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5β-HYDROXYDIGITOXIGENIN—A METABOLITE OF DIGITOXIGENIN BY RABBIT LIVER HOMOGENATES

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Abstract—^{3}H-digitoxigenin was metabolized *in vitro* by rabbit liver homogenate. A metabolite resulting from this biotransformation was isolated and identified as 5β-hydroxydigitoxigenin. After an incubation period of 1 hr, approximately 6 per cent of the substrate was converted to this metabolite. This is the first report of the 5β-hydroxylation of digitoxigenin in mammalian systems.

MUCH OF OUR present knowledge concerning the metabolism of cardiac glycosides has been reviewed by Repke,[1,2] Okita[3] and Doherty.[4] Digitoxin undergoes a stepwise cleavage of its three sugar moieties, ultimately yielding digitoxigenin.[5–7] In addition, in the rat, dog and man it has been shown that digitoxin can undergo a C-12 hydroxylation to form digoxin,[8,9] with subsequent stepwise cleavage of the sugar moities to form digoxigenin.[10] Perfused livers of guinea pigs have yielded unidentified polar metabolic products of ^{3}H-digitoxin.[11] Griffin *et al.*[12] recovered two polar metabolites of 7α-^{3}H-digitoxin from the urine of rabbits and subsequently demonstrated that these metabolic products were considerably more toxic than the parent compound, but failed to identify them. Okita and Curry[13] have reported the presence of five unidentified polar metabolic products of ^{14}C or ^{3}H-digitoxin in human urine.

The major pathway for the metabolism of digitoxigenin was demonstrated by Herrmann and Repke[14] in ten species (including rabbit and man) to be an epimerization at the C-3 hydroxyl group from the β to the α orientation by way of a 3-keto (3-dehydro) intermediate. The pyridine nucleotide coenzyme apparently serves as hydrogen carrier in these steps.[15,16] Herrmann and Repke[14] also found that small amounts of several more polar and apparently monohydroxylated derivatives were formed. Stohs *et al.*[17] subsequently demonstrated that this hydroxylating system was microsomal in nature and that it could be induced by phenobarbital. Little is known of the identity or significance of these polar, apparently hydroxylated derivatives of digitoxigenin. The present study was undertaken to identify these metabolites.

EXPERIMENTAL

^{3}H-digitoxigenin. An ample supply of randomly labeled ^{3}H-digitoxigenin (I), having a specific activity of 600 μCi/mg, was donated by the Gesellschaft Fur Kernforschung M.B.H., Karlsruhe, Germany. The genin was purified by preparative thin-layer chromatography (TLC) according to a previously reported method,[17] and had a radiochemical purity of greater than 98 per cent. Prior to experimentation, an aliquot

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having a specific activity of 0·5 μCi/mg/0·5 ml of 70% ethanol was prepared from the purified stock solution.

5β-OH-digitoxigenin. Reference amounts of 5β-OH-digitoxigenin (periplogenin) (II) were provided by Dr. Yoshio Nozaki, Shionogi Research Laboratory, Shionogi & Company, Fukushima-ku, Osaka, Japan. The purity of the cardenolide was established by TLC analysis.

Reagents. The Tris buffer, nicotinamide, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, bovine serum albumin (BSA) and digitoxigenin were purchased from the Sigma Chemical Company. All other chemicals were Fisher reagent grade.

Animals. Male rabbits derived from the California strain were obtained from a local breeder and were maintained in our animal colony for 1 week prior to use on a diet of Purina Laboratory Chow and tap H_2O *ad lib.* The animals weighed approximately 2·2 kg, were 10 weeks of age, and were fasted overnight prior to use.

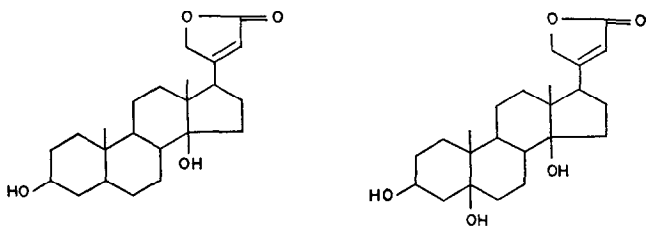

FIG. 1. Structural configuration of (I) digitoxigenin and (II) 5β-hydroxydigitoxigenin (periplogenin).

