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Studies on Digitalis Glycosides.[1] Gitoxin Acetates. 2.[2] Deacetylation of Pentaacetylgitoxin with Liver and Heart Homogenates

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Incubation of pentaacetylgitoxin (I) with liver and heart homogenates of rabbits easily caused the fission of acetyl groups in the terminal sugar moiety and gave 3′,3″, 16-triacetylgitoxin (IIc) as a main product, respectively, accompanied with small quantities of several partial acetates and gitoxin (IIj). IIc was the intermediate in hydrolysis of I to IIj with dilute alkali.

Gitoxin (IIj), one of the main cardiac glycosides of digitalis species, is unfavorable for intestinal absorption due to its insolubility, but it was found that pentaacetylgitoxin (I) is easily absorbed from the intestine owing to its lipophylic property and it exhibits sufficient cardiac activity.[4–7] The cardiac activity of I was shown in *in vitro* tests using the isolated guinea-pig auricles and frog hearts, but it could not be demonstrated by Langendorff's method.[8]

From these results, the revealation of activity of I *in vivo* was suggested to be due to a metabolite of I in body.[6,7] In the oral test of I in rats, it was reported that deacetylation products such as gitoxin (IIj), the major product, and its partial acetates together with the hydrolysis products such as gitoxigenin mono- and bisdigitoxoside were formed, but the details of experiments were not described.[7] On the studies of enzymatic transformation *in vitro*, deacetylation of I to the diacetate[9] having acetyl groups at C–16 and at a sugar moiety was accomplished with intestinal enzyme of the snail and trochoid.[10] Furthermore, transformation of I to gitoxin (IIj) by incubation with liver homogenate of rats was reported in brief.[11] For the purpose of clarifying the process of cardiac activity revealation of I in body, two of the authors (J. M. and D. S.) studied the deacetylation of I with diastase and potassium hydrogen carbonate as a reference experiment.[2] This paper concerns the later studies on transformation of I with liver and heart homogenates of rabbits.

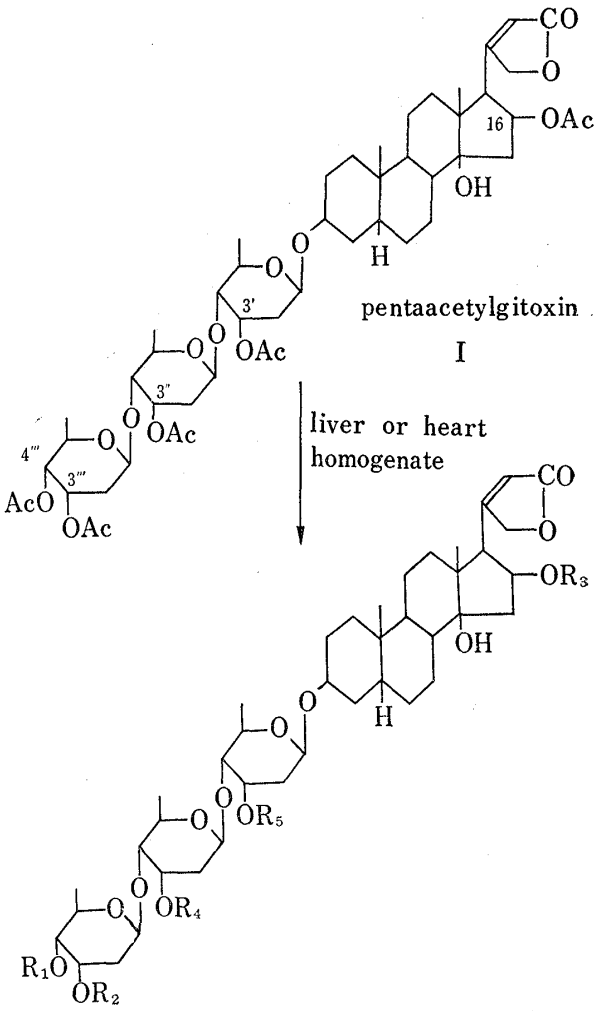
Pentaacetylgitoxin (I) was incubated with liver homogenate of rabbits at 37° and the progress of hydrolysis was followed by thin-layer chromatography (TLC) referring to the partial acetates prepared by acetylation of gitoxin (IIj) as well as by deacetylation of I as described in the preceding paper.[2] Deacetylation arose immediately after incubation to

