Mechanisms of the Liebermann–Burchard and Zak Color Reactions for Cholesterol

R. W. Burke, B. I. Diamondstone, R. A. Velapoldi, and O. Menis

Correlation of SO₂ and Fe²⁺ measurements with new spectral data indicates that the Liebermann–Burchard (L-B) and Zak color reactions for cholesterol have similar oxidative mechanisms, each yielding, as oxidation products, a homologous series of conjugated cholestapolyenes. These studies further suggest that the colored species observed in these two systems are enolic carbonium ions formed by protonation of the parent polyenes. Thus, the red (λ_{max}, 563 nm) product typically measured in the Zak reaction is evidently a cholestatrienylcation, and the blue-green product in the L-B reaction (λ_{max}, near 620 nm) is evidently the pentanevinyl cation. The effects of rate of carbonium ion formation and sulfuric acid concentration on sensitivity and color stability are discussed. A solvent extraction procedure is described for specifically converting cholesterol to 3,5-cholestanediene. Incorporating this step into the typical L-B method can increase the L-B sensitivity for cholesterol by several fold.

Additional Keyphrases: cholestapolyenes • conjugated double bonds • carbonium ions from polyenes • factors affecting color • SO₂ and Fe²⁺ measurement • equivalent ratios

Cholesterol reacts with various strong acids of the Brensted and Lewis types to give colored products. Although these reactions have been used empirically for many years for the qualitative and quantitative determination of cholesterol, their mechanisms still are not clearly understood.

Among the many color reactions for cholesterol, the Liebermann–Burchard procedure is perhaps the most widely used. This reaction was described initially by Liebermann (1) in 1885 and applied to cholesterol analysis shortly after by Burchard (2). Chloroform was used as a solvent in the early studies, but the Liebermann–Burchard (L-B) reaction, as performed today, is carried out in an acetic acid-sulfuric acid-acetic anhydride medium. The other widely used method, the Zak reaction, which was first applied to cholesterol analysis by Zlatkis, Zak, and Boyle (3) in 1953, is carried out in acetic acid-sulfuric acid in the absence of acetic anhydride. In this reaction, however, Fe³⁺ must be added to obtain the desired colored species.

In one of the earliest mechanistic studies, Lange et al. (4) showed that treating chloroform solutions of cholesterol with equivalent amounts of perchloric or hexafluorophosphoric acid resulted in the formation of colorless sterolium salts. These salts hydrolyzed instantaneously on contact with an excess of water, giving quantitative recovery of the added cholesterol. Addition of acid in excess of the stoichiometric amount resulted in slow dissolution of the colorless crystals, followed by formation of purple products that were considered to be halochromatic salts of cholestadiene. Degradation of these products was thought to occur through formation of polymerized dieneoid hydrocarbons, with the trimer being the highest polymer observed. Subsequent studies by Dulou et al. (5) and by Brieskorn and Capuano (6) suggested that the initial step in the L-B reaction was the dehydration of cholesterol to form cholesta- diene, which dimerized to bis-cholestadene. The final product was believed to be the monosulfonated dimer. Later studies by Watanabe (7) supported this hypothesis; he isolated a 3,3′-bis-3,5-cholestadiene by column chromatography from a concentrated L-B reaction mixture. However, a subsequent paper by Brieskorn and Hofmann (8) indicated that dimer formation was not the principal reaction in the L-B system and that a more probable mechanism involved the oxidation of cholesterol to a conjugated pentene. It is at this stage that efforts to elucidate the mechanism of the L-B reaction appear to have stopped.

Received Jan. 4, 1974; accepted May 7, 1974.
In contrast to the investigations of the L-B reaction, little, if any, systematic study has been made of the mechanism of the Zak reaction. The brief mention (9) that the reaction appears to be oxidative is apparently the only reported information on the nature of this color reaction.