Liver homogenates. The rabbits were stunned by a blow to the back of the head and exsanguinated. The livers were quickly excised and the desired amount of tissue was placed in an ice-cold solution of 0·25 M sucrose plus 0·05 M Tris chloride, pH 7·4, 0·005 M $MgCl_2$ and 0·01 M NaCl (TMN). A 10% liver homogenate was prepared, employing a Potter–Elvehjem homogenizer fitted with a Teflon pestle.

Incubation procedure. One mg of ^{3}H-digitoxigenin (0·5 μCi/mg in 70% ethanol) was added to a 150-ml Erlenmeyer flask. All reagents were dissolved in 0·25 M sucrose (TMN) and maintained at 0° over ice. A volume of 0·25 ml each of nicotinamide (73·2 mg/ml), $NADP^+$ (12 mg/ml), glucose 6-phosphate (56 mg/ml) and 0·1 ml of BSA (500 mg/ml) was added to the flask containing the substrate. Five units of glucose 6-phosphate dehydrogenase (10 units/ml) and 7·5 ml of the 10% liver homogenate were added to the flask immediately before incubation. Tris chloride (0·05 M, pH 7·4) was added in an amount sufficient to render a final volume of 10 ml. A control flask was prepared in the above manner by substituting 7·5 ml of 0·25 M sucrose (TMN) in place of the liver homogenate. The flasks were incubated in an Eberbach water bath shaker at 37° with constant shaking under O_2–CO_2 (95 : 5) for 60 min. A typical incubation experiment consisted of one control flask and three or more homogenate-containing flasks.

Extraction. The incubation was terminated by pouring the contents of each flask into 250-ml glass-stoppered Erlenmeyer flasks containing 40 ml of methylene di-

chloride. Small aliquots of distilled H_2O and acetone were used to rinse the incubation flasks. The 250-ml flasks were stoppered and placed on a shaker for 24 hr at room temperature. The resulting emulsions were broken by centrifugation at 7000 *g* for 10 min. The pellets, organic and aqueous phases were separated and pooled. The aqueous phase was placed in a separatory funnel and exhaustively extracted with methylene dichloride until radioactivity could not be recovered from the aqueous material. The pellets were suspended in a mixture of methylene dichloride–methanol (3 : 2), placed in an Erlenmeyer flask and stirred with the aid of a magnetic stirrer for 24 hr. The organic phase was decanted and the process repeated until the pellet material was free of radioactivity. Ultimately all organic phases were pooled, dehydrated over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The resulting extract was taken up in about 3 ml of methylene dichloride–methanol (3 : 2) and transferred to a 10-ml vial.

Chromatography. Thin-layer plates (20×20 cm) of Silica gel H (EM reagents) 0·375 mm thick were routinely employed. Preparative thin-layer Silica gel H plates had a thickness of 0·50 mm. Three TLC systems were used. TLC system I consisted of a methylene dichloride–methanol (85 : 15) solvent system with one development. TLC system II[17] utilized a chloroform–isopropanol (9 : 1) solvent system with two developments, and TLC system III[18] utilized a cyclohexane–acetone–acetic acid (65 : 33 : 2) solvent system with four developments. In all TLC systems, the solvent was allowed to reach the top of the TLC plate during each development. Anisaldehyde reagent,[18] 20% H_2SO_4,[17,18] and Kedde reagent[19] were used as chromogenic agents.

Aliquots of the experimental and control extracts equivalent to 7500 dis./min were initially cochromatogramed with 20 μg of various reference standards in TLC system III. Liquid scintillation counting was employed according to the method of Talcott *et al.*[16] to determine the localization of radioactivity on the resulting chromatograms. The results were expressed as the per cent of total radioactivity ± the standard error of the mean for six chromatographic determinations.

