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-bromohydrins 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-glucopyranose (X) was prepared as a crystalline form. Crystalline, stable bromide (XI) 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 identical with the labile bromide by Fischer, et al.

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

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 solvent, precipitation of free cholesterol with digitonin, and colorimetry of the precipitate by the Liebermann-Burchard reaction or Kiliani reaction.

The quantity of the digitonide may also be estimated by a turbidimetry,[1] a colorimetry with anthrone reagent,[2,3] or by other methods. Digitonin can be substituted either by tomatine[4~7] 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 cholesterol from lipid fraction,[9~11] and gas chromatography might be a new technique.[12,13]

*[1] Part LV : Clin. Chem., in press.
*[2] Katakasu, Fukuoka (百瀬　勉, 上田　陽, 中倉千春).
1) A. F. Krinitskii : Lab. Delo, **7**, 27 (1961); C. A., **56**, 1706 (1962).
2) G. V. Vahouny, C. R. Borja, R. M. Mayer, C. R. Treadwell : Anal. Biochem., **1**, 371 (1960).
3) J. R. Goodman, L. P. Jarnagin, R. M. Meier, I. A. Shonley : Anal. Chem., **35**, 760 (1963).
4) J. J. Kabara, J. T. McLaughlin, C. A. Riegel : *Ibid.*, **33**, 305 (1961).
5) R. K. Rinehart, S. E. Delaney, H. Sheppard : J. Lipid Res., **3**, 383 (1962); C. A., **58**, 1716 (1963).
6) T. C. Huang, V. Wefler, A. Raftery : Anal. Chem., **35**, 1757 (1963).
7) C. H. Edwards, G. A. Edwards, E. L. Gadsden : *Ibid.*, **36**, 420 (1964).
8) T. Kawasaki, I. Nishioka, J. Nagai, et al. : Shishitsu-Seikagaku Kenkyu, **1964**, 49.
9) R. Pierre, M. Pierre : Repert. Med. Prat. Ser. Biol., **1**, 13 (1961); C. A., **56**, 5048 (1962).
10) B. G. Creech, B. W. Sewell : Anal. Biochem., **3**, 119 (1962); C. A., **56**, 15757 (1962).
11) D. Webster : Clin. Chim. Acta, **8**, 19 (1963); C. A., **59**, 9065 (1963).
12) C. J. W. Brooks, J. S. Young : Biochem. J., **84**, 53p (1962); C. A., **57**, 10130 (1962).
13) L. P. Cawley, B. O. Musser, C. S. Campbell, W. Faucette : Am. J. Clin. Pathol., **39**, 450 (1963); C. A., **59**, 11862 (1963).

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.[14~18] A chemical treatment of free cholesterol and successive colorimetry of the resultant is also reported to give good results.[19]

In a previous paper[20] 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,[21] and the results are now described.

Experimental

Reagents[*3]——Solvents : Me_2CO-EtOH, 1:1; Me_2CO-EtOH-H_2O, 12:12:1; Me_2CO; glac. AcOH.

$FeCl_3$, 0.2%, aq. AcOH solution, and the color reagent are the same as previously described.[20]

Standard cholesterol stock solution, 0.05 mg. per ml. : Dissolve 25.0 mg. of pure cholesterol[22] in 500 ml. of Me_2CO-EtOH-H_2O.

Working standard cholesterol solutions : Dilute 20.0, 40.0, 60.0, and 80.0 ml. of standard cholesterol stock solution with Me_2CO-EtOH-H_2O 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 1.00 g. of digitonin " Merck " in 100 ml. of 50% EtOH (by volume) at 50~60°.

Procedure——Add slowly 1.00 ml. of serum into about 15 ml. of Me_2CO-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 Me_2CO-EtOH to the mark, mix the content thoroughly, and filter through a rapid filter paper (Toyoroshi, 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 Me_2CO to the tube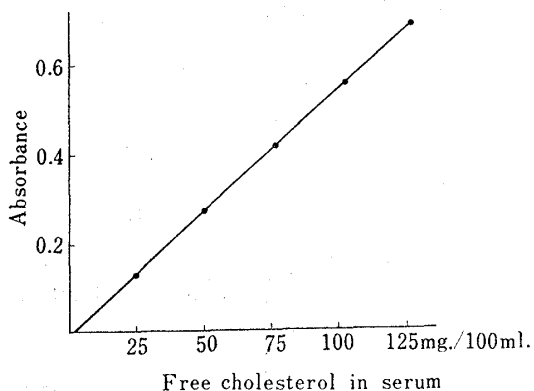, and stir the content of the tube with the same stirring rod. Remove the rod, and centrifuge the tube again. Remove the supernatant liquid as before, and dry the tube with the rod by placing in a dryer at 110° for about 30 min. Add 2.00 ml. of glac. AcOH into the hot tube, and stir the content to bring a clear solution. Then add successively 2.00 ml. of 0.2% $FeCl_3$ aq. AcOH solution and 2.00 ml. of the color reagent. Stir the mixture thoroughly with the rod, and transfer to a test tube.[*4] At the same time, prepare a blank tube with a mixture of each 2.00 ml. glac. AcOH, 0.2% $FeCl_3$ 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.

Fig. 1. Calibration Curve for Cholesteryl Digitonide

[*3] Reagent grade chemicals were used.