We have quantitatively measured SO₂ and Fe³⁺ formation as a function of reaction time, to demonstrate that the L-B and Zak reactions have similar oxidative mechanisms. These data, in conjunction with solvent extraction, uv-visible spectrophotometry, and mass spectrometric measurements show that these oxidation reactions lead to the formation of a series of conjugated cholesterolpolycenes. Moreover, evidence is presented that shows that the colored species comprising these two systems are the corresponding enolic carbonium ions of the respective conjugated polycenes. On the basis of these studies, we propose that the red product typically measured in the Zak reaction with λ<sub>max</sub> at 563 nm is a cholestetraenylc cation while the blue-green product in the L-B reaction with λ<sub>max</sub> near 620 nm is a pentaenylc cation.

**Materials and Methods**

**Reagents**

Solutions of cholesterol were prepared from National Bureau of Standards high-purity cholesterol (NBS SRM 911).

3,3'-bis-3,5-Cholestadiene was obtained from Columbia Organic Chemicals Co., Columbia, S. C. 29209.

2,4- and 3,5-Cholestadiene, 5,7-cholestadien-3β-ol, and 7-cholesten-3β-ol were purchased from Steraloids, Inc., Pawling, N. Y. 12564.

Purified pararosaniline hydrochloride for the determination of SO₂ was obtained as a 2 g/liter concentrate (Item No. 64327) from the Hartman-Leddon Co. (Harleco), Philadelphia, Pa. 19143.

Bathophenanthroline was obtained from the G. Frederick Smith Chemical Co., Columbus, Ohio 43223.

Cyclohexane used in the extraction studies was "Spectro" quality.

All other chemicals were ACS reagent grade.

**Apparatus**

Absorbances were measured with a Cary Model 14 recording spectrophotometer (Varian/Instrument Division, Palo Alto, Calif. 94303).

Mass-spectrometry was done with a Du Pont Model 21-491 medium-resolution mass spectrometer (E. I. du Pont de Nemours, Instrument Products Division, Wilmington, Del. 19898).

Eppendorf pipets (Brinkmann Instruments, Westbury, N. Y. 11590) were used for all microliter-scale transfers.

Extractions were performed in 60- and 125-ml separatory funnels equipped with Teflon stopcocks and stoppers (Kontes Glass, Vineland, N. J. 08360).

The 30-ml midget impingers for collection of SO₂ were also obtained from Kontes Glass, Vineland, N. J. 08360.

**Procedures**

**Determination of sulfur dioxide.** Sulfur dioxide produced in the L-B reaction was determined spectrophotometrically with pararosaniline. This procedure, developed by West and Gaeke (10), was subsequently perfected by Scaringelli et al. (11) for determinations of SO₂ in air. Figure 1 shows a schematic diagram of the apparatus used in our study for generating and collecting SO₂.

The pear-shaped reaction vessel was made from a 10-ml separatory funnel to which was attached a side-arm bubbling tube that extended to approximately 3 mm from the bottom of the vessel. This vessel was connected in series with two midget impingers, each filled with 10 ml of potassium tetrachloromercurate SO₂-absorbing solution (40 mmol/liter) (11). To determine SO₂, we added 5 ml of freshly prepared L-B acid mixture [acetic anhydride: glacial acetic acid:sulfuric acid (10:5:1 by vol), cooled to 25°C] to the reaction vessel followed by 100-300 μl aliquots of a 5 mg/ml solution of cholesterol in acetic acid. The vessel was quickly closed and its contents thoroughly mixed by swirling. Sulfur dioxide was then continually swept from the L-B mixture by purging with nitrogen at the rate of 25 ml/min. At designated intervals ranging from 0.5 to 22 h, purging was discontinued and the contents of the two midget impingers were combined and diluted with the absorbing solution to 25 ml, in volumetric flasks. Appropriate aliquots were then analyzed spectrophotometrically for SO₂ by the procedure of Scaringelli et al. (11).

![Fig. 1. Schematic diagram of apparatus for generation and collection of SO₂ in the Liebermann-Burchard reaction](CLINICAL CHEMISTRY, Vol. 20, No. 7, 1974 795)
Initial experiments in which three impingers were used indicated that 98% of the SO$_2$ was collected in the first impinger while no SO$_2$ was found in the third impinger. Accordingly, we used only two impingers in subsequent experiments.

