) may be attributable to a chemical change from the protonated species of VI to that of VII, namely from XV to XIX. The spectral change of VI in TFA mentioned before suggests that the maximum at 464 nm is due to an intermediate chemical species in the transformation from XV to XIX, *e. g.* perhaps XVIII.

An H_2SO_4 solution showed an intense absorption band having a maximum at 408 nm and a very weak absorption band having a maximum at about 530 nm at 4 min after the preparation of the solution. On standing, the former band became gradually weaker (Fig. 5-c). The chemical species absorbing at 408 nm is considered to be XV.

Compound VI is scarcely soluble in 80% H_2SO_4.

VII——A TFA solution showed an intense absorption band having a maximum at 491 nm at 10 min after the preparation of the solution. This spectrum was found to change slowly, and at 30 hr it had two absorption bands with maxima at 491 and 550 nm (Fig. 6-a).

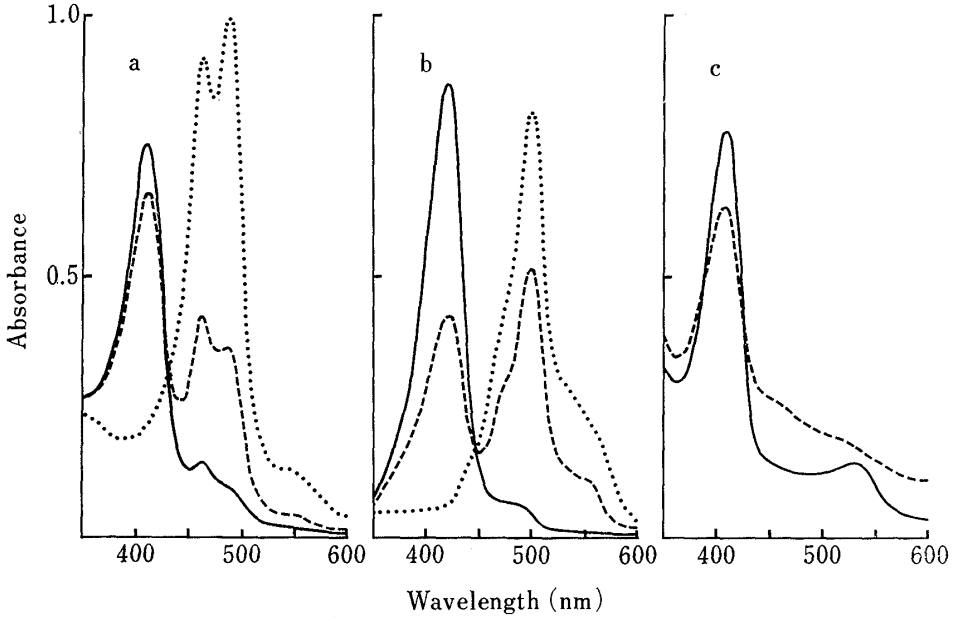

Fig. 5. Spectral Changes of VI in Strong Acid[a)]

(a) — (TFA), ——: 15 min, – – –: 1 hr, ·····: 5 hr.
(b) 1.8×10^{-5} M ($CHCl_3$–TFA), ——: 1 min, – – –: 3 hr, ·····: 20 hr
(c) — (H_2SO_4), ——: 4 min, – – –: 20 hr.
a) In cases (a) and (c) the concentrations of VI could not be calculated (see "Experimental").

A $CHCl_3$–TFA solution showed an intense absorption band having a miximum at 500 nm accompanied by a shoulder at about 470 nm and a weak absorption band having a maximum at 560 nm at 2 min after the preparation of the solution. On standing, the former band became weaker and the latter band became more intense (Fig. 6-b).

An H_2SO_4 solution showed an intense absorption band having a maximum at 494 nm at 2 min after the preparation of the solution. Two hr later, the spectrum had changed to show a very broad band having a maximum at 455 nm (Fig. 6-c). The chemical species absorbing at 500 nm in $CHCl_3$–TFA (or at 491 nm in TFA, or at 494 nm in H_2SO_4) is considered to be the protonated species of VII, namely XIX.

Compound VII is scarcely soluble in 80% H_2SO_4.

