color of the solution remained. The result from this experiment was completely the same as that from agar plate method, as shown in Table IV.

TABLE IV.

Compound	Tumor	MED in rat mg./kg.	Resistance index of tumors	Concn. in mM					
				100	50	20	10	5	0
Methyl-bis(2-chloroethyl)amine N-oxide	AH-130	1	1	+	+	−	−	−	−
	AH-7974	50	50	+	+	−	−	−	−

Conclusion

The non-parallelism between two biological activities of a compound, the tumor growth retardation and inhibition on its enzymic action, is worth a careful consideration when selecting the most effective among the anti-tumor agents for clinical application by experiments based on the estimation of dehydrogenase activity of the tumor specimen from a patient.

The authors are much indebted to Dr. H. Satoh for his collaboration in animal experiments and also wish to thank Dr. D. Mizuno of National Institute of Health, Tokyo, for his kind advice concerning the CAP-method.

Summary

The fact was presented in this paper that there was no quantitative parallelism between the tumor-growth retarding effect of a compound on animal tumors and its inhibiting effect on dehydrogenase activity *in vitro* of the living cells of the same kind of tumor. An attention should therefore be paid to this fact when a drug is to be selected for clinical purpose from the anti-cancer agents by comparing their inhibiting activity on this enzyme of tumor piece taken from a patient.

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99. **Masao Uchibayashi**: Studies on Steroids. XII.* Isolation of Gitorin from *Digitalis lanata.*

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The cardiac glycoside, gitorin, was isolated from the leaves of *Digitalis lanata* by Tschesche and his co-workers[1] in 1952 and was formulated as gitoxigenin glucoside on the ground that it gives dianhydrogitoxigenin and glucose on acid hydrolysis and gitoxigenin on enzymatic hydrolysis. They found that the material obtained through the usual procedures of isolation was a mixture of gitorin and digitalinum verum, and that separation of the two was difficult, the isolation of gitorin being achieved only through its acetyl derivative. Hasegawa and his collaborators[2] obtained gitorin, without deriving it to its acetate, by repeated chromatography on charcoal and through counter-current distribution method. It is the purpose of this paper to report an efficient method for obtaining gitorin from the leaves of *Digitalis lanata* and to present the results of the Mannich hydrolysis of gitorin.

Dried leaves of *Digitalis lanata* were submitted to extraction referring to the known method.[3] Somewhat differing from description in the literature, however, concentration of

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* The paper which appeared in this Bulletin, **6**, 255(1958), constituted the last in a series entitled "Studies on the Components of *Rhodea japonica* Roth.", and the title is changed with this report to "Studies on Steroids" by Hayao Nawa.

** Juso-nishino-cho, Higashiyodogawa-ku, Osaka (内林政夫).

[1] R. Tschesche, G. Grimmer, F. Neuwald: Chem. Ber., **85**, 1103(1952).

[2] H. Hasegawa, K. Inoue, J. Ishii, H. Iijima: This Bulletin, **4**, 319(1956).

[3] A. Stoll, W. Kreis: Helv. Chim. Acta, **16**, 1049(1933).

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the aqueous solution gave first a crude glycoside mixture as a pale yellow precipitate and then digilanide (mixed crystals of lanatosides A, B, and C). The crude glycoside mixture was found by paper partition chromatography to contain, besides lanatosides A, B, and C, an appreciable amount of a substance which was supposed to be gitorin or digitalinum verum, judging from its Rf value (Fig. 1-i).

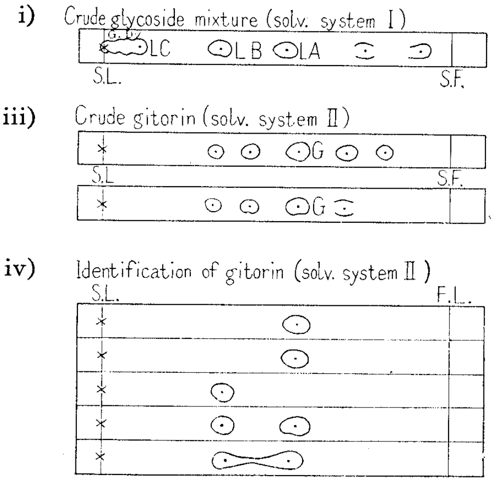
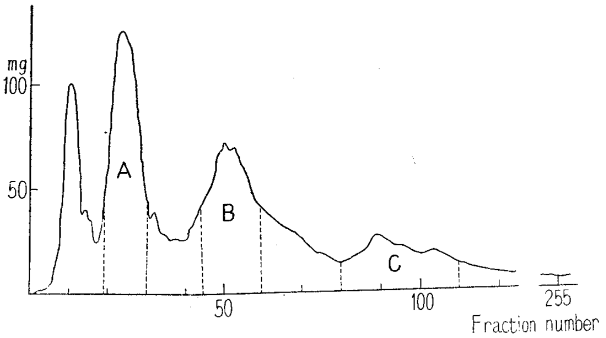