**Determination of Fe$^{2+}$ formed in the Zak reaction.**

The acid conditions and Fe$^{3+}$ concentration used for carrying out the Zak reaction were those specified by Boutwell (12): a glacial acetic acid to sulfuric acid ratio of 1.5:1 and a 4.4 x 10$^{-3}$ mol/liter Fe$^{3+}$ concentration. A 50-200 µl aliquot of a standard solution of cholesterol (1 mg/ml in acetic acid) was added to 13 ml of this mixture in a 60-ml separatory funnel. The contents of the funnel were quickly mixed by vigorous shaking. One-milliliter aliquots were taken at prescribed intervals over a period of 0.5-5 h, to be analyzed for Fe$^{2+}$.

The spectrophotometric bathophenanthrolone procedure described by Pollock and Miguel (13) was used to determine Fe$^{2+}$ in the presence of Fe$^{3+}$. The key feature in this modified procedure is the addition of phosphate ion which complexes Fe$^{3+}$ and so effectively removes it from the Fe$^{2+}$-Fe$^{3+}$ equilibrium. Failure to use this masking reaction will lead to high results for Fe$^{2+}$, because the reagents used in the spectrophotometric procedure also reduce Fe$^{3+}$ to Fe$^{2+}$.

**Extraction studies.** After initial tests with a series of solvents, cyclohexane was selected for subsequent study of the extraction of cholesterol and its reaction products because of its superior transmission in the ultraviolet. We used two variations in the fundamental procedure. In the first, cholesterol in acetic acid was added directly to various mixtures of acetic and sulfuric acids, which were then extracted with cyclohexane. In the second, cholesterol was dissolved in cyclohexane and this solution was equilibrated with the acid mixtures by mechanical shaking.

**Results and Discussion**

In both the L-B and Zak reactions, our data indicate that cholesterol is oxidized in steps, with each oxidation step yielding a cholestapolyene having one more double bond than the compound from which it was derived. Further evidence is presented to show that the active oxidants in these two systems are SO$_2$ and Fe$^{2+}$, respectively, and that measurement of the corresponding reduction products—i.e., SO$_2$ and Fe$^{2+}$—can yield valuable information regarding the rates and relative efficiencies of these two reactions. Such measurements have now been carried out using the analytical procedures and conditions described in the Materials and Methods section and constitute one of the significant aspects of this report.

Some typical results obtained for SO$_2$ and Fe$^{2+}$ are presented in Tables 1, 2, and 3. The last column in each of these tables is headed "equivalent ratio," which is defined as the ratio of the number of equivalents of SO$_2$ or Fe$^{2+}$ found to the number of equivalents of cholesterol initially added. Because a two-electron transfer is required for generation of a double bond, the equivalent weight of cholesterol in this instance is one-half its molecular weight. The calculated values of the equivalent ratios are numerically equal to the average number of double bonds formed per cholesterol molecule, and they will be used in this context throughout this paper. In Table 1, for example, it is seen that after 0.5 h the equivalent ratio obtained for the L-B reaction with cholesterol is 0.62. As shown later, although this reaction time is optimum for spectrophotometric measurement under

<table>
<thead>
<tr>
<th>Reaction time, h</th>
<th>SO$_2$, µg</th>
<th>SO$_2$/chol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>52</td>
<td>0.62</td>
</tr>
<tr>
<td>1</td>
<td>144</td>
<td>1.73</td>
</tr>
<tr>
<td>2</td>
<td>221</td>
<td>2.65</td>
</tr>
<tr>
<td>3</td>
<td>258</td>
<td>3.10</td>
</tr>
<tr>
<td>4</td>
<td>279</td>
<td>3.34</td>
</tr>
<tr>
<td>6</td>
<td>304</td>
<td>3.65</td>
</tr>
<tr>
<td>22</td>
<td>349</td>
<td>4.18</td>
</tr>
</tbody>
</table>

SO$_2$ blank = 10µg/22h

* In Tables 1-3, "equivalent ratio" refers to the ratio of number of equivalents of SO$_2$ or Fe$^{2+}$ found to the number of equivalents of cholesterol added.