1) Part XXVI: J. Morita and D. Satoh, *Chem. Pharm. Bull.* (Tokyo), **16**, 1056 (1968).
2) Gitoxin Acetate 1: J. Morita and D. Satoh, *Chem. Pharm. Bull.* (Tokyo), **16**, 1056 (1968).
3) Location: *Sagisu, Fukushima-ku, Osaka.*
4) A. Okano, K. Hoji, T. Miki and K. Miyatake, *Chem. Pharm. Bull.* (Tokyo), **5**, 171 (1957).
5) D. Satoh, H. Ishii, Y. Oyama and S. Takahashi, Japan. Patent Pub. 6982/60.
6) T. Minesita, R. Hirota and S. Kimoto, unpublished.
7) R. Megges and K. Repke, "Proc. First Intern. Meeting, Stockholm 1961," Vol. III, Pergamon Press, Oxford, 1962, p. 271; K. Repke and R. Megges, *Dtsch. Ges. Wesen.*, **18**, 1325 (1963).
8) The coronary vessel of the isolated rabbit heart was perfused with Ringer-Lock's solution and the glycoside solution was injected directly into rubber tube from Malliot vessel just above the joint to the heart.
9) This diacetate was reported to be identical with diacetate prepared by partial acetylation of gitoxin,[10] and the latter diacetate was proved to be 4‴,16-diacetylgitoxin.[2] Therefore the sugar moiety bearing an acetyl group was considered to be a terminal one.
10) K. Miyatake, A. Okano, K. Hoji, T. Miki and A. Sakashita, *Chem. Pharm. Bull.* (Tokyo), **8**, 1144 (1960).
11) R. Megges and K. Repke, *Arch. Exptl. Path. Pharmacol.*, **243**, 330 (1962).

form 3′, 3″, 3‴, 16-tetraacetylgitoxin (IIa). After one hour, 3′,3″,16-triacetylgitoxin (IIc) became a main product, and after five hours IIc remained as major besides several by-products. Incubation was stopped at this point and transformation products were extracted and isolated by silica gel chromatography to identify with authentic samples[2] by mixed fusions as well as by comparisons of infrared (IR) spectra and *Rf* values, respectively. The main product, mp 150—158°, $[\alpha]_D^{23}$+10.0°, was shown to be a triacetate by the intensities of the acetyl proton signals in nuclear magnetic resonance (NMR) spectrum ($CDCl_3$), 7.91 τ (6H), and 8.03 τ (3H), and was further proved to be 3′,3″,16-triacetylgitoxin (IIc) by identification with an authentic sample.[2] Two of the by-products, a diacetate, mp 244—247°, and a monoacetate, mp 225—233°, were clarified to be 3′,3″-diacetylgitoxin (IId) and 16-acetylgitoxin (IIg) by similar identification, respectively. Three other by-products were presumed to be 3′,3″,3‴,16- and 3′,3″,4‴,16-tetraacetylgitoxin (IIa and IIb) as well as gitoxin (IIj), a totally deacetylated product, by thin-layer chromatography. The last by-product, mp 148—160°, was shown to be a diacetate based on the chemical shifts of acetyl protons, 7.91 τ (3H) and 8.03 τ (3H), which indicated that one of the acetyl groups is located at a sugar moiety and the other at the 16-position,[2] and the acetyl groups did not migrate by treatment with silicagel.[2] This product exhibited a positive *cis*-glycol test with periodate-benzidine reagent which corresponded to 3‴, 4‴-glycol. As the hydrolysis of this product with a dilute solution (0.06%) of potassium hydrogen car bonate gave IIj[12] through 3′-acetylgitoxin (acetylgitoxin-γ,[2,13] IIh) and 3″-acetylgitoxin (acetylgitoxin-σ,[14] IIi), this product was presumed to be a mixture of 3′,16-diacetylgitoxin (IIe) and 3″,16-diacetylgitoxin (IIf), but the further experimentation could not be done due to the lack of material.

Incubation of I with heart homogenate of rabbits for five hours also gave 3′,3″,16-triacetylgitoxin (IIc) as a main product analogously to that with liver homogenate, together with small amounts of IIa and two unidentified by-products.