Fig. 1. Paper Chromatograms of Gitorin
Solvent system I: AcOEt-benzene-EtOH-H_2O =86 : 14 : 2 : 50
II: AcOEt-MeOH-H_2O=8 : 2 : 5
Ascending method. S. L.=starting line, S. F.=solvent front,
LA=lanatoside A, LB=lanatoside B,
LC=lanatoside C, G=gitorin,
DV=digitalinum verum.

For the purpose of isolating this substance, the crude mixture was chromatographed on a silica gel column and the eluate collected in fractions was examined by the Legal test, the Keller-Kiliani test, and by paper partition chromatography. As is seen in Fig. 2, the desired substance was present in the fractions eluting after lanatosides A, B, and C, which were separately isolated. The fractions in question were combined and evaporated to give a crude powder (Fig. 1-ii). The substance was subjected to solvent-partition method[3] to remove impurities more soluble in organic solvents (Fig. 1-iii), and again chromatographed on silica gel, yielding a white powder which showed a single spot on paper chromatograms. The product was identified as gitorin by elemental analysis, melting point, optical rotation (Table I), and by paper partition chromatography (Fig. 1-iv). The yield of the product was remarkably high, i.e. 0.0123% on the basis of the dried leaves, as compared with that obtained by Tschesche[1] of 0.0005~0.001%, therefore, the content of gitorin seems to have been particularly high in the leaves of *Digitalis lanata* used for this study.

Fig. 2. Separation of the Crude Glycoside Mixture



As is well known, cardiac glycosides containing gitoxigenin as the aglycone are divided into two groups; one is glycosides in which a 2-desoxy sugar is linked to the aglycone and the other is those in which a 2-hydroxy sugar is attached. Whereas the former undergoes acid hydrolysis very easily to give gitoxigenin and one or more sugars, the latter resists the action of acids, and drastic conditions necessary to cleave the glycoside linkage usually cause additional change in the aglycone with production of dianhydrogitoxigenin. It is a matter of general knowledge, therefore, that in order to obtain an aglycone intact, which is susceptible of change, from the glycosides of 2-hydroxy sugar without the aid of enzymes, application is required of the method employed by Mannich in the preparation of ouabagenin from ouabain, known as the Mannich hydrolysis method.[4] Example of the application of the

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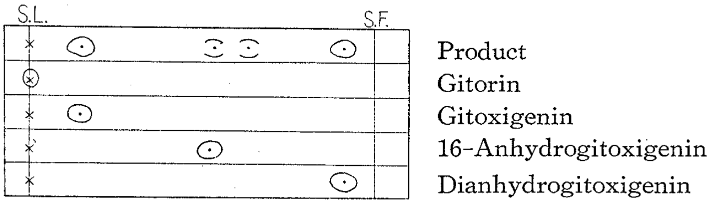
[4] C. Mannich, G. Siewert: Chem. Ber., **75**, 737(1942).

Mannich hydrolysis to the gitoxigenin glycosides of 2-hydroxy sugar is found only in the case of strospeside (gitoxigenin digitaloside).[5] Taking these facts into consideration, the Mannich method was applied to gitorin with the concurrent purpose of confirming its structure.

