Sample of Gas Chromatographic Determination——Take 50 ml. from extracted (100 ml.) sample solution accurately, remove the solvent by distillation and dissolve in a solution containing suitable amounts of the internal standard.

Colorimetric Determination of Malathion——In a separatory funnel place 20 ml. of abs. EtOH, then add 5 ml. of sample extract, add 2 ml. of 0.5*N* NaOH solution. Shake to mix them for 15~20 seconds and let the solution stand for 3 min. To this solution add 75 ml. of ferric reagent (dissolve 0.2 g. of $FeCl_3$ in a small quantity of distilled water, add 8 ml. conc. HCl and adjust the volume to 1000 ml. with distilled water), shake throughly and let the solution stand for 5 min. Then add 25 ml. of carbon tetrachloride (freshly redistilled) and 2 ml. of $CuSO_4$ solution (dissolve 1 g. of $CuSO_4 \cdot 5H_2O$ in distilled water and dilute to 100 ml.), immediately shake vigorously for 1 min., let the solution stand for 3 min. to permit the layers to separate, draw off the CCl_4 layer and filter the separated CCl_4 layer through a piece of dried filter paper. Determine the absorbance of the filtrate at 418 mμ within 10~25 min. after addition of $CuSO_4$ solution. The absorbance determined is defined as E_1. On the other hand, weigh about 0.22 g. of standard Malathion accurately, dissolve in abs. EtOH and make up to 100 ml. Treat a 5 ml. portion of this solution in the same manner as that for sample solution. The absorbance obtained by this standard is defined as E_2. The quantity of Malathion is obtained by the following equation :

The percentage of Malathion in sample

$$\frac{E_1}{E_2} \times \frac{\text{wt. of Malathion in standard (mg.)}}{\text{wt. of sample (mg.)}} \times 0.1 \times 100$$

The authors wish to express their deep gratitude to Dr. M. Matsui, Director of this laboratory, and Dr. A. Ito, group leader, for their warm encouragement during the course of this work.

Summary

Gas chromatography was successfully applied to the separation of a mixture of nine organophosphorous insecticides using three different column under varying conditions. This method was applicable to the quantitative analysis of commercial Malathion dusts and provided results comparable to the colorimetric estimation.

(Received May 8, 1964)

[Chem. Pharm. Bull. 12(11)1319~1328(1964)]

UDC 547.92 : 543.41

184. Kazukichi Kato : A New Color Reaction of Steroid with Anhydrous Aluminum Chloride and Anisaldehyde. IV.*1 Studies on the Reaction Mechanism.

(*Shinagawa Factory, Sankyo Co., Ltd.**2)

The new color reaction of steroid with anhydrous aluminum chloride and anisaldehyde was proposed by the present author at first for the colorimetric determination of ethylestrenol,[1] and was employed in the determination of allylestrenol and cholesterol.[2]

As reported in the second paper of this series,[2] a conclusion drawn from the results of an investigation on the selectivity of this color reaction was that a double bond in steroidal molecule may be responsible for coloration; the steroid having an isolated double bond or conjugated double bonds between four carbon atoms gave a positive

*1 Part III : This Bulletin, 12, 824 (1964).
*2 Nishi-shinagawa, Shinagawa-ku, Tokyo (加藤寿吉).
1) K. Kato : This Bulletin, 12, 578 (1964).
2) *Idem* : *Ibid.*, 12, 582 (1964).

result. On the contrary, results were negative for the steroids possessing a C–C double bond conjugated with a carbonyl group. As a support of the above conclusion, it was reported in the same paper, that positive result on cyclohexanol is due to cyclohexene produced from it by anhydrous aluminum chloride.

The purpose of the work reported here was to study the mechanism of the color reaction on steroid itself. As an object of the investigation, cholesterol was chosen because of its availability. In the course of the study on the determination of cholesterol, it was remarked that by settting an interval between addition of anhydrous aluminum chloride solution and of anisaldehyde solution a higher absorbance of the colored solution was obtained than by simultaneous addition of both reagents. This fact was investigated in further detail and reaffirmed. For example, at the reaction temperature of 40°, the relation between the reaction time with anhydrous aluminum chloride and absorbance of the solution colored by the second reagent, anisaldehyde, was shown in Fig. 1. The absorbance at the wave length of the absorption maximum, 536 mμ, increased with the increasing reaction time with anhydrous aluminum chloride and reached its maximum at the reaction time of about 60 minutes. This result suggests that cholesterol reacts at first with anhydrous aluminum chloride alone to afford an intermediate which undergoes a next reaction with anisaldehyde. This interpretation was strengthen by an observation that cholesterol gave no coloration when both reagents were added in the reversed order, *i.e.*, anisaldehyde was added before anhydrous aluminum chloride. Anisaldehyde is assumed to disturb the reaction between cholesterol and anhydrous aluminum chloride.