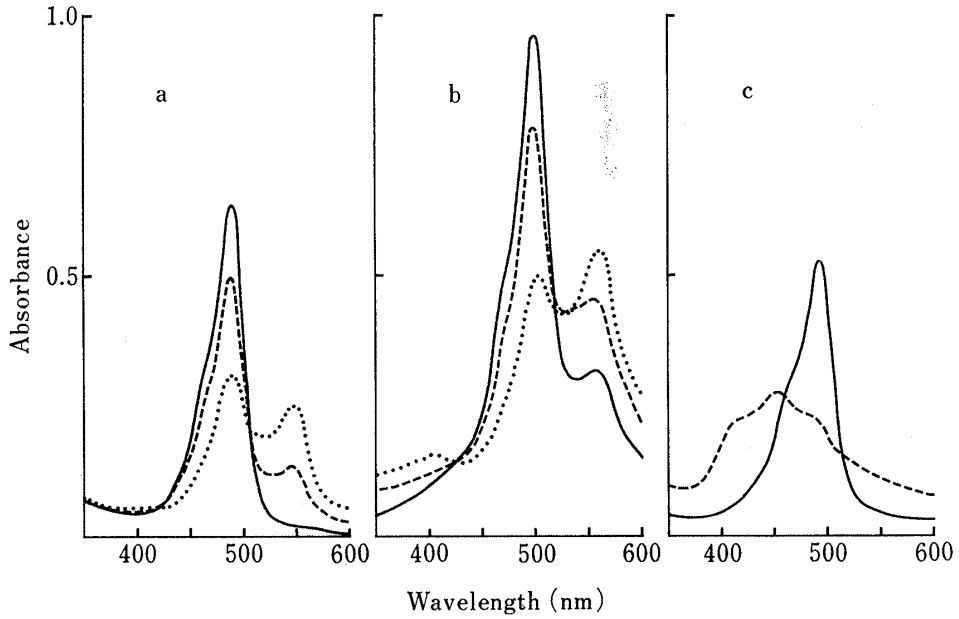

Fig. 6. Spectral Changes of VII in Strong Acid[a)]

(a) — (TFA), ——: 10 min, – – –: 10 hr, ·····: 30 hr.
(b) 2.3×10^{-5} M ($CHCl_3$–TFA), ——: 2 min, – – –: 20 min, ·····: 1.5 hr.
(c) 3.1×10^{-5} M (H_2SO_4), ——: 2 min, – – –: 2 hr.
a) In case (a) the concentration of VII could not be calculated (see "Experimental").

VIII——A TFA solution showed three absorption bands having maxima at 490, 580 and 631 nm at 2 min after the preparation of the solution; the band at 631 nm was most intense. On standing, the bands at 490 and 580 nm scarcely changed, but the band at 631 nm became more intense (Fig. 7-a).

A $CHCl_3$–TFA solution showed an intense absorption band having a maximum at 646 nm and two weak absorption bands having maxima at 500 and 590 nm immediately after the preparation of the solution. On standing, the band at 646 nm slowly became more intense and the other two bands became weaker (Fig. 7-b).

Compound VIII showed a much more rapid spectral change on standing in a dilute $CHCl_3$ solution of TFA. For example, VIII showed two absorption bands of almost the same intensity at 587 and 648 nm in a dilute $CHCl_3$ solution of TFA (25: 1, v/v) immediately after the preparation of the solution, while 2 hr later the former band was weak and the latter band was intense.

A $CHCl_3$–TFA solution was neurtalized immediately after the preparation of the solution. The ultraviolet spectrum of the $CHCl_3$ layer of the reaction mixture was essentially the same as that of a solution of VIII in $CHCl_3$. This result and the presence of two bands having almost the same intensity in the initial spectrum determined in a dilute $CHCl_3$ solution of TFA suggest that the chemical species absorbing at 590 and 646 nm in $CHCl_3$–TFA are protonated species

of VIII, *i. e.*, XX and XXI. XX has a homoannular diene system while XXI does not, so XX and XXI may be the species absorbing at 646 and 590 nm, respectively. The absorption maximum at 500 nm in $CHCl_3$–TFA coincides with that observed in the spectrum of VII in the same solvent. Therefore, it may be assumed that the chemical species absorbing at this wavelength has a conjugated double bond system similar to that of XIX, for example XIX′.