<table>
<thead>
<tr>
<th>Reaction time, h</th>
<th>Fe$^{2+}$, µg</th>
<th>Fe$^{2+}$/chol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>90.1</td>
<td>3.07</td>
</tr>
<tr>
<td>1.0</td>
<td>102.6</td>
<td>3.50</td>
</tr>
<tr>
<td>1.5</td>
<td>114.2</td>
<td>3.92</td>
</tr>
<tr>
<td>2.0</td>
<td>122.5</td>
<td>4.19</td>
</tr>
<tr>
<td>2.5</td>
<td>131.7</td>
<td>4.50</td>
</tr>
<tr>
<td>3.0</td>
<td>136.3</td>
<td>4.69</td>
</tr>
<tr>
<td>4.0</td>
<td>143.0</td>
<td>4.92</td>
</tr>
<tr>
<td>5.0</td>
<td>146.5</td>
<td>5.02</td>
</tr>
</tbody>
</table>

Fe$^{2+}$ blank = 0.5 µg

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Cholesterol, µg</th>
<th>Time, h</th>
<th>SO$_2$, µg</th>
<th>SO$_2$/chol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-B</td>
<td>505</td>
<td>22</td>
<td>348</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>1010</td>
<td>22</td>
<td>711</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>2020</td>
<td>22</td>
<td>1396</td>
<td>4.17</td>
</tr>
</tbody>
</table>

Zak

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Cholesterol, µg</th>
<th>Time, h</th>
<th>SO$_2$, µg</th>
<th>SO$_2$/chol.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50.5</td>
<td>5</td>
<td>73.5</td>
<td>5.04</td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>5</td>
<td>146.5</td>
<td>5.02</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>5</td>
<td>289</td>
<td>4.95</td>
</tr>
</tbody>
</table>

Table 3. SO$_2$ and Fe$^{2+}$ Formation as a Function of Cholesterol Concentration (25 °C) in the Liebermann-Burchard and Zak Reactions
the conditions used, the conversion of cholesterol to the requisite cholestapolyene (four generated double bonds) is, at best, only one-seventh complete. Similar data are shown for the Zak reaction in Table 2. Again, a 0.5-h reaction time is optimum for spectrophotometric measurement. In this case, however, the conversion of cholesterol to the appropriate cholestapolyene (three double bonds generated) is potentially 100% efficient.

The data in Table 3 show that the equivalent ratios calculated for the L-B and Zak reactions are constant over fourfold changes in cholesterol concentration. Such reproducibility definitely implies that the reaction pathways are more explicit than previously expected. In addition, the constancy of these equivalent ratios also indicates that these color systems should obey Beer's law; that they in fact do so has been well established.

As further evidence of the stepwise nature of the oxidation processes, it will be helpful to examine more closely the absorbance characteristics of the Zak reaction. This reaction was chosen in preference to the L-B system because it better illustrates the significant points. A series of absorbance spectra, obtained over a period of about 1 h for a typical Zak reaction, is shown in Figure 2. Three absorbance peaks are observed, with \( \lambda_{\text{max}} \) at 412, 478, and 563 nm. The peaks at 478 and 563 nm have been described in an earlier paper (12); the 412-nm peak, however, has not been reported previously. This initial peak disappears after the first few minutes of the reaction and can best be observed by making either of the following modifications to the fundamental procedure: (a) by carrying out the reaction in a solution containing a lower proportion of acetic to sulfuric acid, i.e., 1:1 HOAc/H\(_2\)SO\(_4\) rather than the 1.5:1 ratio usually used, or (b) by lowering the temperature of the reaction to 10°C. The serial nature of peak formation, together with the well-defined isosbestic points at 427 and 503 nm, clearly show that the final steps in the Zak color reaction consist of a product that absorbs maximally at 412 nm converting to a 478-nm absorbing species which, in turn, yields the peak at 563 nm that is routinely measured. As discussed in a subsequent paper (14), the rates of these reactions are strongly temperature dependent, increasing rapidly with increasing temperature.

