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Tsutomu Momose, Yo Ueda, Mitsuyoshi Kageura, Toshiko Masumura, and Kiyoko Ohta : Color Reaction Mechanism of Cholesterol in Zak-Henly's Method. (Organic Analysis. LVII.*1)

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Cholesterol in biological fluids has been determined colorimetrically by several coloring reagents. Those involve conc. sulfuric acid (Liebermann-Burchard reaction,[1] or Salkowski-reaction[2]), ferric chloride and conc. sulfuric acid (Zak-Henly reaction),[3] benzoyl peroxide and conc. sulfuric acid (Lifschutz reaction),[4] perchloric acid (Schaltegger reaction),[5] trichloroacetic acid (Rosenheim reaction),[6] zinc chloride and acetylchloride (Tschugaeff reaction),[7] and perchloric acid-phosphoric acid-ferric chloride.[8]

Extensive works on the reaction mechanism of cholesterol in the Liebermann-Burchard (L. B.) reaction,[9] the Tschugaeff (T.) reaction,[10] and the Salkowski (S.) reaction[11] clarified that cholesta-3,5-diene (in L. B. and T. reaction), 3,3′-bi[cholesta-2,4-diene] (in L. B., T. and S. reaction), and 3,3′-bi[cholesta-3,5-diene] (in L. B. and S. reaction) were the only crystalline products which revealed colors with the respective color reagent. These results may indicate that the crystalline reaction products of various color reactions are essentially the same, but the colors developed by these reactions are frequently quite different with each other. In order to ascertain the point and to look for other products than those mentioned above, an investigation of the color reaction mechanism of cholesterol has been undertaken. In this paper the color reaction mixture of cholesterol developed by Zak-Henly's method is studied.

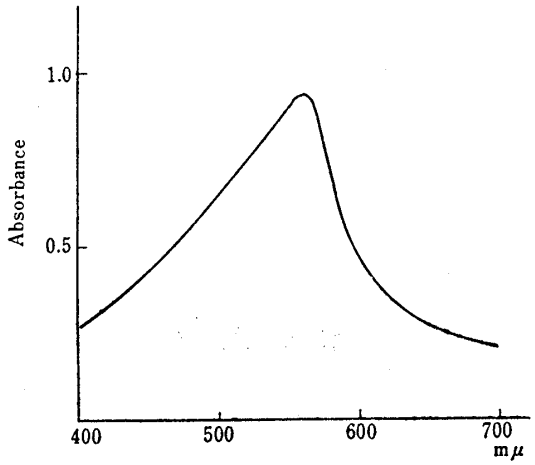

Fig. 1. Absorption Spectrum of the Color developed by Cholesterol with Zak-Henly's Reagent

The Zak-Henly's method for the determination of cholesterol in blood serum is widely used in clinical chemistry, and it gives a purple coloration (λ_{max} 560 mμ). The absorption spectrum of the reaction mixture is shown in Fig. 1. Since it was assumed that the coloration could occur by the presence of strong acids and the reaction products might be a mixture of almost colorless substances, the isolation of these substances was the main purpose of our investigation. Along

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with this direction, the reaction mixture was diluted with water, extracted with chloroform, and separated to their components by their solubilities. Thereby a crystalline product was isolated with a rather good yield, and it was identified as 3,3′-bi[cholesta-2,4-diene] from its physical properties. The remaining brownish resinous substance was submitted to a chromatography on alumina, however, it was rather difficult to isolate an appreciable amount of pure compound. A repeated chromatography of it is now under investigation and the result will be published later.

In spite of the fact that 3,3′-bi[cholesta-2,4-diene] is colorless plates, its chloroform solution develops instantaneously an intense purple red color by shaking with a mixture of acetic acid, ferric chloride and conc. sulfuric acid, which has exactly the same composition as that of Zak-Henly reaction. And the acid layer remains with no appreciable color. The absorption spectrum of the chloroform layer is shown in Fig. 2, and it has an absorption maximum at 550 mμ.

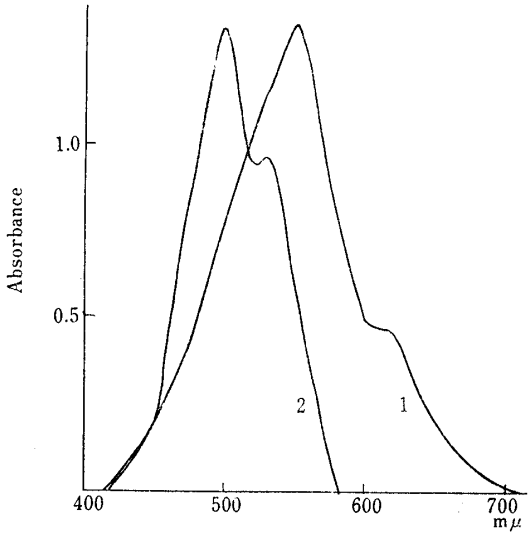

Fig. 2. Absorption Spectra of the Colors developed by 3,3′-Bi[cholesta-2,4-diene] with (1) Zak-Henly's Reagent, and (2) a Mixture of Acetic Acid and conc. Sulfuric Acid

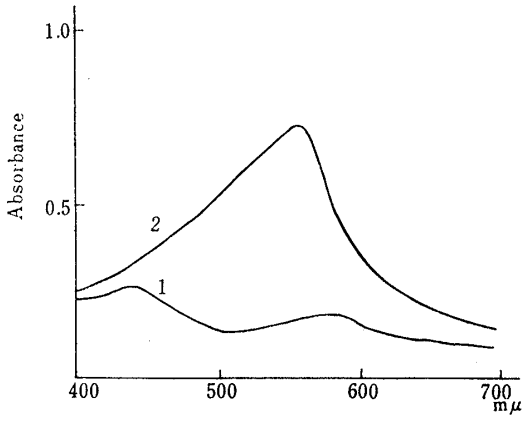

Fig. 3. Absorption Spectra of the Colors of (1) Chloroform, and (2) Acid Layer

On the other hand, the developed color of Zak-Henly reaction can be partially extracted with chloroform, and the acid layer keeps a rather intense purple color. Absorption spectra of both layers are shown in Fig. 3.