An H_2SO_4 solution showed a very broad absorption band having a maximum at 670 nm accompanied by a shoulder at about 620 nm and a weak absorption band having a maximum at 492 nm. This spectrum did not show any marked change on standing (Fig. 7-c).

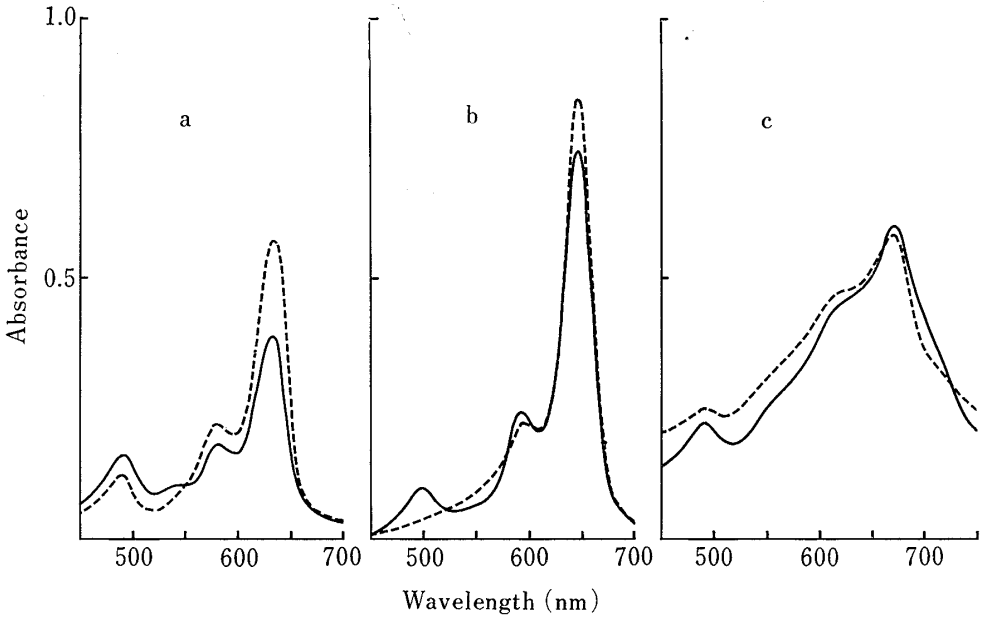

Fig. 7. Spectral Changes of VIII in Strong Acid

(a) 9.8×10^{-6} M (TFA), ——: 2 min, ----: 10 hr.
(b) 8.3×10^{-6} M ($CHCl_3$–TFA), ——: 1 min, ----: 12 hr.
(c) 2.0×10^{-5} M (H_2SO_4), ——: 3 min, ----: 1.5 hr.