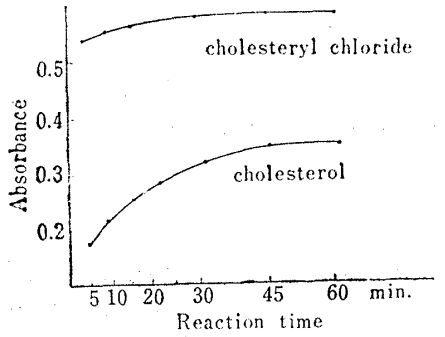

Fig. 1 Relation between Absorbance of Colored Solution and Reaction Time with Aluminum Chloride

For above reason it appeared of interest to investigate the first intermediate derived from cholesterol by anhydrous aluminum chloride. Chloroform solution of cholesterol was heated with nitrobenzene solution of anhydrous aluminum chloride according to the procedure for the colorimetric determination. The reaction mixture was poured into petroleum ether, and dried diatomaceous earth (Celite 545) was added to adsorb the unreacted halide. After removal of the solvents by distillation *in vacuo*, the residue was submitted to thin-layer chromatography over silica gel developed by hexane, in which three spots were detected with antimony trichloride. The main spot of 0.6 was accompanied with the minor one of Rf 0.7, and a small amount of unreacted cholesterol was found on the starting point. The residue was then chromatographed over silica gel, and each fraction successively eluted by hexane was examined by the thin-layer chromatography described above. From the fractions giving a single spot of the main product, white needles, m.p. 97°, were obtained by recrystallization from alcohol, and characterized as cholesteryl chloride, $C_{27}H_{45}Cl$, undepressed by admixture with a sample prepared by Diels'method.[3] This result coincides with the report by Broome, *et al.*[4] that 3β-hydroxy-5-ene-steroid and its ester gave 3β-chloro-derivative when they were refluxed with anhydrous aluminum chloride suspended in ether for several hours.

In the above experiment a nonaqueous treatment was adopted to separate unreacted aluminum chloride from the reaction mixture for the purpose of preventing the formation of hydrochloric acid which would give a secondary effect on the reaction product. However, the usual aqueous treatment also gave the same result, and furthermore it

3) O. Diels, P. Blumberg : Ber., **44**, 2847 (1911).
4) J. Broome, B. R. Brown, G. H. R. Summers : J. Chem. Soc., **1957**, 2071.

was found that chlesteryl chloride was obtained also by shaking cholesterol with anhydrous aluminum chloride suspended in chloroform at a room temperature for a few hours.

Cholesterol chloride gave the same color as cholesterol with nitrobenzene solution of anhydrous aluminum chloride and of anisaldehyde which were added according to the procedure for the colorimetric determination of cholesterol. As shown in Fig. 2, absorption curve of the colored solution was in good agreement with that of the colored solution obtained from cholesterol, and absorbance at the wave length of absorption maximum, 536 mμ, markedly higher than that obtained from cholesterol.

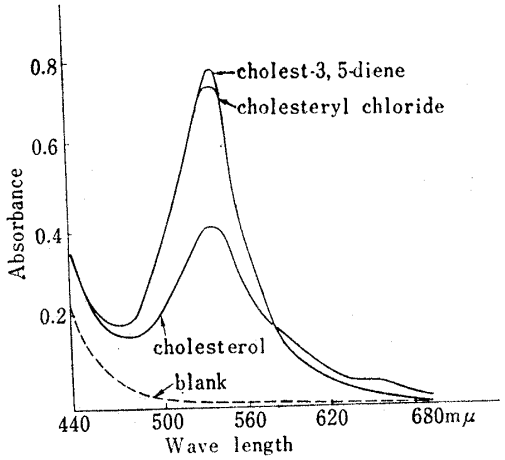

Fig. 2. Absorption Spectra of Colored Solutions

The reaction time with anhydrous aluminum chloride required for maximum absorbance of the colored solution was distinctly shorter on cholesteryl chloride than on cholesterol (Fig. 1). On the basis of these data it seemed reasonable to assume that cholesterol is changed by anhydrous aluminum chloride to cholesteryl chloride, through which the color reaction proceeds to give a colored species with anisaldehyde.

Accordingly, the next experiment was undertaken to investigate the reaction of cholesteryl chloride with anisaldehyde in the presence of anhydrous aluminum chloride. Nitrobenzene solution of anhydrous aluminum chloride was added to cholesteryl chloride, and after the mixture was allowed to stand at 50° for 30 minutes, anisaldehyde was added. The reddish violet reaction mixture thus obtained was mixed thoroughly with dried Celite 545 and repeatedly washed with petroleum ether to remove unreacted cholesteryl chloride and nitrobenzene. The Celite layer and petroleum ether solution were separated by centrifugation. During these treatments, the Celite layer retained the initial reddish violet color. Then it was mixed with a fresh portion of petroleum ether, and as soon as a small quantity of alcohol was dropped in this mixture with stirring, the reddish violet color disappeared. This treatment was taken to have a reaction product liberated from a coupling with anhydrous aluminum chloride and removed to petroleum ether portion. Meanwhile anhydrous aluminum chloride remained in the Celite layer. The yellow colored petroleum ether solution was evaporated under reduced pressure in nitrogen atmosphere at a temperature as low as possible and gave a deep orange oily residue. When the residue was developed by cyclohexane-benzene (3:1) on thin-layer chromatography over silica gel, a main spot of Rf 0.7 was detected by antimony trichloride besides other minor spots. The residue was dissolved in hexane and chromatographed on a column of silica gel. Fractions containing the main product were eluted by hexane-benzene (9:1) and evaporated as described above. The column chromatography was repeated several times until a deep orange oily substance was obtained which gave a single spot of the main product on the thin-layer chromatography. Attempts with various organic solvents to crystallize it were unsuccessful.

It was freely soluble in various organic solvents but very slightly soluble in alcohol and insoluble in water. Though unstable in the air, it was reasonably stable in nitrogen atmosphere or in solution. High vacuum distillation of this substance resulted in giving numerous spots on thin-layer chromatography suggesting that some structural change took place. Considering such behavior, further investigation were carried out on the substance carefully purified by the column chromatography.

