Determination of Glucose—A mixture of 28.95 mg. of the crystalline substance A-Ⅷ (m.p. 251～254°) in a solution of 1 : 1 mixture of dioxane and water, containing 3.5% of HCl, was refluxed in CO_2 atmosphere for 6 hrs. on a water bath of 90～95°, the solution was concentrated to one-half the original volume under a reduced pressure and below 50°, and extracted with $CHCl_3$ to remove the aglycone. The $CHCl_3$ extract was washed with water, washing was combined with the aqueous layer, and treated with Amberlite IR-4B. The effluent was concentrated under a reduced pressure and the syrupy residue was dissolved in water in a 2-cc. measuring flask and diluted to the mark. This solution was submitted to paper chromatography by spotting 0.1 cc. of this solution on Toyo Roshi No. 50 and developed by the ascending method with the same solvent as that used for the development of sugars. The spot of glucose was cut out, extracted, and the extracted solution was concentrated. This concentrated extract was treated by the method of Borel and others and its optical density was measured at 520 mμ by a spectrophotometer. The same measurement was carried out with digitalinum verum and the results obtained are listed in Table II.

Summary

Of the unknown cardioglycosides contained in the seeds of *Digitalis purpurea* reported in the preceding paper, substance A-VIII was isolated in crystalline state. This substance occurs as a very bitter needle crystals, m.p. 252～254° (Kofler, uncorr.); $[\alpha]_D^{21}$ −3.1°, $C_{42}H_{66}O_{19} \cdot 2H_2O$; negative to the Keller-Kiliani reaction, sulfuric acid layer in carmine red, and positive to Legal and Raymond reactions; U.V. λ_{max}^{EtOH} 218 mμ (log ε 4.21). Its hydrolysis with 3.5% hydrochloric acid afforded dianhydrogitoxigenin and the sugar portion was found to be digitalose and glucose by paper partition chromatography. Determination of glucose by the Borel-Hostetteler-Devel method showed the presence of two moles. This substance was found to be a new glycoside, a triglycoside, of gitoxigenin series, and was named gitostin. A structure of gitoxigenin-glucosido-glucosido-digitaloside for gitostin as the gitoxigenin-series glycoside corresponding to odoroside-G was forwarded.

(Received January 11, 1957)

U. D. C. 547.918 : 582.951.6

28. Atsuji Okano, Kazuhiko Hoji, Tōsaku Miki, and Kazuo Miyatake :

Studies on the Constituents of *Digitalis purpurea* L. IV.[1] Enzymatic Decomposition of Gitostin.

(*Yanagishima Research Laboratory, Daiichi Seiyaku Co., Ltd.**)

It was shown in the preceding paper[1] that a new cardioglycoside, gitostin, had been isolated from the leaves of *Digitalis purpurea* L., and that this glycoside is a triglycoside possessing gitoxigenin as the aglycone and one mole of digitalose and two moles of glucose as the sugars. It was assumed from the bonding order of the sugars in odorotrioside-G[2] that the structure of gitostin would be gitoxigenin-glucosido-glucosido-digitaloside, with one more mole of glucose bonded to the terminal glucose in digitalinum verum.

Reichstein and others utilized enzymatic hydrolysis in their structural studies on cardioglycosides and revealed the structure of the sugar portion. Stoll also used the autoenzyme[3] taken out of fresh digitalis leaves for the enzymatic decomposition of true glycosides such as digilanid and purpurea glycoside and also examined the manner

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1) Part Ⅲ : This Bulletin, **5**, 163(1957).
2) A. Rheiner, A. Hunger, T. Reichstein : Helv. Chim. Acta, **35**, 687(1952).
3) A. Stoll, W. Kreis : *Ibid.*, **16**, 1390(1933).

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of hydrolysis by the use of enzymes[4] and molds[5] present in various digestive organs of animals. Nawa[6] also used autoenzyme for the glycosides of *Rhodea japonica*. There are also several reports on the isolation and hydrolysis of scillarenase[7] and strophanthobiase.[8]

In selecting the enzyme for the present case, considerations were made on the absence of specificity to various glycosides composed of structurally different aglycones and easy availability. For such reasons, Schneckenferment[9] used by Reichstein and others was selected. These workers employed this enzyme for numerous glycosides but the example of its use in glycosides of gitoxigenin series was the isolation of strospeside and digitalinum verum by its application to digitalinum verum monoacetate.[10] They reported that this enzyme effected rapid deacetylation, followed by gradual liberation of glucose.