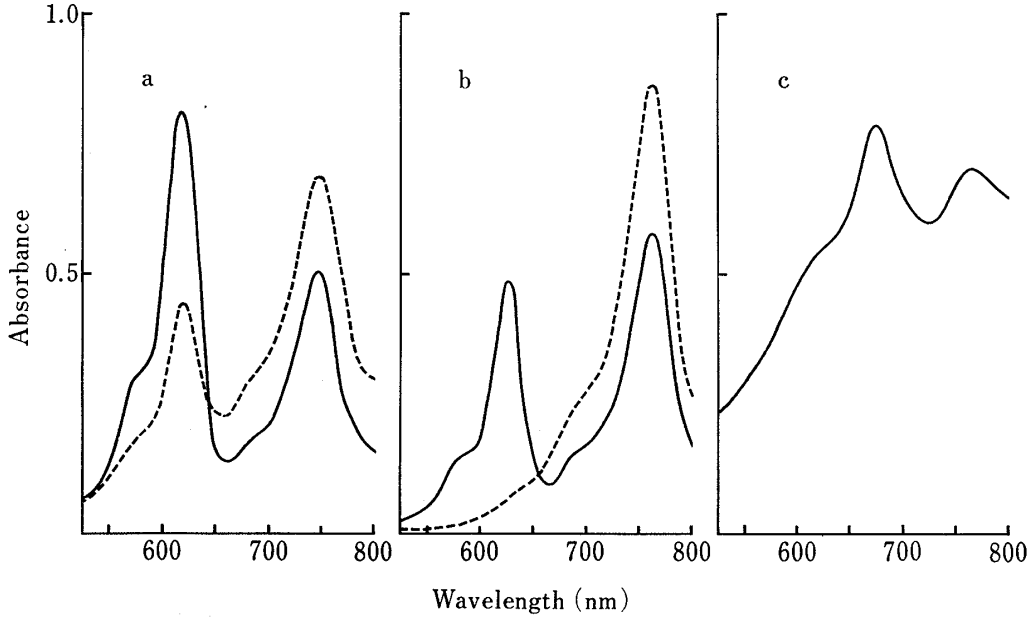

Fig. 8. Spectral Changes of IX in Strong Acid

(a) 9.1×10^{-6} M (TFA), ——: 5 min, ----: 4 hr.
(b) 8.4×10^{-6} M ($CHCl_3$–TFA), ——: 1 min, ----: 5hr.
(c) 1.5×10^{-5} M (H_2SO_4), ——: 1 min.

Compound VIII is scarcely soluble in 80% H_2SO_4.

IX——A TFA solution showed an intense absorption band having a maximum at 618 nm accompanied by a shoulder at about 580 nm and an absorption band having a maximum at 746 nm accompanied by a shoulder at about 680 nm at 5 min after the preparation of the solution. On standing, the former band became weak and the latter band became intense (Fig. 8-a).

A $CHCl_3$–TFA solution showed two absorption bands of almost equal intensity, one with a maximum at 629 nm accompanied by a shoulder at about 580 nm and the other with a maximum at 763 nm accompanied by a shoulder at about 690 nm, immediately after the prepration of the solution. On standing, the former band disappeared and the latter band became intense (Fig. 8–b). The chemical species absorbing at 763 nm is presumed to have a structure such as XXII. The chemical species absorbing at 629 nm is considered to have a shorter conjugated double bond system than XXII.

An H_2SO_4 solution showed a very broad absorption band having two maxima at 672 and 765 nm and a shoulder at about 620 nm immediately after the preparation of the solution (Fig. 8-c).

Conclusion

The absorption maxima observed and the postulated structures (shown in parentheses) of the protonated species of all the compounds employed (except for V) can be summarized as follows:

