product which had been reported by Fischer, Bergmann, and Schotte (Ber., 53, 509 (1920)) were restudied. The structures of the two crystalline compounds termed the "triacetyl-
methylglucoside-2-bromohydrcins I and II" were demonstrated as methyl 2-bromo-2-
deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside and methyl 2-bromo-2-deoxy-3,4,6-tri-O-
acetyl-β-D-mannopyranoside, respectively.

2-Bromo-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranosone (X) was prepared as a crys-
talline form. Crystalline, stable bromide (X) was prepared starting from X, and assigned
as 2-bromo-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl bromide which was not identi-
tical with the labile bromide by Fischer, et al.

The nuclear magnetic resonance spectra presented a further proof for the configura-
tions at C1 and C2 in X and XI.

(Received July 22, 1964)

181. Tsutomu Momose, Yo Ueda, and Chiharu Nakakura: Organic
Analysis. LVI.*1 Determination of Free Cholesterol in
Blood Serum. An Application of Perchloric Acid-
Phosphoric Acid-Ferric Chloride Reagent
to Cholesteryl Digitonide.

(Faculty of Pharmaceutical Sciences, Kyushu University*2)

The determination of free cholesterol in blood serum is usually carried out according
to the following procedures: extraction of lipid fraction from serum with organic sol-
vent, precipitation of free cholesterol with digitonin, and colorimetry of the precipitate
by the Liebermann-Burchard reaction or Killiani reaction.

The quantity of the digitonide may also be estimated by a turbidimetry,*1 a colori-
metry with anthrone reagent,*2,9 or by other methods. Digitonin can be substituted either
tomatoine*7 or by another saponin separated from the leaves of Digitalis Purpurea L.,*8
which are claimed to give more sensitive precipitation with cholesterol.

Column chromatography is also shown to be effective for the separation of free cho-
lesterol from lipid fraction,*2,10 and gas chromatography might be a new technique.13,115

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*2 Katakusu, Fukuoka (百崎 勝, 上田 陽, 中倉千春).
The performance of the Kiliani reaction at a low temperature is shown to be useful for the separate determination of free and esterified cholesterol in serum. A chemical treatment of free cholesterol and successive colorimetry of the resultant is also reported to give good results.

In a previous paper we presented a new color reagent for the determination of total cholesterol in blood serum. Thereafter we have examined the applicability of the reagent to the cholesteryl digitonide precipitated from blood serum by a similar method as Schoenheimer and Sperry's one, and the results are now described.

**Experimental**

**Reagents**—Solvents: Me₂CO–EtOH, 1:1; Me₂CO–EtOH–H₂O, 12:12:1; Me₂CO; glacial AcOH. FeCl₃, 0.2%,aq. AcOH solution, and the color reagent are the same as previously described.

Standard cholesterol stock solution, 0.05 mg. per ml.: Dissolve 25.0 mg. of pure cholesterol in 500 ml. of Me₂CO–EtOH–H₂O.

Working standard cholesterol solutions: Dilute 20.0, 40.0, 60.0, and 80.0 ml. of standard cholesterol stock solution with Me₂CO–EtOH–H₂O to give a total volume of 100 ml., respectively. The working standard solutions thus obtained and the standard stock solution have the equivalent concentrations of 25, 50, 75, 100, and 125 mg. per 100 ml. of free serum cholesterol, respectively.

Digitonin solution: Dissolve 1.0 g. of digitonin "Merck" in 100 ml. of 50% EtOH (by volume) at 50–60°C.