Many reports have been published on Japanese snails but the enzyme was isolated in the present series of experiments according to the method of Reichstein and others.[9] The intestines of comparatively large (diam. 3~5 cm.) snail (*Euhadra quaesita* DESHAYES) were taken out, taking care not to lose intestinal fluid, placed in cold (ca. −30°) acetone, and lyophilized. This was ground, washed several times with cold acetone, repeatedly centrifuged, and the precipitated enzyme powder, including tissue pieces, was stored in a desiccator in a cold, dark place. This crude, powdered enzyme was extracted extemporaneously with acetate buffer (pH 5.4) at room temperature, centrifuged, and the clear supernatant was used as the enzyme solution. This enzyme solution was applied to digitalinum verum and strospeside was formed, and the application to digitalinum verum monoacetate afforded strospeside and digitalinum verum, thereby confirming the fact that this enzyme possessed the same activity as the Schneckenferment from the Weinbergschnecke, described in the literature.[9]

This enzyme solution was applied to gitostin and strospeside was obtained as anticipated. In this case, gitostin is completely hydrolyzed but it was found through paper partition chromatography that a minute amount of digitalinum verum was present in it. Therefore, examinations were made on the conditions for separating digitalinum verum from gitostin.

Reichstein and others[10] found that the velocity of glucose liberation by the snail enzyme was slower than the glucosidase obtained from *Adenium multiflorum*, therefore required longer time for complete hydrolysis, and the decomposition of digitalinum verum monoacetate and of odorobioside-G monoacetate stopped in the stage of deacetylation, affording digitalinum verum and odorobioside-G, rather than strospeside and odoroside-H.

In the present series of experiments, it was also found that the original glycoside was partly recovered during hydrolysis of digitalinum verum, but no such recovery was observed in the case of gitostin. It was therefore assumed that one mole of glucose is rapidly removed from gitostin but it required more time for the hydrolysis of glucose from the digitalinum verum so formed. The state of hydrolysis of gitostin was therefore examined by paper partition chromatography and using a more milder hydrolytic conditions, by carrying out the hydrolysis in distilled water at pH 5.6~6.4 during a short period, digitalinum verum was obtained in a good yield of approximately 70%.

4) A. Stoll, J. Renz: Helv. Chim. Acta, **34**, 782(1951).
5) A. Stoll, J. Renz, A. Brack: *Ibid.*, **34**, 397(1951).
6) H. Nawa: J. Pharm. Soc. Japan, **72**, 989(1952).
7) A. Stoll, W. Kreis, A. Hoffmann: Z. physiol. Chem., **222**, 24(1933).
8) J. A. Jacobs, A. Hoffmann: J. Biol. Chem., **69**, 153(1926).
9) O, Schindler, T. Reichstein: Helv. Chim. Acta, **34**, 68(1951).
10) W. Rittel, A. Hunger, T. Reichstein: *Ibid.*, **35**, 434(1952).

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From such a fact, it was learned that the hydrolysis of gitostin proceeds stepwise, first by the rapid hydrolysis of one mole of glucose to form digitalinum verum and further liberation of one mole of glucose to give strospeside. Reichstein and others used strophanthobiase for such stepwise hydrolysis but the present writers found that similar hydrolysis can be effected by the snail enzyme, as shown above.

The foregoing results have proved that gitostin has a structure of gitoxigenin–glucosido–glucosido–digitaloside and is a glycoside formed by the addition of two moles of glucose to strospeside or one mole of glucose to digitalinum verum.

Nerium odorum contains odorotrioside–G but not odorobioside–G and in its stead, contains odoroside–F[11] (graciloside) formed by the addition of glucose to C_2 of digitalose. Since the partial enzymatic decomposition of gitostin afforded only digitalinum verum and not a diglycoside that differs in properties, it may be assumed that the sugar bonding seen in the glycosides of *Nerium odorum* does not exist in those of digitalis. Assuming that the terminal glucose in gitostin is bonded to the terminal glucose in digitalinum verum, relative structures shown in Chart 1 are forwarded. Mutual bonding of glucose is not known but, considering the conditions used for acid hydrolysis of gitostin,[1] a pyranoside and not furanoside was thought to be the case, and the glucose in β–bonding, using the calculations of Klyne indicated in Table I.