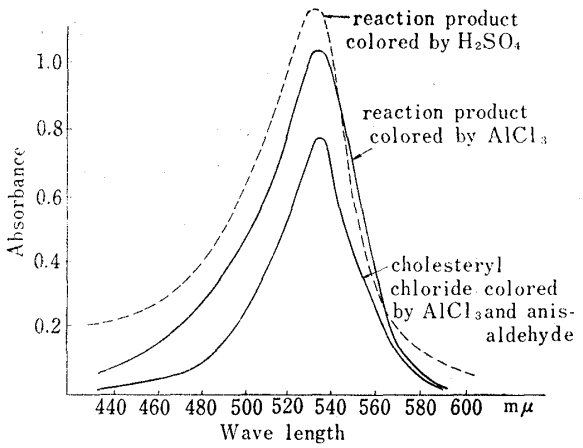

Fig. 3. Absorption Spectra of Colored Solutions

When nitrobenzene solution of anhydrous aluminum chloride was added to this substance, an intense reddish violet color was instantly produced, absorption curve of which is given in Fig. 3. In view of the wave length of absorption maximum and the absorbance at this wave length, the color is assumed to be identical with that produced from choresterol or cholesteryl chloride by adding nitrobenzene solution of anhydrous aluminum chloride and anisaldehyde. From this result it may be concluded that the color which is obtained from cholesterol by this reaction is due to a coupling of this substance with anhydrous aluminum chloride.

Infrared absorption spectrum of this substance exhibited the bands due to aromatic type structure at 1508, 1570, and 1605 cm^{-1} and the bands due to aromatic ether at 1030 and 1242 cm^{-1} in addition to other peaks (Fig. 4). Especially, the band at 1570 cm^{-1} indicates the presence of an unsaturated bond conjugated with the aromatic ring.[5] These data and the lack of the absorption bands which is characteristic for carbonyl group suggest that anisaldehyde has reacted with the steroid at the carbonyl group. It must be also remarked that the C-Cl band found at 764 cm^{-1} in cholesteryl chloride is absent in this reaction product.

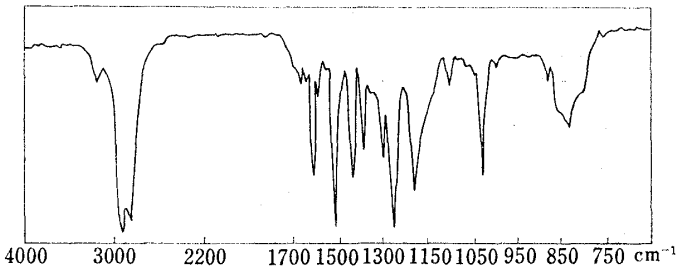

Fig. 4. Infrared Absorption Spectrum of Reaction Product from Cholesteryl Chloride and Anisaldehyde

Liquid film/0.5 mm.

This substance was submitted to microanalysis at every time when it was purified by the column chromatography described above, and at last it gave constant analytical figures consistent with $C_{35}H_{50}O$. These data offer a basis for supposing that anisaldehyde condensed with a methylene group of cholesteryl chloride while the chlorine was eliminated from the steroid and as a whole the reaction product was formed with loss of each one molecule of water and hydrogen chloride. On the other hand, absorption maximum of this substance in the ultraviolet region was found at a wave length of 336 mμ in alcohol as shown in Fig. 5, and molar absorptivity at this wave length was 39,600 calculated on the molecular formula of $C_{35}H_{50}O$. They indicate the exi-

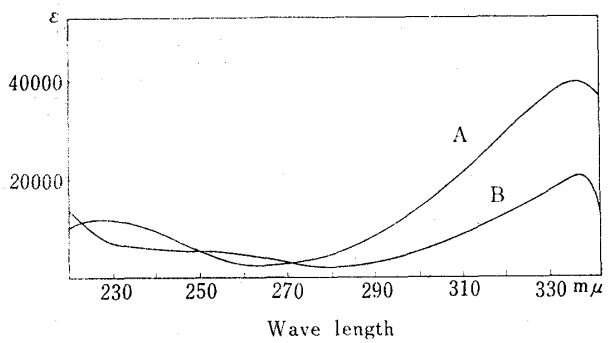

Fig. 5. Absorption Spectra of Reaction Products

A : Reaction product from cholesteryl chloride and anisaldehyde
B : Reaction product from cholest-5-ene and anisaldehyde

5) L. J. Bellamy : "The Infrared Spectra of Complex Molecules," 72 (1958), Methuen & Co., London.

stence of a conjugated system of a considerable degree in the molecule.

Before further discussing on the structure of this substance, it seemed of importance to clarify the eliminating process of chlorine from cholesteryl chloride. To cholesteryl chloride dissolved in chloroform, nitrobenzene solution of anhydrous aluminum chloride was added, and the mixture was allowed to stand at 50° for 30 minutes. Then it was treated in the same manner as described in the preceding experiment on the reaction of cholesterol with anhydrous aluminum chloride. In thin-layer chromatogram of the reaction mixture, a main spot was detected at the position of Rf value greater than cholesteryl chloride while the spot of the latter disappeared. Furthermore, it was found that Rf value of the main spot and its coloration by antimony trichloride were in good agreement with those of cholest-3,5-diene.[6] The residue obtained by removal of the solvents solidified in alcohol on cooling. Purification by column chromatography over silica gel and repeated recrystallizations from alcohol afforded white needles, m.p. 78.8°, which was identified with cholest-3,5-diene from elementary analysis, absorption maximum in the ultraviolet region and mixed melting point with a sample prepared by Mauthner's method.