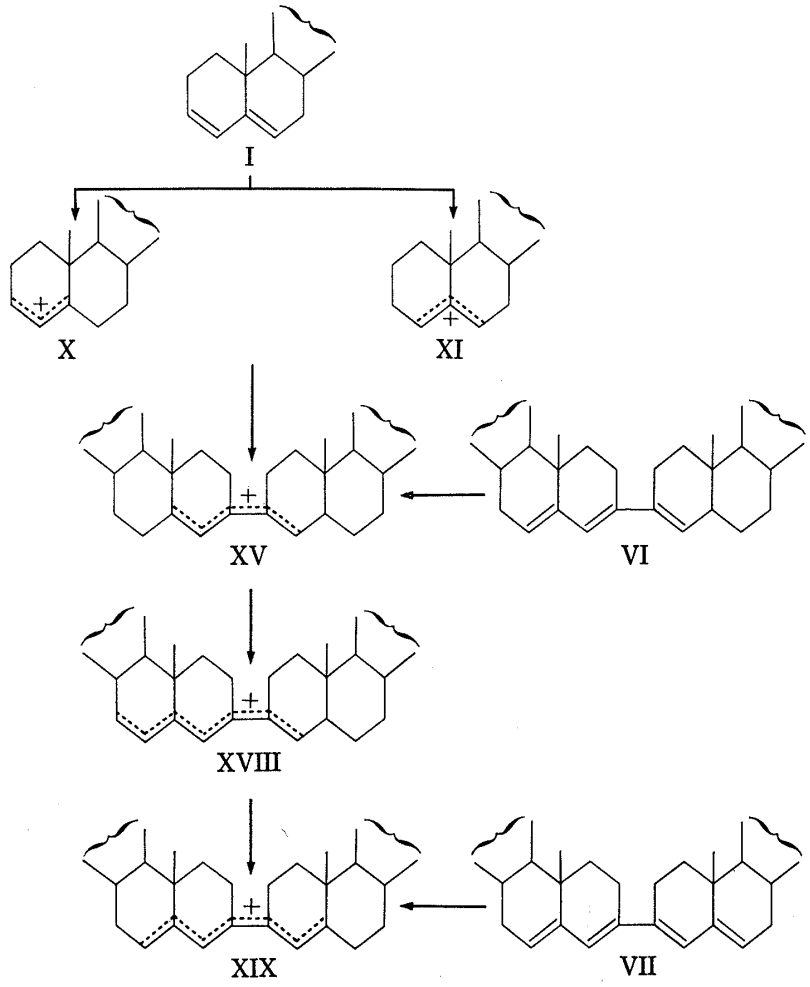

Chart 1

I: 320 nm in H_2SO_4 (X or XI)
II and III: 453 nm in TFA (XII)
IV: 504 nm in TFA (XIII)
VI: 413 nm in TFA (or 408 nm in H_2SO_4) (XV)
VII: 491 nm in TFA (or 494 nm in H_2SO_4) (XIX)
VIII: 580 and 631 nm in TFA (XXI and XX)
IX: 746 nm in TFA (XXII)

Chemical conversions and dimerizations of monomeric molecules in strong acid were found to take place as follows:

Compound I dimerizes fairly easily to form VI and VII.
Compound II is converted to III very quickly.
Dimerization of III (and II) takes place to a small extent.
Compound IV is converted to III very quickly.
Compound V dimerizes fairly easily to form a compound having a conjugated five-double-bond system.
Compound VI is converted to VII rather slowly.

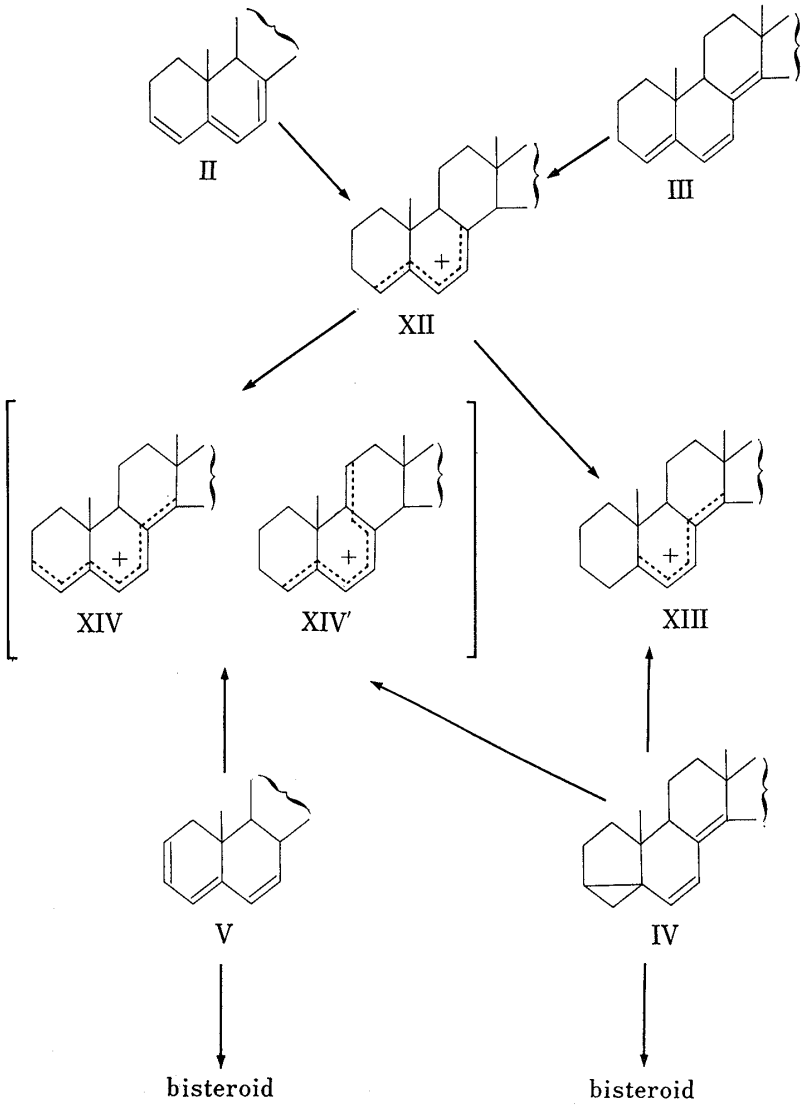

Chart 2

The principal relationships between spectral changes of compounds and presumed structural changes of protonated species in strong acid can be summarized as shown in Charts 1, 2 and 3.

