Deglycosylated Products of Endogenous Digoxin-like Immunoreactive Factor in Mammalian Tissue*

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Digoxin-like immunoreactive factor (DLIF) from adrenal cortex is an endogenous molecule with structural features remarkably similar to those of digoxin, a plant-derived cardiac glycoside (Shaikh, I. M., Lau, B. W. C., Siegfried, B. A., and Valdes, R., Jr. (1991) J. Biol. Chem. 266, 13672-13678). Two characteristic structural and functional features of digoxin are a lactone ring and three digitoxose sugars attached to a steroid nucleus. Digoxin is known to undergo deglycosylation during metabolism in humans. We now demonstrate the existence of several naturally occurring deglycosylated components of DLIF in human serum. The components are identified as DLIF-genin, DLIF-mono, and DLIF-bis, corresponding to the aglycone, and the aglycone with one and two sugars, respectively. Similar components are produced by acid-induced deglycosylation of DLIF isolated from bovine adrenal cortex. The elution pattern and sequence of DLIF-deglycosylation was identical to that of digoxin suggesting identical sugar stoichiometry. However, analysis of these newly discovered congeners by reverse-phase chromatography, spectrophotometry, antibody reactivity, and kinetics of deglycosylation, demonstrates that subtle structural and physical differences do exist when compared to digoxin. DLIF was chromatographically distinct from digoxin, and interestingly, the mobility of the DLIF-genin was shifted toward increased polarity relative to digoxigenin. DLIF and DLIF-bis, -mono, and -genin congeners have absorbance maxima at 216 nm, whereas digoxin and its congeners absorb at 220 nm. Reaction with specific antibodies directed at the lactone portion of these molecules shows DLIF and its deglycosylated congeners to be 10-fold less reactive than digoxin. Kinetics of sugar removal suggests that DLIF is 8-fold more susceptible to deglycosylation than is digoxin. Two less polar DLIF components produced from the DLIF-genin have λmax at 196 nm and are 4-fold less immunoreactive than DLIF. Our data suggest that subtle structural differences exist between DLIF and digoxin at or near the lactone ring as well as in the nature of the sugars. The presence of deglycosylated congeners of DLIF in human serum, including the less polar components, suggests in vivo deglycosylation of these factors. This is the first demonstration of the existence of naturally occurring deglycosylated derivatives of DLIF and establishes the likelihood of active metabolism of DLIF in mammals.

Endogenous digoxin-like immunoreactive factors (DLIFs) present in mammalian blood (1, 2) were discovered in part as a consequence of their cross-reactivity with antidigoxin antibodies. Considerable evidence suggests that endogenous DLIF (3) and ouabain-like factors (OLF) (4) are produced in the adrenal glands and may be linked to modulation of Na,K-ATPase activity in a manner analogous to the family of cardiac glycosides, digoxin or ouabain (5). DLIF, a steroid-like molecule (780 Da), has structural and molecular properties remarkably similar to those of the cardiac glycoside digoxin (3). These properties include a characteristic five-ring structure (aglycone) to which is attached three linearly linked sugar residues (digitoxoses, in case of digoxin) extending from the aglycone C-3 position (Fig. 1). The aglycone portion of the glycoside consists of a steroid skeleton with an unsaturated lactone ring attached at position C-17. The drug digoxin undergoes removal of its digitoxose sugars during metabolism, forming several deglycosylated species (6). The metabolic products of digoxin are important because they interact in a very structure-specific manner with sodium pump isoforms (7) as well as with highly specific antibodies raised against digoxin (8). However, to date no study has identified the presence of naturally occurring metabolic products of DLIF. With improved chromatographic techniques we recently identified the presence of a DLIF with a chemically reduced lactone ring, dihydro-DLIF, analogous to the metabolic product dihydrodigoxin (9).