Gitorin dissolved in acetone containing hydrogen chloride was allowed to stand at room temperature and the progress of the reaction was examined by paper partition chromatography. On the fifteenth day disappearance of gitorin and formation of dianhydrogitoxigenin, gitoxigenin, and two unidentified substances were observed (Fig. 3). Treatment of the reaction mixture gave an aglycone and sugar fractions, the presence of glucose in the latter being verified by paper partition chromatography. The aglycone fraction was passed through a column of powdered cellulose to yield dianhydrogitoxigenin and the desired gitoxigenin as crystals in approximately equal quantities. The two compounds were identified by mixed melting point determination with respective authentic samples. The two unidentified substances on the paper chromatogram of the hydrolysis product were assumed to be 14-anhydro- and 16-anhydrogitoxigenins judging from their Rf values, but their isolation in pure state has not yet been accomplished due to their small amount.

Fig. 3 Paper Chromatograms of the Hydrolysis Products of Gitorin
Solvent system: benzene-MeOH-H_2O=5 : 3 : 2, ascending method



Thus, a method for obtaining gitorin efficiently from the leaves of *Digitalis lanata* using a combination of column chromatography and solvent-partition method was established and the structure of gitorin was confirmed by the Mannich hydrolysis, which realized the first preparation of gitoxigenin from gitorin without the aid of enzymes.

The author is sincerely grateful to Dr. S. Kuwada, Dr. S. Tatsuoka, and Mr. Y. Sasakawa for their encouragement, and to Dr. H. Nawa for his helpful suggestion and kind cooperation. The author also wishes to thank Mr. H. Kamio and his associates for physical measurements, Mr. M. Kan and his colleagues for microanalyses, and Messrs. T. Imai, T. Kamiya, and A. Okabori for technical assistance in paper chromatography.

Experimental*

Extraction of the Crude Glycoside Mixture from the Leaves of *Digitalis lanata*—Referring to the method reported by Stoll and Kreis,[3] 4 kg. of ground leaves of *Digitalis lanata,* which had been stored for some years after harvest and desiccation, was treated with $(NH_4)_2SO_4$ and extracted with AcOEt. After treatment with PbO, the aqueous solution was concentrated and left standing to give 6.66 g. of a crude glycoside mixture, as a pale yellow powder.

Separation of Gitorin from the Crude Glycoside Mixture—To a column of 2 kg. of silica gel (E. Merck & Co., 'for chromatography') holding 1 L. of water, 6.66 g. of the crude glycoside mixture dissolved in a small volume of MeOH was added and the column was developed with water-saturated AcOEt containing 0.5% of MeOH. The eluate was collected in 100-cc. portions and examined by the Legal and Keller-Kiliani tests, and by paper partition chromatography (solvent system, AcOEt : benzene : EtOH : H_2O= 86 : 14 : 2 : 50, and AcOEt : MeOH : H_2O=8 : 2 : 5; reagent: CCl_3COOH). The fractions 19∼30 gave lanatoside-A, the fractions 45∼60, lanatoside B, and the fractions 80∼110, lanatoside-C. The fractions 111∼255 were combined and concentrated to yield crude gitorin (1.9070 g.) (Fig. 2), the paper chromatogram of which showed, besides a spot corresponding to gitorin, two spots travelling ahead of gitorin and another two spots running behind gitorin (Fig. 1-ii). To remove the substances which moved in front of gitorin on this chromatogram, a method of partition between solvents, i.e. MeOH, $CHCl_3$, and water, was adopted. One part of the material was shaken with a mixture of 100 parts of MeOH and 500

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[5] O. Schindler, T. Reichstein: Helv. Chim. Acta, **35,** 442(1952).

* All melting points are uncorrected. The paper-chromatographic conditions mainly referred to those reported in the following paper unless otherwise noted: Y. Sasakawa: Yakugaku Zasshi, **75,** 946(1955).

parts each of $CHCl_3$ and water, and the aqueous layer was concentrated to dryness *in vacuo*. With the residue, the same procedure as above was repeated four times. The product (0.7840 g.) was found by paper partition chromatography to contain almost no substances which travelled ahead of gitorin (Fig. 1-iii). Then, partition chromatography on a silica gel column was employed so as to remove the substances which run behind gitorin on the chromatogram. One kilogram of silica gel was mixed with 500 cc. of the aqueous layer of a mixture of AcOEt : MeOH : H_2O=8 : 2 : 5** and packed into a column. After the organic layer of the above mixture passed through the column, the crude gitorin in a small volume of MeOH was added to the column, and the column was developed with the same organic solvent mixture as above. The eluate collected in 50-cc. portions was examined by paper partition chromotagraphy (AcOEt : MeOH : H_2O=8 : 2 : 5). The fractions 19～50 were combined (0.5881 g.), concentrated, treated with charcoal in MeOH, and ether was added to the concentrated MeOH solution, by which 0.4929 g. of a white powder was obtained. The yield was 0.0123% on the basis of the dried leaves.