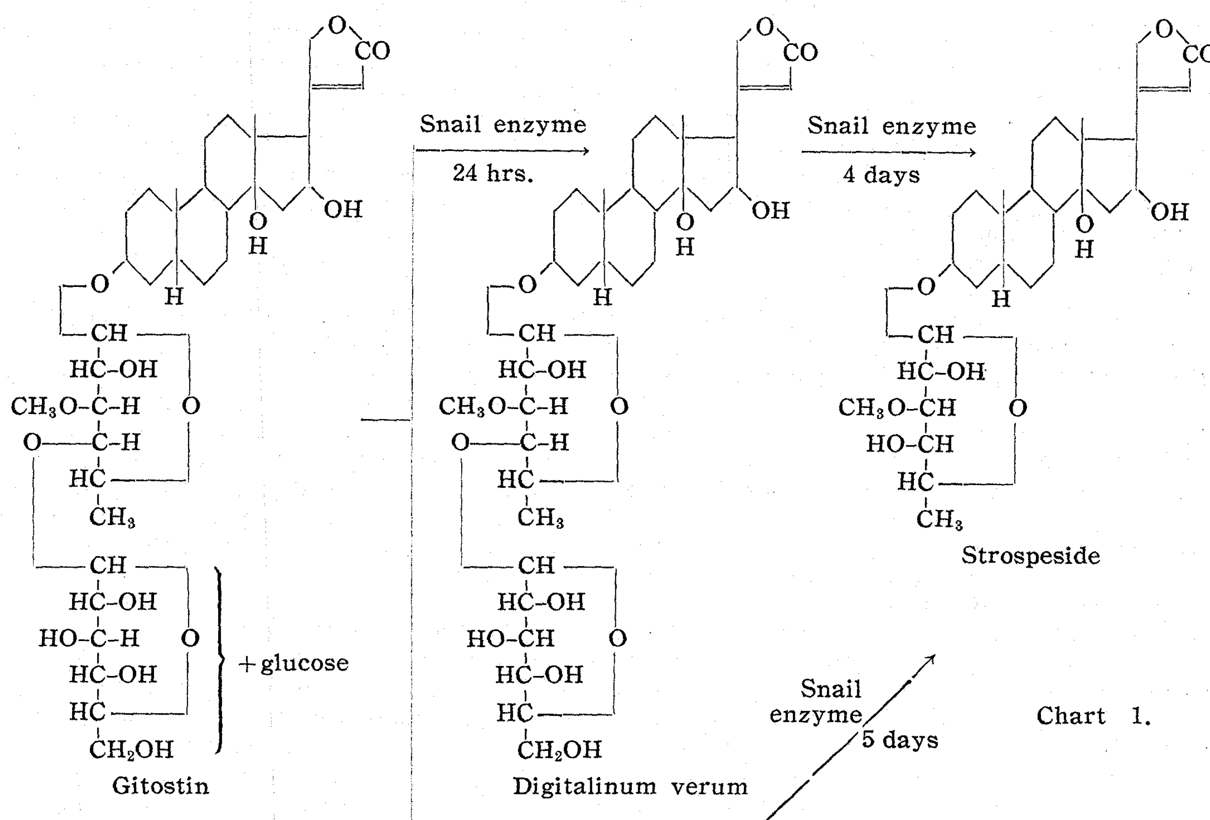

Chart 1.

TABLE I.

Substance	$[\alpha]_D$	$[M]_D$ Calc'd
Gitostin	$[\alpha]_D^{21}$ $-3.4°\pm2°$	$-30.9°\pm18.2°$
Digitalinum verum	$[\alpha]_D^{28}$ $+0.9°\pm2°$	$+6.5°\pm14.6°$
$\Delta[M]$		$-37.4°\pm32.8°$
α-Methyl-*d*-glucoside-(1,5)	$[\alpha]_D^{20}$ $+158.9°$	$+270°$
β-Methyl-*d*-glucoside-(1,5)	$[\alpha]_D^{20}$ $-34.2°$	$-58°$

11) J.P. Rosselet, T. Reichstein : Helv. Chim. Acta, **36**, 787(1953).
12) W. Klyne : Biochem. J.(London), **47**, xli(1950).