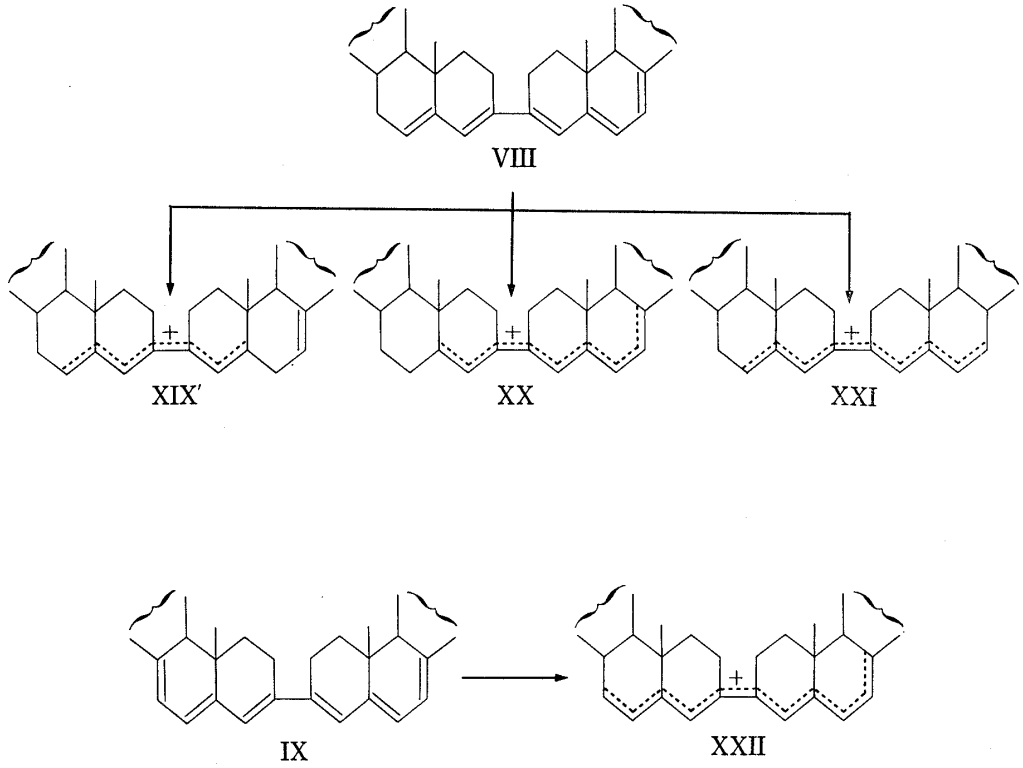

Chart 3

From the results mentioned so far, the color change of cholesterol initiated by the formation of protonated species of I in strong acid can be considered to be mainly due to the formation of protonated species of dimeric molecules, VI and VII, and to their chemical conversions. Thus, the iaolation of VI and VII from reaction mixtures of the Lieberman-Burchard, the Salkowsky and the Zak-Henly reactions of cholesterol can now be readily understood, because these reactions are thought to be initiated by the formation of protonated species of I. Colors developed by these reactions of cholesterol can be considered to be due to the protonated species of dimeric molecules having conjugated systems longer than those of VI and VII.

Experimental

Electronic spectra were recorded (scanning speed: 10 nm/sec) on Union SM 401 and Hitachi 556 spectrometers at room temperature. Mass spectra were recorded on a JMS-OISG spectrometer. High-speed liquid chromatography was carried out on a Hitachi 635 liquid chromatograph equipped with both a UV monitor and a Union SM 303 spectrometer. Melting points are uncorrected.

Preparation of Solutions in Strong Acid——A solution of a compound in EtOH (I, II, III, IV and V) or in *n*-heptane (VI, VII, VIII and IX) was evaporated to dryness *in vacuo*, and TFA or H_2SO_4 or 80% H_2SO_4 was added to the residue. In the cases of VI and VII, which cannot be dissolved in strong acid in a short time, remaining insoluble material was removed by filtration. $CHCl_3$–TFA solutions were made by adding one volume of TFA to two volumes of a $CHCl_3$ solution of a compound.

