Table V. Recovery Test on Serum

Serum	Free cholesterol value (mg./100 ml.) initial	added	total free cholesterol found	% of recovery
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.

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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.

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

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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).

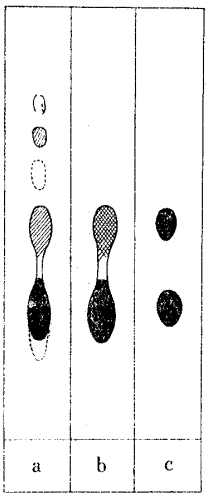

Fig. 1. Paper Chromatograms

Solvent: BuOH-AcOH-H_2O (4:1:5)
a: BuOH extract
b: Crude saponin
c: Commercial "digitonin" (reference)

of tigogenin and gitogenin and the absence of digitogenin in the acid hydrolyzate of the leaves extract and Weiss and Manns[3] also described that no digitogenin was found. Therefore the existence of digitonin seems unlikely and tigogenin- and gitogenin-glycosides are expected.[2,3]

We should like now to describe the survey of steroid saponins in the *Dig. purpurea* leaves and the isolation and the characterization of two new saponins, tigogenin tetraglycoside and gitogenin tetraglycoside which is not identical with gitonin.*1

When butanol extract of the remainings from the manufacture of cardiac glycosides from *Dig. purpurea* leaves was subjected to an usual paper chromatography two saponins (I) (less polar, much smaller amount) and (II) were detected besides unknown substances (Fig. 1), and the sapogenin fraction of hydrolyzate of the extract was found to contain tigogenin (about 15%) and gitogenin (about 85%). The butanol extract was subsequently treated with cholesterol in ethanol and the resultant insoluble molecular complex was decomposed with pyridine to afford a crude saponin. This crude saponin was shown by paper chromatography (Fig. 1) to be composed of the above two, I and II, and the respective yield of tigogenin and gitogenin from the hydrolyzate was almost same as that from the original butanol extract.*4 In comparing the Rf values of I and II on a paper chromatogram with those of seed saponins*1 run in paralell they seemed tetraglycosides and pentaglycosides, respectively, but the crude saponin was shown by thin-layer chromatography[4] on silica gel G to be a mixture of eight saponins (four pairs) (Fig. 2), and when I and II were separated with the aid of chromatography on alumina in the same way as for the seed saponins*1 and examined by thin-layer chromatography*5 (Fig. 2) it was found that I contains the least polar pair of saponins, I-1 and I-2, and II a mixture of other three pairs. The hydrolyzates

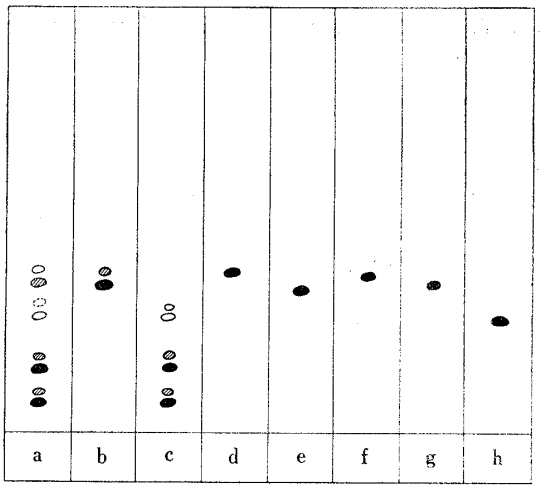

Fig. 2. Thin-layer Chromatograms on Silica Gel G

Solvent: $CHCl_3$-MeOH-H_2O (65:35:10)
a: Crude saponin
b: Saponin I
c: Saponin II
d: Desgalactotigonin
e: F-gitonin
f: Gitonin (reference)
g: Desglucodigitonin (reference)
h: Digitonin (reference)

*4 At first I was assumed to be gitonin*1 and II to be tigonin*3 and since cholesterol-tigonide is known to have fifteen times as much solubility in 95% EtOH as gitonide (P. Bladon: "Cholesterol", Ed. by R.P. Cook, 81 (1958), Academic Press Inc., New York), it was hoped that the saponin obtained *via* molecular complex might be almost pure gitonin, but it was not the case. However, the relative amount of I compared with II (on a paper chromatogram) in the crude saponin was apparently much increased than that in the BuOH extract indicating that the complex of I has less solubility than that of II.

*5 By the Tschesche method (chromatography on formamide impregnated paper)[5] I was separated into the component saponins but II gave no satisfactory result.

*6 The rotation ($[\alpha]_D^{25}$ −100° (C=0.15, pyridine)) described previously[4] should be corrected.