As mentioned above, liver and heart homogenates of rabbits more easily hydrolysed acetyl groups in the terminal suger moiety of I, that is, 3‴- and 4‴-positions, than those at the other positions, and the main product after five hours incubation was



IIa: R_1=H, R_2–R_5=Ac } tetraacetate
IIb: R_2=H, R_1=R_3–R_5=Ac
IIc: R_1=R_2=H, R_3–R_5=Ac triacetate
IId: R_1–R_3=H, R_4=R_5=Ac
IIe: R_1=R_2=R_4=H, R_3=R_5=Ac } diacetate
IIf: R_1=R_2=R_5=H, R_3=R_4=Ac
IIg: R_1=R_2=R_4=R_5=H, R_3=Ac
IIh: R_1–R_4=H, R_5=Ac } monoacetate
IIi: R_1–R_3=R_5=H, R_4=Ac
IIj: R_1–R_5=H, gitoxin

12) Detected by thin-layer chromatography.
13) K. Hoji, *Chem. Pharm. Bull.* (Tokyo), **9**, 296 (1961).
14) 3″-Acetylgitoxin (IIi) will be reported in the forthcoming paper.

3′,3″,16-triacetylgitoxin (IIc) which corresponded to the main product in two days hydrolysis of I with 0.06% potassium hydrogen carbonate.[2] The velocities of fissions of acetyl groups in the various positions were estimated with this reagent, and it was found that acetyl groups in the 3‴- and 4‴-positions are most easily eliminated, and that in the 16-position shows a moderate reactivity, and those in the 3′- and 3″-positions considerably resist hydrolysis. Though the hydrolysis with enzyme may not always proceed analogously to that with alkali, it can be presumed that there is a close connection between the variety in reactivities of acetyl groups and the dissimilarity[15–17] in cardiac effects of partial acetates and pentaacetylgitoxin (I).

Experimental[18]

Thin-Layer Chromatography (TLC)——Analytical and preparative TLC were performed with the following systems:

A = SiO_2, pyridine–$CHCl_3$ (1:4, v/v)
B = SiO_2, AcOEt
C = SiO_2, $CHCl_3$–acetone (1:1, v/v)

Incubation of Pentaacetylgitoxin (I) with Liver Homogenate of Rabbits——To a homogenate made of 300 g of rabbit liver and 2500 ml of phosphate buffer (pH =7.0), a solution of 1000 mg of I in 500 ml of EtOH was added dropwise under agitation and the mixture was incubated at 37° for 5 hr. The incubated mixture was extracted with $CHCl_3$[19] and the $CHCl_3$ solution was washed with H_2O, dried over Na_2SO_4 and evaporated under reduced pressure to give 9223 mg of residue. As the extract was contaminated with a large quantity of impurities from liver components, the residue was dissolved in 300 ml of benzene and chromatographed on a silica gel column (SiO_2=200 g) to separate into the following fractions.

i) First fraction eluted with benzene=liver components.

ii) Second fraction eluted with $CHCl_3$-benzene (1:1, v/v), $CHCl_3$, and $CHCl_3$–MeOH (99:1, v/v)=liver components.

iii) Third fraction (1278 mg) eluted with $CHCl_3$–MeOH (98:2 and 75:25, v/v) = transformation products of I.

The third fraction was further separated in the order of *Rf* values into the following fractions by repeated preparative TLC (SiO_2, $CHCl_3$–MeOH (9:1, v/v), and AcOEt).

i) The first fraction (21 mg) was shown to be a mixture of 3′,3″,3‴,16- and 3′,3″,4‴,16-tetraacetylgitoxin (IIa and IIb) by TLC (system A, B, C).

ii) The second fraction (261 mg) was recrystallized from acetone–ether–petroleum ether to give 199 mg of 3′,3″,16-triacetylgitoxin (IIc) as colorless crystals, mp 150—158°. *Anal.* Calcd. for $C_{47}H_{70}O_{17}\cdot H_2O$: C, 61.02; H, 7.85. Found: C, 60.78; H, 7.74. IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3612, 3540, 1744, 1631, 1620. Admixture with the authentic sample of IIc did not show any depression of melting point and TLC (system A, B, C) indicated the identity of both substances.