Reagents and Materials——TFA of guaranteed reagent grade and $CHCl_3$ for spectroscopic analysis were purchased from Wako Pure Chemical Industry, Ltd. and Tokyo Kasei Kogyo Co. Ltd., respectively. H_2SO_4 for quantitative analysis of cholesterol was purchased from Ishizu Pharmaceutical Co. Ltd.. All other chemicals used were of guaranteed reagent grade, and were purified by distillation or recrystallization from

suitable solvents before use. Compounds I,[13] II,[12] III,[14] IV,[14,15] VI,[5] and VII[16] were prepared by the cited methods.

Syntheses of V, VIII and IX——A solution of $LiAlH_4$ (230 mg) in Et_2O (180 ml) was added gradually to a solution of ergosta-4,6,22-trien-3-one (2.2 g) in dry Et_2O (100 ml), and the mixture was refluxed for 2 hr. After the decomposition of excess $LiAlH_4$ by adding Et_2O saturated with H_2O, an aq. saturated solution of potassium sodium tartrate was added. The Et_2O layer was washed with H_2O and dried over anhyd. Na_2SO_4. The crystals left on removal of the solvent were recrystallized from acetone to afford colorless needles of ergosta-4,6,22-trien-3ξ-ol (1.8 g). A solution of this substance (700 mg) in Ac_2O (18 ml) was treated with NaOAc (700 mg), and the reaction mixture was refluxed in an oil-bath under an atmosphere of N_2 for 2.5 hr. After cooling, the reaction mixture was poured into an ice-cold aq. solution of $NaHCO_3$. The separated crystals were collected by filtration, washed with H_2O and dried *in vacuo* over silica gel. An *n*-hexane solution of the crystals was subjected to chromatographic separation on Al_2O_3 using *n*-hexane as an eluant. Recrystallization from EtOH of the crystals left on removal of the solvent from the first fraction afforded colorless prisms of V, mp 86—88°. *Anal.* Calcd for $C_{28}H_{44}$: C, 88.82; H, 11.18. Found: C, 88.52; H, 11.27. UV λ_{max}^{EtOH} nm (ε): 290 (sh), 315 (15800), 340 (10340). MS *m/e*: 378 (M^+).

To a solution of cholesta-4-en-3-one (2.39 g) and ergosta-4,7,22-trien-3-one (3.0 g) in a mixture of HOAc (220 ml) and propylalchol (220 ml), 3% Na(Hg) (700 g) was added gradually with stirring on an ice bath for 4 hr. The reaction mixture was heated in a boiling water bath for a few min, then the mixture was poured into ice-water. The separated amorphous precipitate was collected by filtration, washed with H_2O and dried *in vacuo* over silica gel. The product was washed with acetone and dissolved in cyclohexane, and the solution was subjected to chromatographic separation on Al_2O_3 using cyclohexane as an eluant. A mixture of Ac_2O (20 ml) and HOAc (20 ml) was added to a benzene solution of the residue (*ca.* 400 mg) left on removal of the solvent from the first fraction, and the reaction mixture was refluxed under an atmosphere of N_2 for 3 hr. The reaction mixture was poured into an aq. $NaHCO_3$ solution and the product was extracted with benzene. The benzene layer was washed with H_2O, dried over anhyd. Na_2SO_4 and concentrated *in vacuo*. Repeated recrystallization of the residue from benzene afforded pale yellow needles of VIII, mp 287—289° (in an evacuated capillary). *Anal.* Calcd for $C_{55}H_{84}$: C, 88.64; H, 11.36. Found: C, 88.70; H, 11.32. UV $\lambda_{max}^{n\text{-heptane}}$ nm (ε): 348 (38600), 365 (56100), 386 (47500). MS *m/e*: 744 (M^+).