In the experiment on the reaction of cholesterol with anhydrous aluminum chloride described already in this report, a minor spot of Rf 0.7 corresponding to cholest-3,5-diene was detected in thin-layer chromatogram. In that case, however, the molar ratio of anhydrous aluminum chloride/cholesterol was 4.4, and it was assumed too small to afford cholest-3,5-diene as a main product.

In order to make clear the effect of this molar ratio on products, the reaction mixtures prepared under various molar ratios were investigated by thin-layer chromatography over silica gel with hexane. The result is given in Fig. 6, indicating that cholest-3,5-diene predominated as the molar ratio of anhydrous aluminum chloride/cholesterol increased.

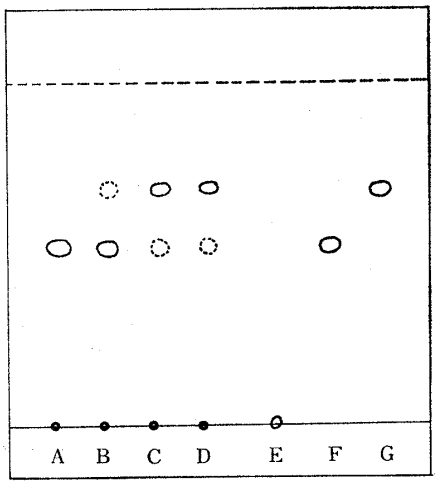

Fig. 6. Chromatogram of Reaction Mixtures prepared from Cholesterol and Aluminum Chloride

A : Molar ratio of $AlCl_3$/cholesterol 2.2
B : Molar ratio of $AlCl_3$/cholesterol 4.4
C : Molar ratio of $AlCl_3$/cholesterol 8.8
D : Molar ratio of $AlCl_3$/cholesterol 17.6
E : Cholesteryl
F : Cholesteryl chloride
G : Cholest-3,5-diene

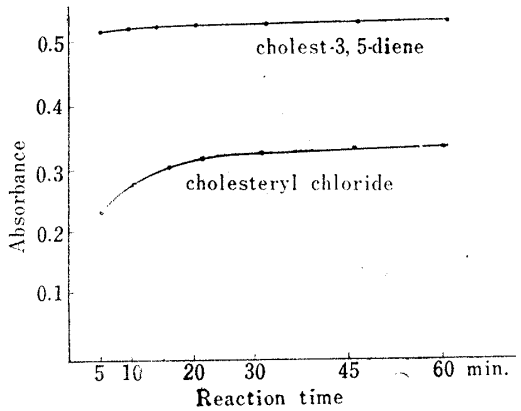

Fig. 7. Relation between Absorbance of Colored Solution and Reaction Time with Aluminum Chloride

Cholest-3,5-diene gave an absorption curve similar to that obtained from cholesteryl chloride when it was submitted to the color reaction with anhydrous aluminum chloride and anisaldehyde according to the procedure for the determination of cholesterol[2] (Fig. 2). On the other hand, Fig. 7 shows the relations between the reaction time with anhydrous aluminum chloride and absorbance of the colored solution obtained from cholest-3,5-diene or from cholesteryl chloride. As the reaction temperature with anhydrous aluminum chloride, 0~2° was

6) J. Mauthner : Monatsh. Chem., 24, 648 (1903).

chosen in order to make clear the difference between absorbances of the colored solutions at each reaction time while other conditions were the same as those in the preceding experiment on cholesterol and cholesteryl chloride (Fig. 1). On cholest-3,5-diene, absorbance of the colored solution reached its maximum in a reaction time far shorter than on cholesteryl chloride.

On the basis of these data, it may be concluded that cholesteryl chloride derived from cholesterol by anhydrous aluminum chloride is changed to cholest-3,5-diene by an excess of the metal halide and the diene undergoes the reaction with anisaldehyde in the presence of the metal halide. Further evidence on this interpretation was found in the fact that cholest-4-ene,[2] cholest-5-ene as well as cholest-3,5-diene gave a reddish violet color in a sufficient intensity even if both reagents were added in the reversed order; cholesterol and cholesteryl chloride gave no color unless the reagents were added in the normal order. In this experiment, the general procedure for the determination of the selectivity of this color reaction described in the previous paper[2] was employed with the normal or reversed addition order of the reagents. Thus, chlorine is not essential for this color reaction, and rather it must be eliminated before the steroid reacts with anisaldehyde.

The condensation of olefins with aldehydes catalysed by Lewis acid is referred to generally as the Prins reaction.[7] Yang, *et al.*[8] clarified mechanism of this reaction as shown in Chart 1. Aromatic aldehydes were also employed in some cases.[9,10] By analogy with this mechanism, the reaction of cholest-3,5-diene with anisaldehyde can be explained as shown in Chart 2.

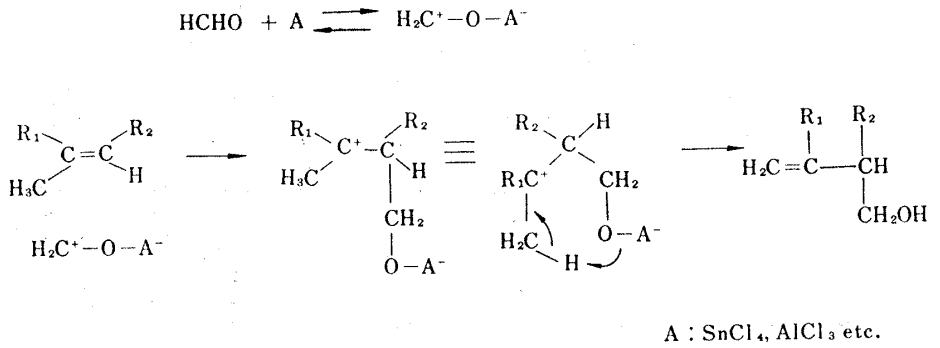

Chart 1.