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The writers express their deep gratitude to Dr. Junzo Shinoda, President of this Company, and to Mr. Isamu Nakano, Director of the Yanagishima Factory, for their kind guidance and encouragement and for permission to publish the present work.

Experimental

Preparation of Enzyme Solution—Snails (*Euhadra quaesita* DESHAYES) collected during the rainy season in June to July were bred on cabbage for a few days, then on a filter paper, and treated after dipping instantly in hot water (80°). The intestine is orange red in color, about 4 cm. in length, and filled with digestive fluid. This was taken out cautiously, both ends pinched with a pincette to avoid loss of the fluid, and dipped in cold acetone (−30°), and precipitated as a white solid. The precipitate was collected, rapidly ground up in a mortar, and centrifuged with cold acetone. Acetone was again added to the precipitate, centrifuged, and the procedure was repeated twice with cold ether. The precipitate was dried in vacuum desiccator and stored in a cold, dark place. This was weighed, mixed with about 200 volumes of acetate buffer (pH 5.4) or distilled water, mixed thoroughly, while disintegrating the lumpy mass, and centrifuged. The clear supernatant was used as the enzyme solution. The enzyme prepared by this method was found to maintain its activity even after 6 months.

Enzymatic Decomposition of Digitalinum verum—To a solution of 100 mg. of digitalinum verum crystals (m.p. 240~244°)[13] dissolved in 200 cc. of distilled water, enzyme solution prepared by digestion of 200 mg. of enzyme powder with 40 cc. of acetate buffer (pH 5.4) was mixed, 5 cc. of toluene added, and the mixturs was allowed to stand in a thermostat of 32° for 4 days. The white turbid liquid was concentrated to 40 cc. under a reduced pressure at a bath temp. of 45° and about 5 vols. (200 cc.) of EtOH was added to the concentrated solution. The enzyme that precipitated out on standing was removed by the use of Hyflo Super Cel (Johns-Manville product), the filtrate was concentrated to 40 cc. under a reduced pressure, and the concentrate was extracted by a 2:1 mixture of $CHCl_3$ and EtOH. $CHCl_3$ layer was evaporated under a reduced pressure and the residual extract was chromatographed on 3 g. of alumina (Merck product). The residue from $CHCl_3$ effluent was recrystallized from 2% MeOH and 41 mg. of prismatic crystals, m.p. 233~235°, were obtained, which showed no depression of m.p. on admixture with strospeside, m.p. 242~246°. They were also identified by paper partition chromatography.[14]

Elution of the foregoing alumina column with water-saturated BuOH afforded the recovery of a small amount of digitaliunm verum.

Enzymatic Decomposition of Digitalinum verum Monoacetate—A solution of 10 mg. of digitalinum verum monoacetate,[15] m.p. 235~240°, dissolved in a small amount of MeOH was added to 30 cc. of distilled water, and MeOH was distilled off under a reduced pressure. The enzyme solution prepared from 10 mg. of enzyme powder and 2.5 cc. of distilled water as above was added to this solution, 1 cc. of toluene added, and the mixture was allowed to stand at 32°. A part of the solution was taken out after 14, 24, and 72 hrs. and examined by paper partition chromatography as described in Part II of this series.[15] Formation of digitalinum verum was observed already after 14 hrs., the majority changed to digitalinum verum after 24 hrs., and the spot for the monoacetate completely disappeared 72 hrs. later, indicating that deacetylation had been effected. At the same time, indistinct spot of a minute amount of strospeside was detected besides digitalinum verum.