3) F. Weiss, O. Manns: Pharm. Zentralhalle, **98**, 437 (1959).
4) T. Kawasaki, K. Miyahara: This Bulletin, **11**, 1546 (1963).

of I and II showed in either case the presence of both tigogenin and gitogenin suggesting that they are respectively a mixture of the glycosides of these two sapogenins. The preparative separation of the component saponins of I was then carried out by partition on formamide impregnated cellulose powder column*1 principally according to the Tschesche method[5] and I-1 and I-2 were isolated in pure state (Fig. 2). I-1 was further purified by recrystallization from methanol-chloroform or from dilute ethanol to give a crystalline solid, m.p. 284~286° (decomp.), $[\alpha]_D^{28}$ −64.0°,*6 and I-2 was recrystallized from butanol saturated with water to provide fine needles, m.p. 252~255° (decomp.), $[\alpha]_D^{25}$ −58.5°. Complete hydrolyses of I-1 and I-2 followed by qualitative and quantitative determination of the products indicated that the former is a tigogenin tetraglycoside and the latter a gitogenin tetraglycoside and that their sugar moieties are equally composed of one mole each of D-galactose and D-xylose and two moles of D-glucose. Since I-1 is a new saponin having one mole less galactose than tigonin and I-2 being different in its sugar composition from that (2 galactose+glucose+xylose) of gitonin*1 in the *Dig. purpurea* seeds, they are named desgalactotigonin and F-gitonin, respectively. It is interesting to note that these two saponins have the same sugar composition which is identical with those of tomatine and demissine[6] and is different*7 from those of tetraglycosides*1 in the seeds of the same plant species.

Although the isolation and characterization of the six component saponins of II are still under investigation, taking into account their Rf values on thin layer the least polar pair (vary small amounts) is assumed to be pentaglycosides, the next one (major saponins) to be hexa- and the most polar one to be hepta-glycosides, and on the basis of their coloration on silica gel G with sulfuric acid and by analogy to the tetraglycosides each pair is presumed to consist of tigogenin- (less polar) and gitogenin-glycosides, both of which might have the same sugar composition.

Experimental*8

Examination of the Butanol Extract*9 of the Remainings from the Manufacture of Cardiac Glycosides from *Digitalis purpurea* Leaves——By paper chromatography (P. C.) two saponins, Rf 0.51 (I)±,0.27 (II) ++, and five accompanying compounds, Rf 0.70, 0.60, 0.22 (visualized under UV light without spray reagent) and Rf 0.77, 0.70 (in trace amounts, giving a similar color reaction to that of saponin), were detected (Fig. 1). The sapogenin fraction of the hydrolyzate showed two spots on a paper chromatogram (Rf 0.76±, 0.35 +). Sapogenins were separated by chromatography on alumina into Fr. 1 (Benzene-$CHCl_3$ (3:1)), Rf 0.75 (yield : 10~15%) and Fr. 2 ($CHCl_3$-MeOH (50:1)), Rf 0.35 (yield : 85~90%). Each fraction was recrystallized from MeOH to give needles, m.p. 208~210° and m.p. 276~278°, respectively, and identified with authentic samples*9 of tigogenin (m.p. 207~210°) and gitogenin (m.p. 274~278°) by mixed melting point and co-chromatography.

Crude Saponin——Above BuOH extract (50 g.) in MeOH (700 ml.) was boiled with decolorizing carbon for 30 min., filtered and the filtrate was evaporated *in vacuo* to dryness. The residue was treated with $CHCl_3$ (200 ml.) and insoluble substance was dissolved in 90% EtOH (500 ml.). Cholesterol (10 g.) in 99% EtOH (150 ml.) was added to the above solution, boiled on a water bath for 10~20 min. and left overnight

*7 The question of the respective identity of tigonin and "gitonin" from the leaves of other digitalis species*3 with "tigonin" and gitonin of the purpurea seeds still remains open. Recently it has been reported (R. Tschesche, G. Balle : Tetrahedron, **19**, 2323 (1963)) that a tigogenin pentaglycoside, lanatigonin I, from the lanata seeds is not identical with tigonin from the leaves of the same plant.

*8 Paper chromatography (P. C.) of sugars, sapogenins and saponins, hydrolysis of saponins and qualitative and quantitative determinations of the products were all carried out in the same ways as described in Part I*1 of this series. Thin-layer chromatography (T. L. C.) of saponins was conducted as reported before[4] using $CHCl_3$-MeOH-H_2O (65:35:10). A relative amount of each substance detected on a chromatogram is represented as +++, ++, +, ± by visual comparison of the intensity and the area of the spots.