The data presented thus far suggest that the products observed in the Zak reactions are carbonium ions formed by the successive protonation of the homologous series of cholestapolyenes proposed previously in this report. This theory was strengthened by noting the close similarity between our observations and the spectral data reported by Sorensen (15) for a homologous series of aliphatic polyenyl cations. In the latter study, the structures of the parent polyenes and their corresponding carbonium ions were proven unequivocally by NMR and ultraviolet spectroscopy. These data in conjunction with our equivalent ratio, solvent extraction, ultraviolet–visible and mass-spectrometric measurements have led to the mechanisms proposed in Figure 3 for the Zak and L-B color reactions with cholesterol. According to these two mechanisms, both reactions have a common initiation step, i.e., protonation of the -OH group in cholesterol and subsequent loss of water to

---

**Fig. 2.** Absorbance spectra of a Zak reaction as a function of time, showing, initially, decrease in the 412-nm peak as a peak appears at 478 nm, and subsequent conversion of 478-nm-absorbing material to the product absorbing at 563 nm where the color is typically measured

**Fig. 3.** Proposed mechanisms of the L-B and Zak reactions

give the carbonium ion of 3,5-cholestadiene. Evidence will be presented subsequently that indicates that formation of this species is the first step in the color reactions. Serial oxidation of this allylic carbonium ion by either Fe$^{3+}$ or SO$_2$ yields the cholestapyrene carbonium ions shown, together with equivalent amounts of Fe$^{2+}$ or SO$_2$. Notably, no cationic species are given in Figure 3 for the L-B system, in going from the allylic to the pentaenyl cation, because none were observed spectrally under the experimental conditions. The acidity of the L-B reaction is relatively low, however, and thus would not normally favor stable carbonium ion formation. The ability to observe the pentaenyl species can therefore be attributed to two factors: (a) sufficiently extended conjugation, and, perhaps more importantly (b) the stabilizing effect of the terminal cyclopentyl ring, as reported by Sorensen (15).

Observed and calculated values of the absorbance maxima are reported for each of the structures shown in Figure 3. The calculated values were obtained from Sorensen’s empirical equation (16): $A_{\text{max}} = 319.5 + 76.5n$ where, for our purposes, n ranges from 1 (dienyllic cation) to 4 (pentaenyl cation).

Having established the sequences of reactions in Figure 3, it is worthwhile to re-examine the usefulness of the equivalent ratio measurements discussed previously. To do so it must be noted that maximum absorbances of the peaks at 563 and 620 nm of the Zak and L-B reactions are obtained typically in 30–40 min at 25°C. In the Zak reaction it is seen that the generation of the desired tetraenyl cation requires the formation of three additional double bonds in the cholesterol molecule. Reference to Table 2 shows that after the reaction has proceeded for 30 min, an equivalent ratio of Fe$^{2+}$ to cholesterol of 3.07 is obtained, indicating that within this time the formation of the tetraenyl cation is potentially quantitative. On the other hand, the equivalent ratio of SO$_2$ to cholesterol obtained in the L-B reaction after 30 min is only 0.62 (Table 1). This ratio is substantially less than the minimum value of 4 required for complete conversion of cholesterol to the pentaenyl cation. On this basis the L-B procedure would be expected to yield about one-seventh the concentration of the product desired for spectrophotometric measurement, as compared to the Zak reaction. Assuming similar molar absorptivities for the two colored species, this difference would account for the well-known fact that the Zak procedure is about sevenfold as sensitive as the L-B method. This increased sensitivity may be attributed largely to the stabilizing effect on enylic cation formation of the higher sulfuric acid concentration. In general, increasing the sulfuric acid concentration would be expected to improve the stability of each of the carbonium ions formed in the stepwise oxidation of cholesterol, thereby making it much more likely that one could observe carbonium ion formation in the Zak reaction than in the L-B reaction. This hypothesis agrees well with the fact that we saw no absorbance peaks in the visible spectrum of the L-B mixture preceding the appearance of the 620-nm peak normally measured. The subsequent conversion of the compound absorbing at 620 nm to the product absorbing at 410 nm (Figure 3) can be reasonably justified on the basis of the agreement of the experimental absorption peak with the value calculated by the Fiesers’ rules (17) and from the SO$_2$ data obtained. The formation of this type of structure is further supported by the observation that the intensity of the 410-nm peak increases continually with time and the yellow product formed is not extractable into immiscible organic solvents.