Enzymatic Decomposition of Gitostin

a) **Formation of Strospeside from Gitostin**—Enzyme solution prepared from 200 mg. of enzyme powder and 40 cc. of acetate buffer was added to a solution of 60 mg. of gitostin, m.p. 250~252°, dissolved in 120 cc. of distilled water, 5 cc. of toluene added, and the mixture was allowed to stand at 32° for 5 days. This was treated as in the case of digitalinum verum, chromatographed on alumina, and 41.5 mg. of prisms, m.p. 235~238.5°; $[\alpha]_D^{28}$ +15.5°±2°(c=0.980, MeOH), were obtained. This substance was identified with strospeside through admixture and paper chromatography.[14] A small amount of digitalinum verum crystals, m.p. 232~236°, were obtained by elution of the alumina column with water-saturated BuOH. The original gitostin was not detected even by paper partition chromatography.

b) **Formation of Digitalinum verum from Gitostin**—The enzyme solution prepared from 20 mg. of enzyme powder and 2.5 cc. of distilled water was added to the solution of 60 mg. of gitostin, m.p. 250~252°, dissolved in 120 cc. of distilled water, 3 cc. of toluene added, and the mixture was allowed to stand at 32° for 24 hrs. The reaction product obtained by the usual treatment was submitted to partition chromatography with a column prepared from 30 g. of Celite 535 and developed with water-

13) All m.p.s were measured on Kofler block and are uncorrected.
14) Toyo Roshi No. 50 impregnated with formamide and developed with a mixed solvent of xylene: MeCOEt(1:1) and toluene:BuOH(3:1), both saturated with formamide.
15) K. Miyatake, *et al.*: This Bulletin, **5**, 157(1957).

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saturated MeCOEt. The effluent was fractionated into 25 cc. each. Fraction Nos. 5~10 (50 mg.) revealed a spot of digitalinum verum in paper partition chromatography and its residue was recrystallized to 30 mg. of digitalinum verum, m.p. 238~242°.

This recrystallization mother liquor was acetylated by the usual method and 8 mg. of digitalinum verum hexaacetate was obtained as needle crystals, showing double melting point of 170~175°/220~224°. These crystals were proved to be of identical substances by mixed m.p. and paper partition chromatography (Rf 0.14 with cyclohexane:AcOH:$CHCl_3$:H_2O=100:30:30:1). A very minute amount of gitostin was recovered from fractions below No. 10.

Summary

Gitostin, a new cardiotonic glycoside isolated from the seeds of *Digitalis purpurea*,[1] was hydrolyzed with digestive enzyme from the intestine of a snail (*Euhadra quaesita* DESHAYES) and strospeside was obtained, confirming the structure of gitostin as gitoxigenin-glucosido-glucosido-digitaloside. Partial decomposition of gitostin with the same enzyme afforded digitalinum verum, indicating that this enzyme effected stepwise hydrolysis, liberating one mole each of glucose. From the fact that only digitalinum verum is obtained from gitostin, the extra glucose was found to be bonded to the glucose in digitalinum verum, and by the comparison of molecular rotation of gitostin and digitalinum verum, the bonding of this glucosylglucoside was confirmed to be in β-position to the terminal glucose.

(Received January 11, 1957)

U.D.C. 547.918.582.951.6

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29. Atsuji Okano, Kazuhiko Hoji, Tōsaku Miki, and Kazuo Miyatake : Studies on the Constituents of *Digitalis purpurea* L. V.[1] On the Acetates of Some Cardiotonic Glycosides.

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Prior to the study of cardiotonic glycosides contained in the seeds of *Digitalis purpurea*,[2] leaves of the digitalis cultivated in the Narita Farm of this company were extracted and the known gitoxin, digitoxin, purpurea glycoside-A and -B, digitalinum verum, and strospeside were isolated. It was found that the amount of glycosides belonging to the gitoxigenin series, the so-called B-series, was larger than those of digitoxigenin or A-series, similar to the results obtained by Okada.[3] Further, extraction of the same leaves after natural fermentation indicated about the same qualitative relationship in the amount of digitoxin, gitoxin, and strospeside thereby obtained.

Of these glycosides, purpurea glycoside-A and -B had remained uncrystallizable for a long time since their isolation,[4] but Stoll[5] recently reported obtaining both glycosides in crystalline form and described their properties.

The purpurea glycoside-A and -B isolated by the present workers were not crystallized and were identified through comparison of data on elemental analyses, optical

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1) Part IV : This Bulletin 5, 167(1957).
2) Part II. K. Miyatake, *et al.* : This Bulletin, **5**, 157(1957).
3) M. Okada : J. Pharm. Soc. Japan, **75**, 611(1955).
4) A. Stoll, W. Kreis : Helv. Chim. Acta, **18**, 120(1935).
5) A. Stoll, W. Kreis, A. von Wartburg : *Ibid.*, **37**, 1134(1954).

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