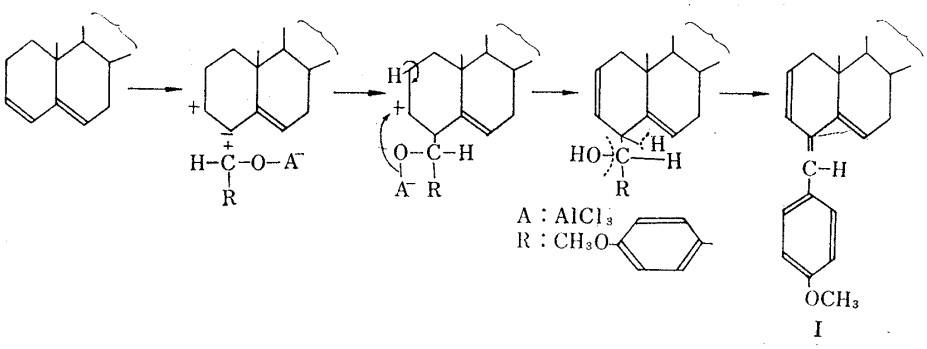

Chart 2.

7) H. J. Prins : Chem. Weekblad, **16**, 1072 (1919).
8) N. C. Yang, Ding-Djung H. Yang, C. B. Ross : J. Am. Chem. Soc., **81**, 133 (1959).
9) R. Lombard, J. Adam : Bull. soc. chim., **1954**, 1216.
10) P. H. Williams, S. A. Ballard : U. S. Pat., 2,452,977 (1948); C. A., **43**, 3042 (1949).

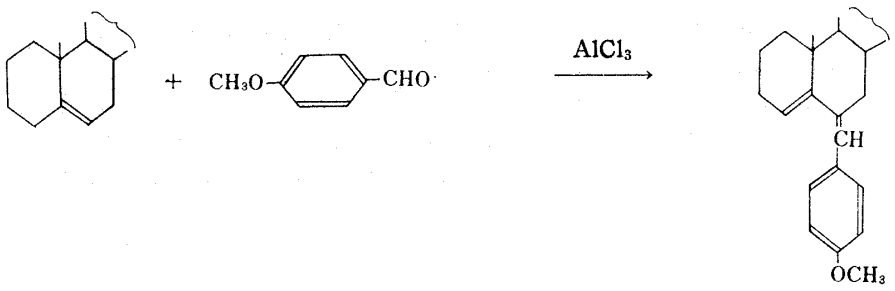

Chart 3. II

In order to give such a condensation product as expected from Yang's mechanism, the carbonium cation derived from aldehyde should attack C_4 of the steroid because C_3 or C_6 may be attacked likewise but in these cases C_5 or C_4 can not afford a proton to transfer to the terminal oxygen of the quasi six-membered ring. The resulting unsaturated alcohol may be easily dehydrated by a large excess of anhydrous aluminum chloride. Analytical figures and infrared absorption spectrum of the reaction product supported wholly these interpretations.

A more strong basis for this postulation was found in the comparison of spectroscopic data between this reaction product and another one obtained from cholest-5-ene and anisaldehyde. According to this mechanism, formula (II) is only expected for the product from cholest-5-ene, and the wave length of absorption maximum of the product (II) must essentially coincide with that of the product (I) because the product (I) has a cross-conjugated triene system which further conjugates with an aromatic ring while the product (II) has a linear conjugated diene system in the corresponding part of the molecule as shown in Chart 3.

Thus, cholest-5-ene[11] was submitted to the reaction with anisaldehyde in the presence of anhydrous aluminum chloride, and the reaction product was isolated in the same manner as described for that from cholesteryl chloride. It was also a deep orange oily substance giving an infrared absorption spectrum essentially identical with that of the reaction product from cholesteryl chloride (Fig. 8).

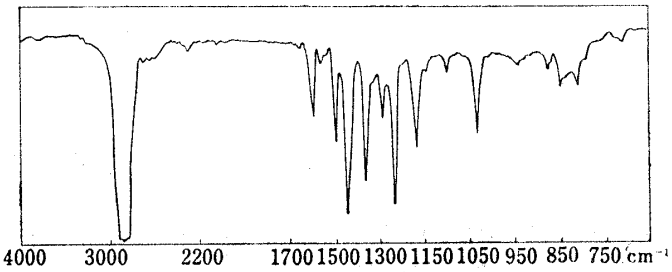

Fig. 8. Infrared Absorption Spectrum of Reaction Product from Cholest-5-ene and Anisaldehyde (Nujol)

As was expected, it gave analytical figures consisted with $C_{35}H_{52}O$ and absorption maximum at a wave length of 336 mμ (Fig. 5). From these results, formula (I) and (II) are postulated for the product from chlolesteryl chloride and cholest-5-ene respectively. The negative result of this color reaction to the steroid which has a double bond conjugated with a carbonyl group is also comprehensible by this mechanism.

Chloroform solutions of these two reaction products gave essentially same color not only with nitrobenzene solution of anhydrous aluminum chloride but also with other Lewis acids, as in the cases of cholesterol and cholest-5-ene which were treated with anhydrous aluminum chloride and anisaldehyde by the procedure for the color reaction.