**Procedure**—Add slowly 1.00 ml. of serum into about 15 ml. of Me₂CO–EtOH in a 25 ml. volumetric flask with continuous rotation. Heat the flask in a water bath to bring just to boil, and cool. Add Me₂CO–EtOH to the mark, mix the content thoroughly, and filter through a rapid filter paper (Toyopaki, No. 5A) into a glass–stoppered flask. Add 1.00 ml. of digitonin solution to 6.00 ml. of the filtrate in a centrifuge tube (12 ml.), and stir thoroughly with a stirring rod. Remove the rod without contact with the upper part of the tube. Cover the tube with aluminum foil, and leave it at room temperature for 30 min. Then centrifuge the tube for about 30 min. at about 3,500 r.p.m. Remove carefully the clear supernatant liquid by a pipette, add about 4 ml. of Me₂CO to the tube, and stir the content of the tube with the same stirring rod. Remove the rod, and centrifuge the tube again. Remove the supernatant liquid as before, and dry the tube with the rod by placing in a dryer at 110°C for about 30 min. Add 2.00 ml. of glacial AcOH into the hot tube, and stir the content to bring a clear solution. Then add successively 2.00 ml. of 0.2% FeCl₃ aq. AcOH solution and 2.00 ml. of the color reagent. Stir the mixture thoroughly with the rod, and transfer to a test tube. At the same time, prepare a blank tube with a mixture of each 2.00 ml. glacial AcOH, 0.2% FeCl₃ aq. AcOH solution and the color reagent. Heat the test tubes in a boiling water bath for 20 min., cool in running water, and read the absorbance of the sample at 450 mμ on a spectrophotometer with a glass cell of 10 mm. sample path length against the reagent blank. The free cholesterol value is calculated from the calibration curve which is described below.

* Reagent grade chemicals were used.
* To avoid the contamination with H₂O while heating in a boiling water bath.

19 T. Nishima, M. Kimura: This Bulletin, 12, 521 (1964).
Calibration Curve—Pipett three aliquots of 6.00 ml. of each working standard solution and standard stock solution into centrifuge tubes, and add 1.00 ml. of digitonin solution to each tube. The subsequent treatment is identical with the one described under procedure. The absorbances are read at 450 mμ against the reagent blank. The calibration curve thus drawn up is a straight line and is shown in Fig. 1.

Results and Discussion

The absorption spectrum of a cholesteryl digitonide developed with perchloric acid–phosphoric acid–ferric chloride reagent is shown in Fig. 2.

![Graph](image)

**Fig. 2. Absorption Spectra of the Colors Developed by (1) Digitonin and (2) Cholesteryl Digitonide**

![Graph](image)

**Fig. 3. Calibration Curve for Digitonin**

In the colorimetric determination of cholesteryl digitonide by the Liebermann–Burchard reaction or the Kilianni reaction the digitonin gives no coloration with the reagent. With the present reagent, digitonin gives a weak coloration (Fig. 2) and the absorbance of the color obeys Beer's law at 450 mμ (Fig. 3). The cholesteryl digitonide is consisted of equivalent moles of cholesterol and digitonin and color intensities developed by both components are proportional to their concentrations, and therefore, the absorbance of color in the present method of procedure may depend on the concentration of cholesteryl digitonide. In this case the important point in question should be an influence which might be caused by the possible presence of excess digitonin in the cholesteryl digitonide precipitated. To check this influence the absorbance of a color developed by digitonide precipitated from a known amount of cholesterol was compared with the value obtained by calculation. The working standard cholesterol solution whose equivalent concentration was 50 mg. per 100 ml. of serum gave a cholesteryl digitonide of absorbance 0.270. The net amount of cholesterol used in this experiment was 0.12 mg. and should have an absorbance of 0.256 on the calibration curve of total cholesterol determination with perchloric acid–phosphoric acid–ferric chloride reagent. The amount of digitonin used to the formation of digitonide with 0.12 mg. of cholesterol might be about 0.383 mg., which had an absorbance 0.050 from the calibration curve (Fig. 3). Hence the total absorbance was calculated as 0.306. This result not only shows that the possible influence of excess digitonin is very small, but rather indicates that the calibration curve should not be drawn by calculation. The reason of this discrepancy (0.270 versus 0.306) is not clear, but the following discussion would prove the validity of the experimental calibration curve.

The formation of cholesteryl digitonide may not be so affected by the reaction temperature, time, or the addition of acetic acid. Those were proved by the fact that the absorbance of the color developed by digitonide showed no appreciable difference by keeping the reaction mixture at room temperature or in an ice–box (Table I), or by
standing the reaction mixture for 30 minutes or 24 hours with or without addition of aqueous acetic acid to the serum extract prior to the addition of digitonin solution (Table II).