Recently obtained mass-spectrometric data further support the mechanisms proposed in Figure 3. In this study, we quenched the reaction of cholesterol with Zak reagent by rapidly dispersing the acid mixture in excess alkali. The sample was then extracted with cyclohexane and mass spectra were obtained directly on the extract. Characteristic peaks were found at mass/charge ratios of 368, 366, 364, and 362—peaks that have been assigned to cholesterol, cholestatriene, cholestetraene, and cholestapentaene, respectively. The largest fractions present were the triene and the tetraene. This distribution was to be expected, because the color reaction was quenched when the absorbance of the 478-nm peak was nearly maximal.

A final observation supporting the proposed mechanisms is found in a recent investigation of the kinetics of the Zak reaction (14). In that study the consecutive pseudo-first-order rate constants are shown to be directly proportional to the square of the Fe$^{3+}$ concentration. This is exactly the expected relationship if the function of Fe$^{2+}$ is to generate double bonds for, in doing so, two electrons must be transferred for each new bond produced.

Finally, we also studied the general applicability of equivalent ratio measurements in determining the extent of the reactions of other steroids in the L-B and Zak procedures. A series of Fe$^{2+}$ and SO$_2$ determinations were made on 5,7-cholestadien-3-ol and 7-cholesten-3β-ol, two compounds that react quite differently in these color systems. Cook (18) has shown that these steroids give about twice and four times as much color, respectively, with the L-B reaction as does cholesterol. Henry (19), on the other hand, has reported that these same compounds give only about a third as much color in the Zak reaction as does cholesterol. As expected, SO$_2$ was generated significantly faster for these compounds than for cholesterol while, conversely, the rates of formation of Fe$^{2+}$ were much slower. In the latter system, minimum reaction times of 5 h are required to obtain the requisite values of 3 for the equivalent ratios. These times can be compared to the 30 min necessary for cholesterol (Table 2). Thus, measurement of equivalent ratios may provide a useful tool for studying why compounds similar to cholesterol react differ-
Table 4. Absorbance Characteristics of Cholesterol Solutions (100 mg/liter) in Various Acetic Acid–Sulfuric Acid Mixtures at 25 °C

<table>
<thead>
<tr>
<th>HOAc: H2SO4 (by vol)</th>
<th>Peak</th>
<th>Shoulder</th>
<th>Isosbestic point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>~300 (very strong); ~450, 500</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.5:1</td>
<td>300, 348*</td>
<td>—</td>
<td>325, 377</td>
</tr>
<tr>
<td>2:1</td>
<td>300 peak showing 269, 280, 295 and 312 fine structure; 348*, 412, 438</td>
<td>438</td>
<td>—</td>
</tr>
<tr>
<td>3:1</td>
<td>269, 280, 295, 312</td>
<td>412, 500</td>
<td>—</td>
</tr>
<tr>
<td>5:1</td>
<td>267, 280, 296, 500</td>
<td>312, 385, 412</td>
<td>450, 610</td>
</tr>
<tr>
<td>10:1</td>
<td>385, 440, 480</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Peak continually decreasing.

ently in terms of the colored products formed.