*9 Supplied by Shionogi and Co., Ltd. The leaves were collected in October~November.

5) R. Tschesche, G. Wulff : Chem. Ber., **94**, 2019 (1961); *Idem* : Tetrahedron, **19**, 621 (1963).
6) R. Kuhn, I. Löw, H. Trischmann : Chem. Ber., **90**, 203 (1957).

in a refrigerator. The precipitates formed were collected by filtration, washed with EtOH (100 ml.) and Et_2O (150 ml.),successively, and dried. Yield : 10～50%. The cholesterol complex (10 g.) in absolute pyridine (60 ml.) was heated on a water bath for 1 hr., cooled, Et_2O (600 ml.) added and the precipitates were collected by filtration, washed with Et_2O and dried. Yield of the precipitates from cholesterol complex : 65～70%. They were extracted with warm $CHCl_3$-MeOH (1:1), the evaporation residue was dissolved in hot MeOH (250 ml.), boiled with decolorizing carbon (3 g.) for 30 min., filtered and the filtrate was evaporated *in vacuo* and dried to give a crude saponin as a brownish white powder. Examined by P.C. : Rf 0.51 (I) ±, 0.27 (II) + (reference compounds run in parallel : purpurea seed saponin (I), 0.51 : (II), 0.30) (Fig. 1). Examined by T.L.C. : Rf 0.38 (dark brown) +, 0.35 (dark purple) ++, 0.30 ±, 0.28 (dark purple) +, 0.18 (dark brown) ++, 0.15 (dark purple) +++, 0.10 (dark brown) +, 0.08 (dark purple) ++ (reference compounds run in parallel : gitonin, Rf 0.37; desglucodigitonin, 0.36; digitonin, 0.27) (Fig. 2). The sapogenin fraction of the hydrolyzate revealed two spots (Rf 0.76, 0.35) on a paper chromatogram and separated into tigogenin (about 15%) and gitogenin (about 85%) by alumina chromatography and identified with authentic samples.

Fractionation of Crude Saponin into Saponins (I) **and** (II)——Crude saponin was chromatographed on alumina in the same way as for seed saponins reported in the preceding paper.*1 An example is as follows. Crude saponin (3.0 g.) was separated into three portions : (A), (I) 340 mg.; (B), (I) +, (II) ±, 290 mg.; (C), (I) ±, (II) +, 1330 mg. (C) was rechromatographed and (A′) ((I) +, (II) ±, 196 mg.), (B′) ((I) ±, (II) +, 230 mg.) and (C′) ((I) ±, (II) ++, 890 mg.) were obtained. (C′) was further chromatographed on alumina to give (A″) ((I) ±, (II) +, 220 mg.) and (B″) ((II), 570 mg.). Portions (A) and (B″) were regarded as paper chromatographically homogeneous saponins, (I) and (II), respectively.

Saponin (I) : Examined by T.L.C. (Fig. 2) : Rf 0.37 ±, 0.35 +. The saponin of Rf 0.37 is tentatively named I-1 and that of 0.35 I-2. Examination by P.C. of the sapogenin fraction of the hydrolyzate : Rf 0.76 ±, 0.35 +.

Saponin (II) : Rf values on thin layer : 0.31 ±, 0.28 +, 0.18 ++, 0.15 +++, 0.10 +, 0.07 ++ (Fig. 2). P.C. of sapogenin fraction : Rf 0.77 ±, 0.35 +.

Preparative Separation of I-1 and I-2——Saponin (I) (150 mg.) was chromatographed on cellulose powder impregnated with formamide in the same manner as reported before*1 using $CHCl_3$-tetrahydrofuran-pyridine (10:10:2)/formamide (4) as the solvent and each fraction (2 ml.) was evaporated *in vacuo*, extracted with $CHCl_3$-MeOH and checked by T.L.C. : Fr. 16～25, I-1, 20 mg.; Fr. 26～30, I-1, I-2, 30 mg.; Fr. 31～50, I-2, 60 mg.