A polar metabolite band was observed which was isolated from pooled incubation extracts, employing two successive passages in TLC system II. The initial polar band was subsequently chromatographed in TLC system III and separated into several metabolites, one of which had an R_fs value identical to 5β-OH-digitoxigenin. The band corresponding to 5β-OH-digitoxigenin was chromatographed three times in solvent system III, detecting the band by the technique of Bhavnani.[20] After each development the metabolite was eluted from the Silica gel with methanol.

Metabolite identification. Aliquots of the metabolite biosynthesized by rabbit liver homogenates and authentic 5β-OH-digitoxigenin were chromatographed in one of the three TLC systems described above, visualized with one of the three chromogenic agents, and the R_fs (digitoxigenin) values calculated. The reported values represent the average of at least three determinations for a given TLC system.

Anhydrous H_2SO_4 absorption spectra of the rabbit-generated metabolite believed to be 5β-OH-digitoxigenin, the authentic 5β-OH-digitoxigenin, 12β-OH-digitoxigenin (digoxigenin), 16β-OH-digitoxigenin (gitoxigenin), Δ^5-digitoxigenin (xysmalogenin and digitoxigenin were obtained by the methods of Brown and Wright,[21] and Herrmann and Repke.[14] Each sample was prepared by adding 3 ml of concentrated H_2SO_4 to approximately 100 μg of cardenolide and developing in darkness for 30 min. A Beckmann DB spectrophotometer equipped with a Sargent SRL recorder was

employed, and each spectrum was recorded between 220 and 500 nm, using quartz cells.

Mass spectra of the metabolite and authentic 5β-OH-digitoxigenin were determined on a Hitachi RMU 6D mass spectrometer.

Authentic 5β-OH-digitoxigenin and the metabolite believed to be 5β-OH-digitoxigenin were converted to the 3-dehydro and 3-epi derivatives by a modification of the method of Tamm and Gubler.[22] Approximately 50 μg of 5β-OH-digitoxigenin or the metabolite was dissolved in 0·5 ml of acetone and cooled to 0°. Jones reagent (1 μl) was added to each with rapid mixing and the mixtures were allowed to stand for 3 min at 0°. Each reaction was quenched with 2 ml of distilled H_2O and the resulting 3-dehydro derivatives were extracted three times with ethyl acetate. The extracts for each were pooled, washed three times with 5 ml of 5% $NaHCO_3$, washed two times with 5 ml of distilled H_2O, dried over anhydrous Na_2SO_4, evaporated to dryness *in vacuo*, and transferred to a 1-ml vial in 100 μl of methanol. The cardenolides thus obtained were subjected to TLC analysis or converted to 3-epi derivatives. The 3-dehydro,5β-OH-digitoxigenin and the 3-dehydro derivative from the metabolite were converted to 3-epi cardenolides by dissolving the 3-dehydro derivatives in 1 ml of dioxane–H_2O (4 : 1), adding 1 mg of $NaBH_4$ (10 mg/ml dioxane–H_2O, 4 : 1), and allowing the mixtures to stand for 16 hr at room temperature. The mixtures were cooled to 0°, 2 ml of diluted H_2SO_4 (pH 4) was added, followed by 2 ml of distilled H_2O, and the mixture extracted three times with chloroform. The $CHCl_3$ was found to remove little of the 3-epi derivatives. Therefore, the aqueous phases were evaporated to dryness with N_2 and transferred to a 1-ml vial in 100 μl of methanol. The 3-dehydro and 3-epi derivatives from an authentic sample of 5β-OH-digitoxigenin and the digitoxigenin metabolite were chromatographed in TLC system III, developing each plate six times. The R_fs values calculated and reported are the average of at least three determinations.

RESULTS AND DISCUSSION

The radioactivity localized in the TLC band corresponding to the 5β-OH-digitoxigenin represented 5·91±0·31 per cent of the ^{3}H-digitoxigenin substrate.

The metabolite biosynthesized by rabbit liver homogenate and standard 5β-OH-digitoxigenin were positive to Kedde reagent, giving a pink color which is indicative of the presence of a lactone ring.[23,24] In the three TLC systems the R_fs (digitoxigenin) values for the authentic 5β-OH-digitoxigenin and the metabolite generated by the rabbit were identical (TLC system I R_fs=0·84, TLC system II R_fs=0·38, and TLC system III R_fs=0·58). Identical colors were obtained with the chromogenic agents for the authentic sample and the isolated metabolic product (20% H_2SO_4=light green; anisaldehyde reagent=blue).