**Table I. Effect of Temperature on the Formation of Cholesteryl Digitonide**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 min. at room temperature</td>
<td>0.780, 0.746</td>
</tr>
<tr>
<td>60 min. in ice-box</td>
<td>0.756, 0.756</td>
</tr>
</tbody>
</table>

**Table II. Effect of Reaction Time on the Formation of Cholesteryl Digitonide, compared in Absorbance**

<table>
<thead>
<tr>
<th>With addition of 3 drops of 10% aqueous acetic acid</th>
<th>Without addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min.</td>
<td>24 hr.</td>
</tr>
<tr>
<td>30 min.</td>
<td>24 hr.</td>
</tr>
<tr>
<td>0.308, 0.315, 0.316, 0.319</td>
<td>0.304, 0.302, 0.322, 0.295</td>
</tr>
<tr>
<td>0.302, 0.302</td>
<td>0.302, 0.314</td>
</tr>
</tbody>
</table>

**Table III. Recovery of Free Cholesterol in the Presence of esterified Cholesterol**

<table>
<thead>
<tr>
<th>Ratio of free to ester</th>
<th>Kind of ester</th>
<th>Equivalent amount of free serum cholesterol (mg./100 ml.)</th>
<th>% of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>present</td>
<td>recovered</td>
</tr>
<tr>
<td>2 : 8</td>
<td>oleate</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>acetate</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>4 : 6</td>
<td>oleate</td>
<td>80</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>acetate</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

A preliminary experiment of the determination of free cholesterol dissolved in acetone-ethanol-water demonstrated a good recovery of 100±2% in the presence of varied amount and kind of esterified cholesterol as shown in Table III.

Table IV presents a result of comparison of the present method to the Schoenheimer and Sperry's method, including the result obtained from the "Hyland Normal Clinical Chemistry Control Serum."

Recovery tests of this method were performed by adding known amounts of cholesterol in serum extract and gave 100±8% recovery as shown in Table V.

**Table IV. Comparison of Present Method to Schoenheimer and Sperry's Method**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Free cholesterol value (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>present method</td>
</tr>
<tr>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
</tr>
<tr>
<td>&quot;Hyland normal clinical chemistry control serum&quot;</td>
<td>44</td>
</tr>
</tbody>
</table>
TABLE V. Recovery Test on Serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Free cholesterol value (mg./100 ml.)</th>
<th>% of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial</td>
<td>added</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>53</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>24</td>
</tr>
</tbody>
</table>

The standard deviation of this method at a level of 46.7 mg. per 100 ml. free cholesterol in serum was 1.63 mg., and the coefficient of variation was 3.50%.

Those data indicate that the new method is also applicable to the separate determination of free cholesterol in serum in a clinical laboratory.

The authors express their gratitude to Dr. Junji Nagai, Central Clinical Laboratory of the University Hospital, for the supply of sera. They are also indebted to Assistant Professor I. Nishioka for helpful discussions.

Summary

Free cholesterol in blood serum was extracted with acetone–ethanol, precipitated with digitonin, and developed with perchloric acid–phosphoric acid–ferric chloride reagent, which was previously used for the determination of total cholesterol in serum.

(Received July 24, 1964)

182. Toshio Kawasaki and Itsuo Nishioka: Digitalis Saponins. II.*

Leaf Saponins of *Digitalis purpurea* L.

(Faculty of Pharmaceutical Sciences, Kyushu University**)

As for the leaf saponins of *Digitalis purpurea* L. digitonin has been the sole one recorded in the literature,*1,2* but recently Akahori and Yasuda*3* reported the presence

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*2 Katakasu, Fukuoka (川崎敏男, 萩田五夫).
*3 It is reported that tigion and ginton are obtained from the leaves of *Digitalis lanata* (R. Tschesche: Ber., 69, 1655 (1936)) and *Dig. ferruginea* (R.M. Appel, O. Gisvold: J. Am. Pharm. Assoc., 43, 215 (1954)), and from those of an unspecified digitalis (F. Kraft: Chem. Zentr., 1911 (1), 1698), respectively.
1 C.C. Keller: Chem. Zentr., 1897 (1), 1211.