Those two phenomena tell us that 3,3′-bi[cholesta-2,4-diene] is not a sole product responsible for the color, but it is obviously one of the main crystalline product.

The remarkable red shift of a chloroform solution of 3,3′-bi[cholesta-2,4-diene] by the Zak-Henly's reagent might be mainly attributable to the formation of a corresponding carbonium ion or cation with a strong acid. It is important to note that a chloroform solution of 3,3′-bi[cholesta-2,4-diene] develops an intense pink color instantaneously with a mixture of acetic acid and conc. sulfuric acid, which has exactly identical composition with that of the Zak-Henly reaction but is lacking in ferric chloride. This color has an absorption maximum at 498 mμ (Fig. 2). Hence it is obvious that ferric chloride plays a considerably important role in the red shift, but no reasonable explanation is known about the nature of this shift in the present step.

Experimental

Color Reaction——In a warm (ca. 60°) mixture of 600 ml. of glac. AcOH and 7.5 ml. of H_2O were

dissolved 0.48 g. of $FeCl_3 \cdot 6H_2O$ and 5.0 g. of cholesterol. With continuous stirring 400 ml. of conc. H_2SO_4 was added gradually with such a rate that the temperature of the reaction mixture could be maintained at 58~60°. Then the mixture was kept in a dark place for about 30 min.

Extraction of the Reaction Product——The reaction mixture was diluted with ice-water to about 3 L., and the reaction product was extracted with $CHCl_3$ three times. The combined $CHCl_3$ layer was washed with H_2O, dried over Na_2SO_4, and evaporated *in vacuo*.

The color reaction and the extraction were repeated three times. The total yield of the extracts was 13.8 g.

Isolation of 3,3′-Bi[cholesta-2,4-diene]——To the brown extract was added 50 ml. of AcOEt, and the mixture was warmed in a water-bath to make a suspension. After cooling, an insoluble faint brownish powder (2.5 g.) was collected by filtration. The filtrate gave 11.3 g. of dark brownish resinous substance after evaporation of the solvent. Repeated recrystallization from $CHCl_3$ and from benzene of the powder gave faint yellow plates. A further purification by a chromatography on alumina and repeated recrystallizations from $CHCl_3$ and from benzene gave an analytical sample as colorless plates. *Anal.* Calcd. for $C_{54}H_{86}$: C, 88.21; H, 11.79. Found: C, 87.84; H, 12.00. This compound was identified as 3,3′-bi[cholesta-2,4-diene] by a direct comparison of its physical properties with those of an authentic sample.[12]

	Obtained from the reaction mixture	Authentic sample
m.p.*3 (in evacuated capillary)	359~361°	359~362°
m.p. of mixture (in evacuated capillary)	359~362°	
$[\alpha]_D$	−39° (c=0.10, $CHCl_3$)	−39° (c=0.10, $CHCl_3$)
$\lambda_{max}^{CHCl_3}$ mμ (log ε)	271 (4.56) 281 (4.64) 292 (4.51)	271 (4.55) 281 (4.61) 292 (4.48)

IR spectra of both compounds were identical.

Absorption Spectra——All spectra were measured in a glass cell of 10 mm. optical path length with a Shimadzu SV-50A Recording Spectrophotometer.

Fig. 1: An absorption spectrum of a color developed by 0.37 mg. of cholesterol using 6.00 ml. of 0.08% $FeCl_3$ solution in AcOH and 4.00 ml. of conc. H_2SO_4 was drawn against the reagent blank.

Fig. 2-1: A solution of 0.406 mg. of 3,3′-bi[cholesta-2,4-diene] in 10.00 ml. of $CHCl_3$ was shaken with the reagent of Zak-Henly's method. The slightly turbid $CHCl_3$ layer was used for the measurement. The spectrum was drawn against a $CHCl_3$ layer prepared similarly from a reagent blank.

Fig. 2-2: A solution of a small amount of 3,3′-bi[cholesta-2,4-diene] in about 3 ml. of $CHCl_3$ was shaken with about 3 ml. of a mixture of 3 volumes of AcOH and 2 volumes of conc. H_2SO_4. The spectrum was drawn against a $CHCl_3$ layer prepared similarly from the same reagent mixture.

Fig. 3: The color developed by cholesterol similarly as in the case of Fig. 1 was extracted with 5.00 ml. of $CHCl_3$, and the slightly turbid $CHCl_3$ layer was used for the measurement. The spectrum was drawn against the corresponding $CHCl_3$ layer of the reagent blank.

The absorption spectrum of the acid layer was also measured against the corresponding acid layer.

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Summary

3,3′-Bi[cholesta-2,4-diene] was isolated from the color reaction mixture of cholesterol in Zak-Henly's method. Its chloroform solution develops a purple red and a pink color with the Zak-Henly's reagent and a mixture of acetic acid and conc. sulfuric acid, respectively.

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*3 uncorrected.

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