Although acetic and sulfuric acids are used in different proportions in the L-B and Zak procedures, the study of cholesterol reactions in these simple acid systems provided valuable insight into the mechanisms of these reactions. One of our first experiments was to measure the absorbance spectra of cholesterol in different mixtures of these two acids. Relative HOAc/H2SO4 ratios ranging from 1:1 to 10:1 were used. In general, those mixtures in which the proportion of sulfuric acid was high led to products that absorbed in the ultraviolet, while those in which the proportion of acetic acid was high showed increased absorbance in the visible range. These observations are summarized in Table 4.

One of the most noteworthy features of this study was the similarity between the absorbance spectra of cholesterol in 1.5:1 HOAc/H2SO4 and in the corresponding Zak mixture, in which the same ratio of these acids is used. Thus the decreasing peak at 348 nm and the increasing peak at 412 nm in HOAc/H2SO4 behave similarly to the decreasing 412-nm and increasing 478-nm peaks in the Zak procedure (Figure 2). According to the mechanisms proposed in Figure 3, the conversion of the compound absorbing at 348 nm to the product absorbing at 412 nm is interpreted as the cation of 3,5-diene being oxidized to dienyl cation. Because no Fe³⁺ is present, we can only suggest that the oxidation is due to residual concentrations of SO₃ present. However, we did not attempt to measure SO₃ formation in this system.

A second distinguishing feature of this study was the finger-like peaks observed near 300 nm, which were especially apparent in 3:1 and 5:1 HOAc/H2SO₄ mixtures. Initially, we thought that these peaks reflected the possible formation of the 3,3'-bis-3,5-cholestadiene dimer. Subsequent experiments with a synthesized dimer, however, showed that its absorbance peaks were at longer wavelengths (λ_max(CH₃H₄) = 298, 312, and 323 nm) than the finger-like absorbance peaks observed (λ_max = 269, 280, 295, and 312 nm). Moreover, in these and similar studies, the dimer was characterized by its very low solubility and reactivity in all of the acid mixtures used. In view of these properties and the fact that previous mechanistic studies (4,5,7) were undertaken by using gram amounts of cholesterol, it is not difficult to understand how the dimer was readily isolated and thus considered to be a reaction intermediate rather than a degradation product. Furthermore, our proposal that the colored species measured in the L-B and Zak procedures are carbonium ions of cholestapolyenes is also consistent with the isolation of dimer, because one of the expected modes of disappearance of these ions would be through dimerization.

To determine if any intermediate products could be isolated by solvent extraction and identified by uv spectrophotometry, we extracted the pre-reacted acid mixtures of cholesterol with cyclohexane. Acetic acid, which is also partially extracted and which has an ultraviolet cutoff at about 250 nm, was removed by backwashing the extracts several times with water or dilute base. The absorbance spectra were then observed to have several characteristic absorbance peaks. Only 2,4-cholestadiene (λ_max = 266, 275, and 287 nm) was identified with certainty. Moreover, the orange color present in the prereacted acid layer before extraction was also retained in that layer after extraction.

In a modification of this procedure, a second series of extraction experiments was performed in which cholesterol was first dissolved in cyclohexane and these solutions were equilibrated with the acetic–sulfuric acid mixtures. In this case, spectrophotometric examination of the extracts showed that only 3,5-cholestadiene was formed (λ_max = 228, 235, and 243 nm), and we saw no color in the acid phases. In both of these studies the excellent review by Dorfman (20) on the ultraviolet absorbance of steroids proved extremely valuable. These two extraction experiments suggest that the precursor to color formation in both the Zak and L-B reactions is the initial formation of the carbonium ion of 3,5-cholestadiene, as shown below:

```
HO
+H⁺ → [ ] → [ ] → [ ]
-H₂O

\[
\begin{align*}
\text{HO} & \quad \quad +H^+ \\
\text{[ ]} & \quad \quad \rightarrow \quad [ ] \\
\text{[ ]} & \quad \quad \rightarrow \quad [ ] \\
\end{align*}
\]
```

The nonclassical resonance structure involving the bridgehead C-5 carbon contributes significantly to the stability of this ion. The fact that this dehydrox-
ylation reaction goes easily is clearly demonstrated in the second series of extraction studies. Even though cholesterol is dissolved initially in cyclohexane, the -OH group is sufficiently polar to be protonated at the interface. Subsequent loss of water and a proton yields the 3,5-diene that is observed.