Saponin (**I-1**) (**Desgalactotigonin**)——Fr. 16～25 was recrystallized from MeOH-$CHCl_3$ to give a crystalline solid, m.p. 284～286° (decomp.) (on a Kofler block, uncorr.), $[\alpha]_D^{28}$ −64.0° (c=0.50, pyridine).*6 Thin-layer chromatographically pure (Fig. 2). *Anal.* Calcd. for $C_{50}H_{82}O_{22}\cdot 2H_2O$ (tigogenin + 3 hexose + pentose, dihydrate) : C, 56.06; H, 8.09. Found : C, 56.38; H, 8.57. Examination of the hydrolyzate by P.C. : aglycone, Rf 0.76; sugar, Rg*1 0.85, 1.00, 1.23. Yields of the hydrolysis products : aglycone, 38%; total sugar, 63% (calcd. : aglycone, 39%; total sugar, 64%). Molar ratio of glucose, galactose, and xylose : 2.0:1.0:1.0. The aglycone was recrystallized from MeOH to give needles, m.p. 207～211°, which was identified with authentic sample of tigogenin.

Saponin (**I-2**) (**F-gitonin**)——Fr. 31～50 was recrystallized from BuOH saturated with H_2O to give fine needles, m.p. 252～255° (decomp.) (on a Kofler block, uncorr.), $[\alpha]_D^{25}$ −58.5° (c=0.53, pyridine). Thin-layer chromatographically pure (Fig. 2). *Anal.* Calcd. for $C_{50}H_{82}O_{23}\cdot 2H_2O$ (gitogenin + 3 hexose + pentose, dihydrate) : C, 55.23; H, 7.97. Found : C, 55.63; H, 8.25. Examination of the hydrolyzate by P.C. : aglycone, Rf 0.35; sugar, Rg 0.85, 1.00, 1.23. Yields of the hydrolysis products : aglycone, 39%; total sugar, 62% (calcd. : aglycone, 40%; total sugar, 64%). Molar ratio of glucose, galactose and xylose : 2.1:1.0:1.0. The aglycone was recrystallized from MeOH to give needles, m.p. 275～278°, which was identified with gitogenin.

The authors thank Shionogi Co., Ltd. for the butanol extracts of the digitalis leaves and the authentic samples of tigogenin and gitogenin, Prof. T. Momose and Dr. Y. Mukai for the kind advices and helps in the micro determination of sugars, Miss J. Korenaga for technical assistances, and Mr. M. Shido for elemental analysis. The work was supported in part by a Grant-in-Aid of Scientific Research from the Ministry of Education, to which the authors are also grateful.

Summary

As the leaf saponins of *Digitalis purpurea* L. two saponins, I and II, were detected on a paper chromatogram and isolated by column chromatography on alumina. With the aid of thin-layer chromatography I was found to be composed of two saponins, I-1 and I-2, while II to be a mixture of six (three pairs). I-1 and I-2 were obtained in pure

state according to the Tschesche method and characterized, respectively, as tigogenin- and gitogenin-tetraglycosides both of which equally have one mole each of D-galactose and D-xylose and two moles of D-glucose as the sugar moieties. I-1, m.p. 284~286° (decomp.), $[\alpha]_D^{28}$ −64.0°, was named desgalactotigonin and I-2, m.p. 252~255° (decomp.), $[\alpha]_D^{25}$ −58.5°, F-gitonin. Three pairs of saponins in II were assumed to be penta-, hexa- and hepta-glycosides and each pair was presumed to consist of tigogenin- and gitogenin-glycosides both of which might have the same sugar composition.

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183. Masaaki Horiguchi, Mitsuo Ishida, and Nobuyuki Higosaki : Determination of Some Organophosphorous Insecticides by Gas-Liquid Partition Chromatography.

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Recently, many reports on gas chromatography of insecticides have been published with advances in gas chromatographic techniques. The electron capture type detector[1] was preferably used for the determination of insecticide residue in these papers.[2~6]

Organochlorine insecticides have been chiefly investigated, while only a few studies on organophosphorous insecticide have been reported.[3,7~11]

Many kinds of organophosphorous insecticides have been widely used in agriculture and in the other fields. The qualitative and quantitative analysis of these compounds required very complicated procedures, so the analysis of mixtures was quite difficult. Therefore, the application of gas chromatographic technique to a rapid and simplified determination of these organophosphorous compounds was carried out. Nine organophosphorous compounds were selected from various kinds of insecticides, and good analytical results were obtained from the following investigation.

The insecticides selected were as follows :

(1) O,O-Dimethyl O-(*p*-nitrophenyl)phosphorothioate. (Methyl Parathion)
(2) O,O-Diethyl O-(*p*-nitrophenyl)phosphorothioate. (Parathion)
(3) O,O-Dimethyl S-[1,2-bis(ethoxycarbonyl)ethyl]phosphorodithioate. (Malathion)
(4) O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl)phosphorothioate. (Diazinon)
(5) O,O-Dimethyl S-(N-methylcarbamoylmethyl)phosphorodithioate. (Dimethoate)
(6) O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)phosphorothioate. (Sumithion)

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