iii) The third fraction (65 mg) was recrystallized from acetone–petroleum ether to afford colorless crystals of a probable mixture of 3′,16-diacetylgitoxin (IIe) and 3″,16-diacetylgitoxin (IIf), mp 148—160°. UV λ_{max}^{EtOH} $m\mu$ (ε): 215 (13050). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3540, 1742, 1634, 1620. TLC of this product showed more polarity than 3‴,16- and 4‴,16-diacetylgitoxin (system B, C).

iv) The forth fraction (51 mg) was recrystallized from MeOH–ether–petroleum ether to give colorless crystals of 3′,3″-diacetylgitoxin (IId), mp 244—247°. IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3530, 3460, 1742, 1632, 1618. NMR ($CDCl_3$): 7.91 τ (6H). Mixed fusion and comparison with an authentic sample of IId in IR spectra and TLC (system A, B, C) proved the indentity of both substances.

v) The fifth fraction (44 mg) was recrystallized from MeOH–ether–petroleum ether to afford colorless crystals of 16-acetylgitoxin (IIg), mp 225—233°. IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3540, 1746. 1634, 1620. Mixed fusion and comparison of IR spectra and TLC (system A, B, C) with an authentic sample of IIg showed the identity of both substances.

vi) The sixth fraction (13 mg) indicated the positive color reaction characteristic for cardiac glycoside such as Raymond test, Legar test as well as Keller–Kiliani test, and TLC (system A, B, C) proved the identity with gitoxin (IIj).

Blanc Test of Incubation of I with Inactivated Liver Homogenate——After a homogenate made of 15 g of rabbit liver and 125 ml of phosphate buffer (pH =7.0) was boiled for 15 min to inactivate the enzymes, a solution of 50 mg of I in 25 ml of EtOH was added. The mixture containing I and an intact homogenate

15) T. Minesita, R. Hirota, S. Kimoto and O. Uno, unpublished.
16) R. Megges and K. Repke, *Monatsh. Deut. Akad. Wiss. Berlin*, **7**, 744 (1965).
17) K.O. Haustein, F. Markwardt and K. Repke, *Arch. Exptl. Path. Pharmacol.*, **252**, 424 (1966).
18) All melting points are uncorrected.
19) Protein from liver could be removed as an intermediate layer by centrifuge.

prepared by the same manner excluding I were shaked at 37° for 5 hr in parallel, and extracted with $CHCl_3$. The $CHCl_3$ solutions were washed with H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. Comparison of TLC of both residues thus obtained showed that the boiled homogenate did not cause transformation of I during incubation.

Incubation of I with Heart Homogenate of Rabbits——Fifteen g of rabbits heart was homogenized with 125 ml of phosphate buffer (pH=7.0) and a solution of 50 mg of I in 25 ml of EtOH was added. After shaking at 37° for 5 hr, the incubation mixture was extracted with $CHCl_3$ and $CHCl_3$ solution was washed with H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. The residue (304 mg) was dissolved in 50 ml of benzene and poured on a silica gel column (SiO_2=10 g) and the heart components eluted with 600 ml of benzene. The fraction containing the transformation products of I eluted with $CHCl_3$–MeOH mixture (75:25) was further separated by preparative TLC (SiO_2, system C) into the following parts.

i) The first fraction (6 mg) was shown to be a mixture of IIa and IIb by TLC (system A, B, C).

ii) The second fraction (30 mg) was proved to be IIc by TLC (system A, B, C).

iii) The third fraction (8 mg) was unidentified.

Estimation of Hydrolysis Velocity of Acetyl Groups with $KHCO_3$——Each sample of partial acetates (1 mg) was dissolved in 0.2 ml of 0.06% $KHCO_3$ in 90% MeOH and the solutions were set aside at room temperature. The progress of hydrolysis was checked by TLC (system A, B, C). The beginning of fission of each acetyl group was shown by appearance of the resulting deacetylated products, and its completion was indicated by disappearance of the original acetates.[2)]

Original acetate	Deacetylated product	Position[2)] of eliminated acetyl group	Time of Fission after dissolved in 0.06% $KHCO_3$	
			Beginning	Completion
I	IIa	4‴	2 hr	2 day
IIa	IIc	3‴	2 hr	2 day
IIc	IId	16	24 hr	35 day
IId	IIj	3″ and 3′	7 day	120 day

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