For example, absorption curve of the colored solution obtained from the reaction product (I) with concentrated sulfuric acid is given in Fig. 3. Seventy per cent perchloric acid, 60% phosphoric acid and 35% hydrochloric acid also gave the similar color to these reaction products.

11) J. Mauthner, W. Suida : Monatsh. Chem., **15**, 85 (1894).

In view of these facts the most reasonable conclusion to be drawn from available data may be that the coloration is due to a coupling of the empty orbital of aluminum chloride with the terminal carbanions in the conjugated systems which gives rise to such resonance structures as shown in Chart 4. These electron displacements may be promoted by the electron-releasing effect of *p*-methoxyl of the aromatic structure.

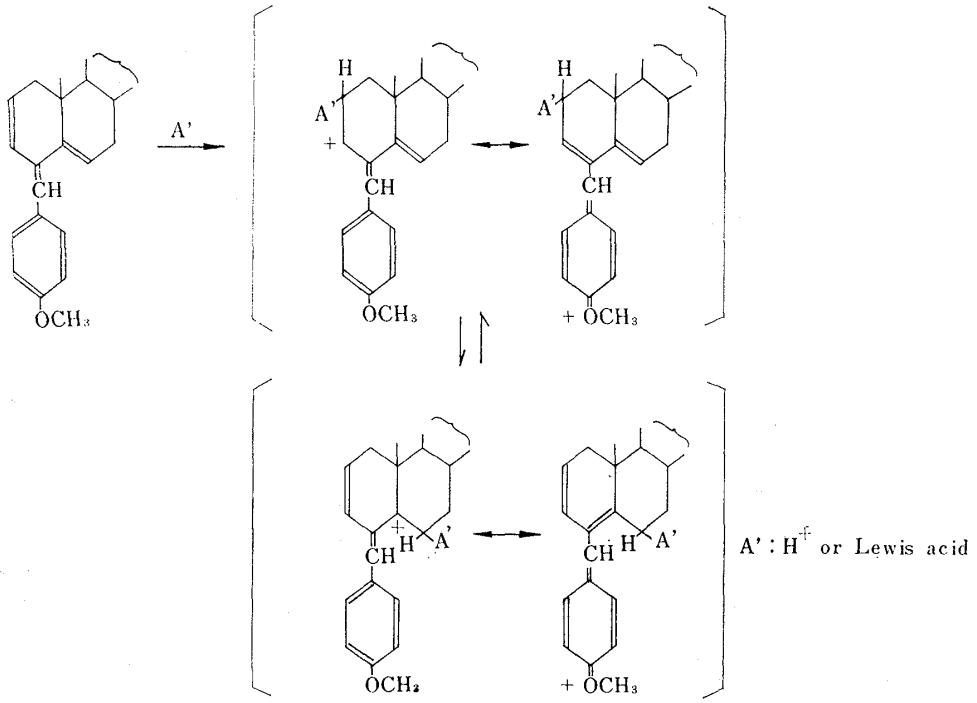

Chart 4.

Experimental

Reaction of Cholesterol with Anhydrous Aluminum Chloride

Absorption Measurement of the Colored Solution of Cholesterol (Fig. 1)——To 1 ml. of $CHCl_3$ solution of cholesterol (70 μg./ml.) in a 10 ml. volumetric flask, 1 ml. of 5% nitrobenzene solution of $AlCl_3$ was added and shaken thoroughly. Being tightly stoppered, it was heated in a water bath at 40° for 5 min. or other reaction times described in Fig. 1. After cooling rapidly to 20~30°, 1 ml. of 5% benzene-nitrobenzene (1:1) solution of anisaldehyde was added, and then the colored solution was treated by the procedure for the colorimetric determination of cholesterol.[2] The relation between the reaction time with $AlCl_3$ and absorbance of the colored solution at a wave length of absorption maximum, 536 mμ, was obtained as shown in Fig. 1.

Isolation of Cholesteryl Chloride——1) A solution of 1.6 g. of cholesterol dissolved in 88 ml. of $CHCl_3$ was heated with 12 ml. of 20% nitrobenzene solution of $AlCl_3$ at 40° for 60 min. The reaction mixture was poured into 750 ml. of petr. ether (b.p. 30°) with stirring, and 12 g. of Celite 545 (Johns-Manville Co.; dried at 105° for 5 hr.) was added. After removal of petr. ether layer by centrifugation, the Celite layer was washed with 200 ml. of petr. ether. The washings were combined with the petr. ether layer. It was evaporated under N_2 and then at 70° *in vacuo* to dryness. The residue was recrystallized from Me_2CO to needles which gave three spots on TLC over silica gel (Kieselgel G, Merck; activated at 105° for 30 min.), the main one of Rf 0.6 and others of Rf 0.7 and 0.0, when it was developed with hexane and detected with $SbCl_3$ dissolved in $CHCl_3$. In order to isolate the pure main product, a solution of these needles in hexane was passed through a column of silica gel and eluted with hexane. Each eluate was checked by the TLC described above, and the fractions containing the main component were collected. They were combined and evaporated to dryness. The residue was recrystallized from EtOH to white needles, m.p. 97° which were positive for Beilstein's halogen test and showed no melting point depression on admixture with cholesteryl chloride prepared by Diels' method. IR : ν_{C-Cl} 764 cm^{-1}(KBr); $\nu_{C=C}$ 1670 cm^{-1}(KBr). *Anal.* Calcd. for $C_{27}H_{45}Cl$: C, 80.05; H, 11.20; Cl, 8.75. Fonnd : C, 80.20; H, 11.19; Cl, 8.87.