The absorption spectra of the 5β-OH-digitoxigenin and the metabolic product generated by liver homogenates were superimposable, with absorption maxima at 236 nm and 411 nm, and absorption minima at 274 nm (Fig. 2), and were identical to the spectrum of Δ^5-digitoxigenin. However, they were different from the spectra of digitoxigenin, 12β-OH-digitoxigenin and 16β-OH-digitoxigenin. Our absorption spectra of Δ^5-digitoxigenin, digitoxigenin, 12β-OH-digitoxigenin, and 16β-OH-digitoxigenin were in agreement with previously reported specta.[14,21] 5β-OH-digitoxigenin is

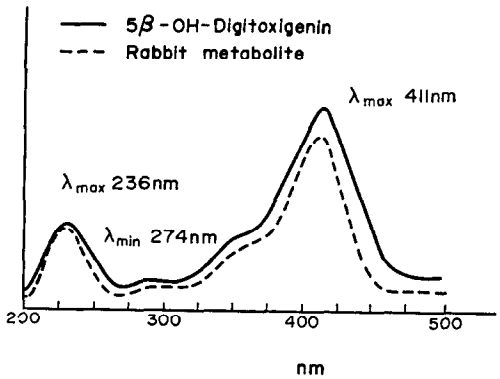

FIG. 2. Absorption spectra of 5β-OH-digitoxigenin and rabbit metabolite.

believed to be dehydrated in H_2SO_4 to Δ^5-digitoxigenin resulting in an absorption spectrum identical to the spectrum of authentic Δ^5-digitoxigenin.[14]

The R_fs (digitoxigenin) values obtained for the 3-dehydro and 3-epi derivatives of 5β-OH-digitoxigenin and the metabolite were identical, but different from 5-OH-digitoxigenin and digitoxigenin in solvent system III (3-dehydro derivatives R_fs=0·70; 3-epi derivatives R_fs=0·845; β-OH-digitoxigenin R_fs=0·76; digitoxigenin R_fs=1·0). The 3-dehydro derivatives of 5β-OH-digitoxigenin and the metabolite gave a gold color to both anisaldehyde reagent and 20% H_2SO_4, and the 3-epi derivatives were yellow to the two chromogenic agents.

Identical mass spectra and fragmentation patterns were obtained for 5β-OH-digitoxigenin and the isolated rabbit metabolite.

The metabolites 3-epidigitoxigenin and 3-dehydrodigitoxigenin were also produced in small amounts, and their presence has been previously observed by other investigators.[3,14,17] A fourth as yet unidentified metabolite is presently under investigation. No oxidation products were seen in control flasks.

These results demonstrate that this metabolite generated by rabbit liver homogenate is indeed 5β-OH-digitoxigenin. This is the first report of the demonstration of the hydroxylation of digitoxigenin at C-5 by a mammalian system. It has been known for some time that micro-organisms can hydroxylate the steroid nucleus of digitoxigenin at various positions including the 5β.[25–27] For example Nozaki[27] obtained hydroxylation products of digitoxigenin at positions 1β, 5β, 7β and two dihydroxy products by the bioconversion of digitoxigenin with *Absidia orchidis*. 5β-OH-digitoxigenin (periplogenin) is the aglycone of the glycoside periplocin which has been isolated from the genus *Strophanthus*,[28] and was biosynthesized by Sauer *et al.*[29] by the administration of progesterone-4-^{14}C to the leaves of *Strophanthus kombe*. Although 5β-OH-digitoxigenin can apparently be synthesized by a diversity of life forms, it should be noted that the mechanisms by which these hydroxylations occur have not been fully elucidated[27,29] and therefore the mechanism may vary with the biological system. Both 5β-OH-digitoxigenin and periplocin are biologically active.[30] Whether 5β-hydroxylation of cardenolides represents a catabolic and excretory route, or whether it plays a significant role in eliciting the pharmacological response of cardenolides is yet to be determined.

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