The α subunit of the sodium pump is the only known functional receptor for digitalis compounds. The stoichiometry of the sugars at the C-3 position on digoxin (8) or of steroids (e.g. progesterone) (10) affect their binding to this receptor. The individual isoforms of Na,K-ATPase have been shown to possess differing affinities for binding of digitalis, ouabain, and their respective deglycosylated derivatives (11, 12). The importance of characterizing the molecular and physical properties of DLIF is further underscored by the linkage observed between increased DLIF concentrations in blood and clinical or pathological conditions associated with altered ion-transport homeostasis such as: renal failure (13, 14), hepatic failure (15), pregnancy (16), neonatal development (17, 18), diabetes- and exercise-induced stress (19, 20), and hypertension (21-23). The source of digitalis-like factors in a hormone-secreting tissue

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¶ The abbreviations used are: DLIF, digoxin-like immunoreactive factor(s); dig, digoxin; dig-bis, digoxigenin bisdigitoxoside; dig-mono, digoxigenin monodigitoxoside; dig-genin, digoxigenin; DLIF-mono, DLIF with one sugar molecule; DLIF-bis, DLIF with two sugar molecules; DLIF-genin, DLIF without its three sugar molecules; SSA, 5-sulfosalicylic acid; HPLC, high performance liquid chromatography; CH₂CN, acetonitrile; OLF, ouabain-like factors.
FIG. 1. Structure of digoxin and its deglycosylated congeners. Digoxin has a characteristic five-ring structure (aglycone) to which are attached three sugars (digitoxoses) at the C-3 position. The aglycone consists of a steroid nucleus with an unsaturated lactone ring attached at the C-17 position. Note that digitoxose residues are sequentially removed to form the bis, mono, and genin derivatives.

such as the adrenal, presence in blood, and the distribution of likely receptors in target tissues suggest the likely possibility of a new hormonal axis linking the adrenal cortex, endogenous digoxigenin, and ouabain-like factors, and sodium pump activity (5, 24).

In this study we demonstrate the existence of several deglycosylated congeners of DLIF in human serum and show that similar derivatives can be produced in vitro by acid-induced deglycosylation of DLIF isolated from bovine adrenal cortex. These congeners are analogous to the formation of bis, mono, and genin components of digoxin by sequential removal of the three sugars at the C-3 position of the aglycone and suggest the likelihood of metabolism of DLIF in mammals. We also describe a technique for purifying these newly discovered DLIF congeners and investigate several physical properties indicating structural differences between DLIF and digoxin at both the sugars and the aglycone portions of these molecules.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals used were reagent grade. 5-Sulfosalicylic acid (SSA), calcium carbonate (CaCO₃), and bovine serum albumin were obtained from Sigma. Digoxin was purchased from Sigma and its related congeners (digoxigenin, mono- and bis-digitoxosides) were donated by Burroughs Wellcome Co. (Research Triangle Park, NC). Pure DLIF (quantitated as dig-equivalent concentration) isolated either by the current or our previous procedures (3) was used as a standard to observe the formation of DLIF-deglycosylated congeners. The most common steroids in the adrenal cortex (cortisone, corticosterone, progesterone, and cortisol) were obtained from Sigma and used as standards to define their chromatographic and cross-reactivity. Acetonitrile (CH₃CN) was HPLC grade and obtained from Aldrich. Equipment and Materials—We used a Polytron PT-3000 (Brinkmann, Westbury, NY) for homogenizing adrenocortical tissue and an Orion pH/SE meter model 710A (Orion, Cambridge, MA) for pH measurements. The Sep-Pak reverse-phase C-18 cartridges were obtained from Waters Associates. The HPLC fractions were dried in a Savant SpeedVac Plus SC210A (Savant, Farmingdale, NY) connected to a Refrigerated Condensation Trap RT 400 and a Virtis Freeze Mobile 12 Lyophilizer (Virtis, Gardiner, NY). A Beckman J-A 2 centrifuge (Beckman) was used for centrifugation in the isolation procedures.