The carbonium ion of 3,5-cholestadiene is shown conclusively to be the reactive intermediate by the following two experiments: (a) If the reactions of cholesterol in acetic–sulfuric acid mixtures are quenched and the cyclohexane extracts then examined spectrophotometrically, at least 80% of the diene formed is apparently the 3,5-derivative. (The quenching was done by rapidly dispersing the acid mixtures into excess base 2–3 min after the cholesterol was added.) (b) Similar studies, starting with 2,4-cholestadiene, show that it also is largely converted to the 3,5-compound, or its carbonium ion, upon dissolution in strong acid. Such acid-catalyzed re-arrangements are not unexpected, however, as evidenced by the work of Allan et al. (21) in the related triterpenoid series, where the mobility of double bonds under acidic conditions has been clearly demonstrated.

The acid-catalyzed dehydroxylation of cholesterol yields predominantly the carbonium ion of 3,5-cholestadiene, together with smaller amounts of 2,4-cholestadiene. All evidence obtained thus far suggests that this carbonium ion is the precursor to the color reactions observed in the Zak and L-B systems. In strongly acidic media such as are used in the Zak reaction, cholesterol is rapidly dehydroxylated, and this is not a limiting factor in the overall rate of the reaction. Consequently, cholesterol, 3,5-cholestadiene, and 2,4-cholestadiene all form color and generate Fe(II) at similar rates under these conditions. If the acidity is decreased, however, as is the case in the L-B system, the rate of dehydroxylation becomes significant. Starting with 3,5-cholestadiene, for example, the amount of product absorbing at 620 nm formed in the L-B reaction is about fourfold that obtained with cholesterol.

To demonstrate the effect of acidity on the rate of dehydroxylation, we equilibrated cyclohexane solutions of cholesterol with acetic–sulfuric mixtures of various proportions and spectrophotometrically determined the 3,5-cholestadiene formed. The results obtained are shown in Figure 4. The rate of formation and the yield of 3,5-diene are greatest at the highest sulfuric acid concentration. The use of an HOAc/H_2SO_4 ratio of 10:1, typical of the L-B conditions, produces dramatic decreases in the rate and the yield of 3,5-cholestadiene. The relative rates obtained on these two-phase systems are believed to be significant, although the absolute rates are not directly applicable to the reactions occurring in the acid phase. Moreover, it should be noted that this two-phase equilibration technique provides a simple means of improving by several fold the potential sensitivity of the L-B reaction. From direct studies of 3,5-cholestadiene in the L-B reaction, we conclude that the maximum increase in sensitivity—assuming complete conversion of cholesterol to the 3,5-derivative—is fourfold.

In conclusion, new evidence has been presented to indicate that the Liebermann–Burchard and Zak color reactions have very similar mechanisms. Following an acid-catalyzed dehydroxylation, the 3,5-cholestadiene and (or) its cation are subjected to a number of step-wise oxidations by either SO_3 or Fe(III), thereby forming a homologous series of cholestapolyene carbonium ions. The stability of each of these new cationic species is primarily a function of the number of conjugated double bonds in the parent polyene and the sulfuric acid concentration. The steroid concentration is also important, because high concentrations can lead to competing reactions such as the dimerization reported by Watanabe (7). Therefore, throughout this study, we tried to use cholesterol concentrations typical of those used in spectrophotometric analyses. This limitation precluded the use of NMR spectroscopy, a technique commonly used to identify intermediates and verify the existence of carbonium ions.

Further work is underway to synthesize and characterize the cholestapolyene intermediates discussed in this paper. Additional studies are intended to examine the relative reactivities and the mechanisms of the reactions of the cholesterol-like compounds routinely found in sera. Of special interest will be those compounds which yield variable amounts of color in the L-B and Zak reactions.

We express our appreciation to Dr. Harry S. Hertz, of the NBS, for making the mass-spectrometric measurements.
References