To a solution of ergosta-4,7,22-trien-3-one (2 g) in a mixture of HOAc (115 ml) and propylalchol (115 ml), 3% Na(Hg) (360 g) was added gradually with stirring on an ice bath for 2 hr. The reaction mixture was heated in a boiling water bath for a few min, then the mixture was poured into ice-water. The separated amorphous precipitate was collected by filtration, washed with H_2O and dried *in vacuo* over silica gel. The residue was washed with acetone and dissolved in $CHCl_3$ (20 ml). The solution was refluxed with a mixture of Ac_2O (10 ml) and HOAc (10 ml) under an atmosphere of N_2 for 5 hr. Upon cooling, the product (500 mg) separated as crystals. Repeated recrystallization from benzene afforded yellow needles of IX, mp 303—305° (in an evacuated capillary). *Anal.* Calcd for $C_{56}H_{82}$: C, 89.06; H, 10.95. Found: C, 89.02; H, 10.96. UV $\lambda_{max}^{n\text{-heptane}}$ nm (ε): 377 (53800), 400 (95800), 426 (94500). MS *m/e*: 754 (M^+).

General Procedure of the Detection of Chemical Species dissolved in Strong Acid by Ultraviolet Spectroscopy——a) For the detection of compounds immediately after preparation: A solution of a compound (II, IV and VIII) in $CHCl_3$ was shaken vigorously with TFA for about 15 sec, then the reaction mixture was neutralized with an aq. $NaHCO_3$ sclution. The $CHCl_3$ layer was washed with H_2O, dried over anhyd. Na_2SO_4 and subjected to the measurement of the ultraviolet spectrum. In the case of II, detection was also performed as follows. An *n*-hexane solution of II was shaken vigorously with H_2SO_4 for 1 min. The reaction mixture was treated as described above.

b) For the detection of compounds after standing for a definite time: A $CHCl_3$–TFA solution of a compound was kept standing at room temperature for a definite time (I, 20 min, 2 hr; II, 10 min; VI, 10 hr), then the solution was treated as described above. The $CHCl_3$ layer was subjected to measurement of the ultraviolet spectrum. The residue left on removal of the solvent from the $CHCl_3$ layer was subjected to measurement of the mass spectrum. In the case of II, the $CHCl_3$ layer was evaporated to dryness and the oily residue was separated by HLC using a 50 cm stainless steel column (I.D., 2.1 mm) packed with Hitachi gel 3011. The operating conditions were as follows: eluant, 33% 2,2,4-trimethylpentane in MeOH; flow rate, 1.0 ml/min (100 kg/cm²); sensitivity, 0.32 AUFS. The chromatogram showed four peaks at retention times of 4.5, 6.0, 9.3 and 13.1 min. The ultraviolet spectra of all effluent fractions were recorded on a Union SM 303 spectrometer.

An 80% H_2SO_4 solution of II or IV was kept standing at room temperature for a definite time (II, 10 min, 3 hr; IV, 2 hr), then the solution was neutralized with an aq. $NaHCO_3$ solution, and substances produced were extracted with $CHCl_3$. The $CHCl_3$ layer was treated as described above.

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Identification of Chemical Species dissolved in Strong Acid by the Isolation of Crystalline Product——TFA (I, 25 ml; V, 100 ml; VI, 25 ml) was added to a solution of a compound (I, 300 mg; V, 60 mg; VI, 100 mg) in $CHCl_3$ (I, 50 ml; V, 600 ml; VI, 150 ml), and the mixture was kept standing at room temperature for a definite time (I, 2 hr; V, 1.5 hr; VI, 10 hr), then the solution was neutralized with aq. $NaHCO_3$ solution. The $CHCl_3$ layer was washed with H_2O, dried over anhyd. Na_2SO_4 and concentrated *in vacuo*. The residue was recrystallized from an appropriate solvent (I, $CHCl_3$; V, *n*-hexane; VI, $CHCl_3$). In the cases of I and VI, colorless prisms (I, 55 mg; VI, 10 mg) of mp 319—321° (in an evacuated capillary) were obtained, and their IR, UV, NMR and MS spectra were found to be identical with those of VII. In the case of V, slightly yellow plates (15 mg) of mp 269—272° (in an evacuated capillary) were obtained. *Anal.* Calcd for $C_{56}H_{84}$: C, 88.82; H, 11.18. Found: C, 88.81; H, 10.99. UV $\lambda_{max}^{n\text{-heptane}}$ nm (ε): 352 (45700), 369 (46300). MS *m/e*: 756 (M^+).

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