Tissue Preparation—Bovine adrenal glands were obtained from Pel-Freeze Biologicals (Rogers, AR) and kept at ~80°C until dissected. Tissues were maintained at 4°C during the isolation procedure (dissection, extraction, and centrifugation), while other steps were performed at 22–25°C. Briefly, after isolating the glands from any surrounding connective tissues and fats, the cortex was separated from the medulla based on the physical appearance of the outer cortical ring. Each gland weighed approximately 15 g and contained twice as much cortex as medulla. The cortical tissue was sliced (3-mm thick sections), chopped, and homogenized in 2 ml of dH₂O/g of cortex. The homogenate was then centrifuged three times at 34,000 × g for 30 min at 4°C in which the pellet from each cycle was resuspended in dH₂O (0.1 ml/g cortex). The pooled supernatants were stored at ~80°C until further use.

Protein Precipitation—The thawed extracts of adrenal cortical tissue were re-centrifuged at 34,000 × g for 30 min at 4°C to remove any remaining cellular debris. Protein was precipitated by incubating the supernatant with 1% SSA (2 g of sulfosalicylic acid/300 ml supernatant) at room temperature for 60 s followed immediately by adding an excess amount of CaCO₃ until the pH increased and stabilized at 5.2 (the pH of the normal adrenal cortex starting homogenate). This extract was then centrifuged at 80,000 × g for 10 min at 4°C followed by vacuum filtration of the supernatant using two layers of Whatman #1 filter paper.

Solid Phase Extraction (C-18 Cartridge Column)—C-18 Waters reversed-phase Sep-Pak solid-phase extraction column (Vac 3 cc) was primed with 1 vol of CH₃CN and rinsed with 2 vol of dH₂O. The protein-free supernatant was passed through the column twice at a rate of 1 ml/min which was then washed with 2–3 vol volumes (typically 10 ml) of dH₂O and eluted with 20 ml of 60% CH₃CN. To remove the CH₃CN from the eluates were evaporated to dryness, reconstituted in H₂O, and filtered through a 0.22-μm Whatman PVDF filter in preparation for HPLC. Reconstitution was made to achieve average yields of immunoreactivity of approximately 10,000 pg/ml by the RIA.

HPLC Reverse-phase Chromatography—We used a C-18 reverse-phase micro-Bondapak column (3.9 × 300 mm, 10-micron particle size) connected with a Waters 600E System Controller and a Waters 966 Photodiode Array Detector to monitor the HPLC output eluent for both UV absorbance and spectral analyses. The eluate was fractionated on HPLC using a linear 20 to 80% CH₃CN in H₂O elution gradient. Unless otherwise indicated, typically, 1-ml (1 min) fractions were collected during each chromatographic run over 40 min using a Waters Fraction Collector (Waters, Milford, MA). The time delay between the detector and the fraction collector was typically 18 s. All fractions were evaporated to dryness, reconstituted in 1 ml of H₂O, and measured by dig-radioimmunoassay. Steroids co-elute with the fractions of interest at 15, 19, 23, and 25 min representing DLIF-genin, DLIF-mono, DLIF-bis, and DLIF, respectively, were separated by re-injected the fractions individually on selected isoatic mobile phase modes (18, 25, 30, and 39% CH₃CN, respectively) for 20 min. Radioimmunoassay measurements showed a single chromatographic peak for each species with immunoreactivity eluted at fraction 6.00 min for DLIF, 8.30 min for DLIF-bis, 12.30 min for DLIF-mono, and 16.30 min for DLIF-genin. The final yield of DLIF and its related deglycosylated congeners were measured by uv spectra and their digoxin equivalent molar absorptivity were determined.

Comparative chromatographic mobility studies between DLIF (and its deglycosylated congeners) and its counterparts digoxin (and its deglycosylated congeners) were conducted. Pure DLIF and digoxin were mixed and co-injected on selected an isocratic mode of 25% CH₃CN mobile phase for 60 min. Similarly, the genin, the mono- and the bis-derivatives of the two parents DLIF and digoxin, were run on 15, 20, and 25% CH₃CN mobile phase, respectively.