[*4] To avoid the contamination with H_2O while heating in a boiling water bath.

14) M. L. Girard, E. F. Assous : Ann. biol. clin. (Paris), 20, 335 (1962); C. A., 57, 4965 (1962).

15) *Idem* : Compt rend., 254, 3752, 3912 (1962); C. A., 57, 6251 (1962).

16) *Idem* : Bull. soc. chim. biol., 44, 1021 (1962); C. A., 58, 4803 (1963).

17) *Idem* : Ann. biol. clin. (Paris), 20, 973 (1962); C. A., 59, 1946 (1963).

18) P. Georgescu, E. Constantinescu : Acad. rep. populare Romine, Studii cercetări Biochim., 5, 595 (1962); C. A., 59, 3072 (1963).

19) T. Nishina, M. Kimura : This Bulletin, 12, 521 (1964).

20) T. Momose, Y. Ueda, K. Yamamoto, T. Masumura, K. Ohta : Anal. Chem., 35, 1751 (1963).

21) R. Schoenheimer, W. M. Sperry : J. Biol. Chem., 106, 745 (1934); " Standard Methods of Clinical Chemistry," 1, 43 (1953), Academic Press, New York.

22) " Organic Synthesis," 35, 43 (1955), Wiley, New York.

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.
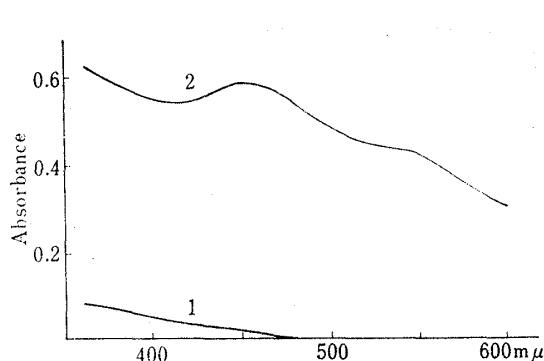

Fig. 2. Absorption Spectra of the Colors Developed by (1) Digitonin and (2) Cholesteryl Digitonide
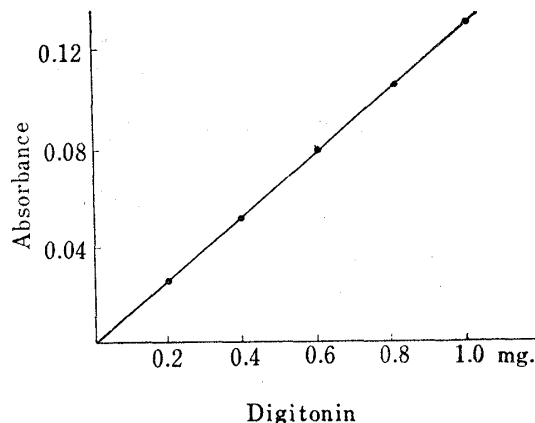

Digitonin

Fig. 3. Calibration Curve for Digitonin

In the colorimetric determination of cholesteryl digitonide by the Liebermann-Burchard reaction or the Kiliani 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.[20] 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

Temperature	Absorbance
60 min. at room temperature	0.780, 0.746
60 min. in ice-box	0.756, 0.756

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

With addition of 3 drops of 10% aqueous acetic acid		Without addition	
30 min.	24 hr.	30 min.	24 hr.
0.308, 0.315, 0.316, 0.302	0.311, 0.319	0.304, 0.302, 0.322, 0.295	0.310, 0.314, 0.302, 0.314

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

Ratio of free to ester	Kind of ester	Equivalent amount of free serum cholesterol (mg./100 ml.)		% of recovery
		present	recovered	
2 : 8	oleate	40	39	98
	acetate	40	41	102
4 : 6	oleate	80	79	99
	acetate	80	80	100

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

Serum	Free cholesterol value (mg./100 ml.)	
	present method	Schoenheimer and Sperry's method
1	47	43
2	55	52
3	59	52
4	53	52
5	58	55
"Hyland normal clinical chemistry control serum"	44	47.5 (from the list)

TABLE V. Recovery Test on Serum

| Serum | Free cholesterol value (mg./100 ml.) | | | % of recovery |
	initial	added	total free cholesterol found	
6	47	24	69	92
		48	94	98
7	40	24	62	92
		48	88	100
8	59	24	81	92
		48	105	96
9	53	24	79	108
		48	105	108
10	58	24	82	100
		48	107	102

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.

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.

182. Toshio Kawasaki and Itsuo Nishioka : Digitalis Saponins. II.[*1]
Leaf Saponins of Digitalis purpurea L.

(Faculty of Pharmaceutical Sciences, Kyushu University[*2])

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

*1 Major part of this work was presented at the Annual Meeting of Pharmaceutical Society of Japan, Shizuoka, Nov. 2, 1962. Part I : T. Kawasaki, I. Nishioka : This Bulletin, 12, 1250 (1964).

*2 Katakasu, Fukuoka (川崎敏男, 西岡五夫).

*3 It is reported that tigonin and gitonin were obtained from the leaves of Digitalis lanata (R. Tschesche : Ber., 69, 1665 (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 (I), 1698), respectively.

1) C. C. Keller : Chem. Zentr., 1897 (I), 1211.

2) A. Akahori, F. Yasuda : Shôyakugaku Zasshi, 15, 149 (1961).