2) The reaction mixture obtained in the same manner as described in 1) was shaken with 500 ml. of H_2O. The upper layer was repeatedly washed with H_2O until the washings became neutral. On evaporation of the solvents *in vacuo* and purification described in 1), cholesteryl chloride was isolated.

3) To 1.2 g. of $AlCl_3$ suspended in $CHCl_3$, 500 mg. of cholesterol was added, and shaken vigorously at a room temperature for 2 hr. Then it was poured into H_2O to decompose excess $AlCl_3$. The $CHCl_3$ layer was repeatedly washed with H_2O, and treated in the same manner as described in 1) to isolate cholesteryl chloride.

Absorption Measurement of the Colored Solution of Cholesteryl Chloride (Fig. 1)——One milliliter of $CHCl_3$ solution of cholesteryl chloride (70 μg./ml.) was treated in the same manner as described in absorption measurement of the colored solution of cholesterol. The results were given in Fig. 1.

Absorption Curves of the Colored Solutions (Fig. 2)——One milliliter of $CHCl_3$ solution (100 μg./ml.) of cholesterol and of cholesteryl chloride were treated by the procedure for the determination of cholesterol[2] respectively. Absorption curves of the colored solutions were obtained as shown in Fig. 2.

Reaction of Cholesteryl Chloride with Anisaldehyde

Isolation of Reaction Product——A solution of 2 g. of cholesteryl chloride dissolved in 8 ml. of nitrobenzene was heated in a stoppered flask with 3.2 ml. of 20% nitrobenzene solution of $AlCl_3$ at 50° for 30 min. After cooling to a room temperature, 0.6 g. of anisaldehyde was added to it and shaken thoroughly. The reddish violet reaction mixture was allowed to stand at a room temperature for 50 min. and then mixed thoroughly with 40 g. of dried Celite 545. After addition of 240 ml. of petr. ether to extract unreacted cholesteryl chloride and nitrobenzene under shaking, the Celite layer was separated from petr. ether layer by centrifugation and washed with another 240 ml. portion of petr. ether. To the Celite layer separated and then suspended in another 240 ml. portion of petr. ether, 2 ml. of EtOH was added dropwise. The reddish violet color of the Celite layer instantly disappeared, and orange yellowish petr. ether layer was obtained. The Celite layer was washed again with 240 ml. of petr. ether, and the washings were combined with the petr. ether-EtOH extract described above. These extracts were evaporated under N_2 *in vacuo* to give 550 mg. of a deep orange oily residue. This residue gave a main spot of Rf 0.7 accompanied with other minor ones when it was submitted to TLC over silica gel developed by cyclohexane-benzene (3:1) and detected with $SbCl_3$ dissolved in $CHCl_3$. After removing a trace of nitrobenzene at 90° under N_2 *in vacuo* (1 mm. Hg) for 10 min. and cooling, the residue was dissolved in 50 ml. of petr. ether and shaken twice with 10 ml. of 50% $NaHSO_3$ solution to remove remaining anisaldehyde. The petr. ether layer was washed with H_2O, dried over anhyd. Na_2SO_4 and evaporated under N_2 *in vacuo* to give 320 mg. of a deep orange oily residue. Rf of the main spot on the TLC did not change during these operations. A solution of this oily residue in hexane was passed through a column of silica gel, and eluted successively with hexane and hexane-benzene mixture. Each fraction was evaporated at about 35° under N_2 *in vacuo*. This column chromatography was repeated successively four times until a pure substance was obtained which gave constant analytical figures after third and fourth chromatography showing a single spot on the TLC. Attempts to crystallize this oily substance were unsuccessful which was insoluble in H_2O, slightly soluble in EtOH and freely in most nonpolar organic solvents. It was negative to Beilstein's halogen test. UV : λ_{max}^{EtOH} 336 mμ (log ε 3.60). $[\alpha]_{546}^{25}$ −40.0°, $[\alpha]_{578}^{25}$ −28.8°, $[\alpha]_{589}^{25}$ −25.1° (c=0.40, hexane). *Anal.* Calcd. for $C_{35}H_{50}O$: C, 86.36; H, 10.35. Found : C, 86.00, 86.08; H, 10.43, 10.41; Cl, 0.00, 0.00.

Absorption Curves of the Colored Solutions Obtained from the Reaction Product (Fig. 3)——1) Coloration by $AlCl_3$: To a solution of 100 μg. of the reaction product dissolved in 1 ml. of $CHCl_3$, 1 ml. of 5% nitrobenzene solution of $AlCl_3$ was added. To this reddish violet mixture, nitrobenzene-benzene (1:1) mixture was added to make 10 ml., and an absorption curve of this solution was measured by an auto-recording spectrophotometer. A blank solution was prepared with 1 ml. of $CHCl_3$, 1 ml. of 5% nitrobenzene solution of $AlCl_3$ and a sufficient quantity of nitrobenzene-benzene (1:1) mixture to make exactly 10 ml.

2) Coloration by H_2SO_4 : To 1 ml. of $CHCl_3$ solution of the reaction product described in 1), 0.1 ml. of conc. H_2SO_4 was added. The reddish violet mixture was treated in the same manner as described in 1), and an absorption curve was obtained.