Identification of Gitorin—m. p. 205～215°, $[\alpha]_D^{24}$ +6.5° (c=2.0%, CH_3OH). *Anal.* Calcd. for $C_{29}H_{44}O_{10}\cdot H_2O$: C, 61.03; H, 8.13. Found: C, 61.05; H, 7.76.

TABLE I. Comparison of Gitorin and Digitalinum verum

	Author's gitorin	Tschesche's gitorin	Reichstein's digitalinum verum
m. p. (°C)	205～215	205～212	241～244
$[\alpha]_D$ in MeOH	+6.5°	+7°	+1.5°±2°

Comparison of data of the product with those of gitorin obtained by Tschesche, *et al.*1) and of digitalinum verum by Reichstein, *et al.*6) is presented in Table I. The product showed a single spot on the paper chromatogram developed with the solvent systems AcOEt : MeOH : H_2O=8 : 2 : 5, and octyl alcohol : isoamyl alcohol : water=7 : 3 : 5. Chromatographic comparison of the product with authentic samples of gitorin and digitalinum verum revealed clear difference between this product and digitalinum verum (Fig. 1-iv).

Mannich Hydrolysis of Gitorin—Gitorin (218.3 mg.) was dissolved in 100 cc. of acetone containing 0.4% of HCl and the mixture was allowed to stand in a closed vessel at room temperature. The progress of the reaction was examined by paper partition chromatography (benzene : MeOH : H_2O= 5 : 3 : 2) and on the 15th day the hydrolysis was found complete with the disappearance of gitorin (Fig. 3). After being neutralized with $KHCO_3$ solution, the reaction mixture was concentrated *in vacuo* at low temperature and the residue was extracted with $CHCl_3$ to give an aqueous and $CHCl_3$ fraction.

(a) Identification of the sugar fraction: The aqueous layer was concentrated *in vacuo* and the residue was extracted with MeOH. The residual substance obtained on concentration of the MeOH extract showed on the paper chromatogram (AcOH : BuOH : H_2O=1 : 4 : 5; ascending method; reagent: NH_4OH solution of $AgNO_3$) Rf values of 0.110 and 0.100, which were undistinguishable from the Rf values (0.107, 0.099) of authentic D-glucose.

(b) Identification of the aglycone fraction: The $CHCl_3$ layer, obtained by the extraction of the hydrolysis products, was washed with dil. $KHCO_3$ solution and water, dried over $MgSO_4$, and the solvent was removed. As the residue dissolved in a small amount of EtOH did not crystallize on standing in the cold, the EtOH solution was chromatographed on powdered cellulose. A column of 70 g. of powdered cellulose was passed with the aqueous layer of a mixture of benzene : MeOH : H_2O=5 : 3 : 2 and then with the organic layer of the mixture until the eluate became homogeneous. The above EtOH solution was added to this column and the column was developed with the same organic solvent mixture. The eluate collected in 10-cc. portions was examined by paper partition chromatography (benzene : MeOH : H_2O= 5 : 3 : 2). The fractions 4～8 were combined and after removal of oily concomitants the crude crystals (28.5 mg.) were purified from MeOH to give colorless needles, m. p. 205～208°. The fractions 81～115 (33.2 mg.) were recrystallized from EtOH to colorless fine crystals, m. p. 225°. The two compounds showed no depression of m. p. when mixed with respective authentic samples of dianhydrogitoxigenin and gitoxigenin, and the Rf values, 0.91 of the former and 0.15 of the latter (the above solvent system; ascending method), agreed with those of the respective samples.

Summary

Gitorin was obtained efficiently from the leaves of *Digitalis lanata* in 0.0123% yield by the method combining column chromatography and solvent-partition method, and its structure was confirmed by the Mannich hydrolysis, which furnished gitoxigenin, dianhydrogitoxigenin, and glucose.

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** Y. Sasakawa: Unpublished data.

6) W. Rittel, A. Hunger, T. Reichstein: Helv. Chim. Acta, **35**, 434 (1952).

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