Isolation of Cholest-3,5-diene

Thin-layer Chromatography of Reaction Mixture (Fig. 6)——To each 1 ml. of $CHCl_3$ solution of cholesterol (16 mg./ml.) in a 10 ml. volumetric flask, 0.06, 0.12, 0.24 and 0.48 ml. of 20% nitrobenzene solution of $AlCl_3$ were added respectively. Thus the molar ratio of $AlCl_3$/cholesterol in each mixture was 2.2, 4.4, 8.8 or 17.6. These mixtures were allowed to stand at 40° for 60 min., and then petr. ether was added to them to make 10 ml. respectively. These solutions were submitted to TLC (Kieselgel G, Merck; 20×20 cm., activated at 105° for 30 min.), developed by hexane and detected with $SbCl_3$ dissolved in $CHCl_3$. Samples of cholesterol, cholesteryl chloride and cholest-3,5-diene were also chromatographed simultaneously on this plate as controls. The results were given in Fig. 6.

Isolation of Cholest-3,5-diene——Two grams of cholesteryl chloride dissolved in 20 ml. of nitrobenzene was heated with 6 ml. of 20% nitrobenzene solution of $AlCl_3$ in a stoppered flask at 50° for 30 min. Then

the reaction mixture was shaken with 100 ml. of H_2O, and washed successively with H_2O until the washings became neutral. The separated upper layer was dried over anhyd. Na_2SO_4 and evaporated *in vacuo* to dryness. The residue solidified in EtOH on cooling and gave a main spot of Rf 0.7 on TLC described above. It was purified by a column chromatography over silica gel and recrystallized from EtOH to white needles, m.p. 78.8°. It showed no melting point depression on admixture with cholest-3,5-diene prepared by Mauthner's method. UV : λ_{max}^{EtOH} 234 mμ. *Anal.* Calcd. for $C_{27}H_{44}$: C, 88.07; H, 11.93. Found : C, 87.90; H, 12.02.

Absorption Measurements of the Colored Solutions (Fig. 7)——To 1 ml. of $CHCl_3$ solution containing 200 μg. of cholesteryl chloride or 200 μg. cholest-3,5-diene in a 10 ml. volumetric flask, 1 ml. of 5% nitrobenzene solution of $AlCl_3$ was added, and this mixture was allowed to stand at 0~2° in an ice bath for 5 min. or other reaction times described in Fig. 7. Then 1 ml. of 5% benzene-nitrobenzene (1:1) solution of anisaldehyde was added, and after the reaction mixture was allowed to stand at a room temperature for 50 min., nitrobenzene-benzene (1:1) mixture was added to make exactly 10 ml. Measurements of the absorbances of these colored solutions were carried out at a wave length of absorption maximum, 536 mμ, by the procedure for the colorimetric determinationn of cholesterol.

Absorption Curve of the Colored Solution obtained from Cholest-3,5-diene (Fig. 2.)——A solution of 100 μg. of cholest-3,5-diene dissolved in 1 ml. of $CHCl_3$ was treated by the procedure for the colorimetric determination of cholesterol.

Reaction of Cholest-5-ene with Anisaldehyde

One gram of cholest-5-ene dissolved in a mixture of 4 ml. of $CHCl_3$ and 2 ml. of nitrobenzene was heated with 1.6 ml. of 20% nitrobenzene solution of $AlCl_3$ in a stoppered flask at 50° for 30 min., and then cooled to a room temperature. To this mixture, 0.3 g. of anisaldehyde was added and allowed to stand for 50 min. From this reddish violet reaction mixture, the main product was isolated by extraction and column chromatography in the same manner as described on the reaction of cholesteryl chloride with anisaldehyde. The main product was eluted with hexane-benzene (9:1) on the chromatography over silica gel, and this treatment for purification was repeated until a deep orange oily substance was obtained which gave constant analytical figures and afforded a single spot on TLC over silica gel (developed by hexane and detected with $SbCl_3$ dissolved in $CHCl_3$). It was considerably unstable in the air and had nearly same solubilities as the reaction product from cholesteryl chloride and anisaldehyde. Attempts to crystallize this substance were unsuccessful. UV : λ_{max}^{EtOH} 336 mμ (log ε 3.36). $[\alpha]_{546}^{25}$ −55.6°, $[\alpha]_{578}^{25}$ −44.5°, $[\alpha]_{589}^{25}$ −41.6° (c=0.36, hexane). *Anal.* Calcd. for $C_{35}H_{52}O$: C, 86.01; H, 10.64. Found : C, 86.19, 86.34; H, 10.59, 10.63.

The author expresses his deep gratitude to Professor Z. Tamura and Assist. Professor T. Nambara, Faculty of Pharmaceutical Sciences, University of Tokyo, for their kind guidance, and Mr. J. Nakahira, Director of this Factory, Drs. H. Negoro, K. Tanabe, and H. Sakurai for their constant encouragement.

Thanks are also due to the members of the Elementary Analysis Laboratory, Sankyo Co., Ltd.

Summary

The mechanism of the new color reaction of steroid has been studied on cholesterol. In this reaction, cholesterol is changed at first by anhydrous aluminum chloride into cholesteryl chloride and then into cholest-3,5-diene. Colorimetric and chromatographic investigations reveal that they are intermediates from cholesterol to the next step of the reaction. A colorless species obtained from cholesteryl chloride and anisaldehyde gives a characteristic color with anhydrous aluminum chloride or other Lewis acid. For this colorless reaction product, a benzal type structure conjugated with double bonds in rings of the steroid is postulated, and a mechanism based on the Prins reaction is proposed, for which a support is obtained from the spectroscopic data in comparison with a reaction product of cholest-5-ene. It is concluded that the coloration may be due to a coupling of the reaction product with Lewis acid which gives rise to resonance structures.

(Received June 15, 1964)