Purification of DLIF from Human Serum—Normal dig-free fresh-frozen human serum obtained from the American Red Cross, Louisville, KY, was treated with SSA (final concentration 1% SSA), vortexed, and centrifuged for 2 min at 9,500 × g. Immediately after centrifugation, we removed the supernatant and the entire supernatant and dried and reconstituted in water. We then applied the entire supernatant to a small primed C-18 reversed-phase Sep-Pak solid-phase extraction cartridge. The column was then washed with 3 ml of H₂O and eluted with 2 ml of 100% CH₃CN. For serum samples not treated with SSA, we applied the whole 1-ml sample directly to the Sep-Pak cartridge. In both cases, to remove the CH₃CN, we evaporated the eluates to dryness, dissolved the residue in 1 ml of dH₂O, and filtered through a Whatman 0.22-μm polyvinylidene difluoride filter in preparation for HPLC.

Molar Absorptivity and Concentration of DLIF—The molar absorptivity of digoxin was determined experimentally by injecting known weighed amounts of digoxin on HPLC and the measured peak area was monitored. At their respective maximum absorbance wavelengths, we assumed comparable molar absorptivity between digoxin and DLIF. Measured amounts of DLIF and its related congeners (by dig-radioimmunoassay equivalence) were injected on HPLC and the apparent molar-immunoreactive concentration of DLIF (or the respective congeners)
was determined. From the above data the percentage cross-reactivity of
DLIF and its congeners (competitive displacement of 125I-digoxin from
polyclonal digoxin-specific antibodies) were obtained.

Protein Assay—Protein concentrations were measured at different
steps of the purification procedure using a Pierce BCA protein assay
reagent (Rockford, IL). Absorbances were measured at 562 nm on a
Beckman DU-64 Spectrophotometer (Beckman) and protein concentra-
tion was estimated using bovine serum albumin as standard.

Digoxin-like Immunoreactivity—DLIF was measured using compet-
titive displacement of 125I-digoxin of polyclonal digoxin-specific anti-
bodies relative to digoxin standards as described elsewhere (25). The
standards consisted of digoxin dissolved in human serum and ranged in
concentration from 25 to 2,000 pg/ml. The zero standard had no digoxin.
The polyclonal antisera and components for the assay were obtained
from DuPont NEN (Wilmington, DE). All radioimmunoassay measure-
ments were done in duplicate.

RESULTS

Acid Hydrolysis of Adrenocortical DLIF—The retention
times of a mixture of pure digoxin and several deglycosylated
digoxin congeners as standards are shown in (Fig. 2, panel A). A
similar HPLC pattern (elution time and order) was observed
when digoxin was treated with SSA (Fig. 2, panel C). Incuba-

Fig. 2. HPLC elution pattern of deglycosylated DLIF and
digoxin molecules. Panel A, standards of digoxin and deglycosylated
congeners, dig-bis, dig-mono, and digoxigenin; panel B, pure adrenocor-
tical DLIF (4.6 ng of digoxin equivalent (d.e.)) incubated without (open
bar) and with (solid bars) 1% SSA for 60 s. Under these conditions,
DLIF is fractionated to components (DLIF-genin, DLIF-mono, DLIF-
bis, and DLIF). Note formation of two compounds less polar than
digoxin eluting at fractions 31 and 34 min, respectively. Panel C shows
digoxin (130 μmol/liter) treated with 1% SSA for 90 s and processed as
described in text.

Acid Hydrolysis of Human Serum DLIF—DLIF was ex-
tracted from human serum (subject not taking digoxin) as
described under “Experimental Procedures.” The initial HPLC
data shows a small peak of endogenous DLIF as seen in Fig. 3A
(open bar) with a retention time (25 min) similar to that of
digoxin or of adrenocortical DLIF. When similar serum was
 treated with SSA (final concentration 1%) for 60 s, the major
part of the 25-min fraction was converted to products with
retention times similar to those after acid exposure of digoxin
or of DLIF from bovine adrenal cortex, including the less polar
product at 31 min (Fig. 3A, solid bar). Fig. 3B shows HPLC
analyses of serum drawn from three human subjects (out of 22
human subjects not taking digoxin) but processed without acid
treatment. The HPLC elution pattern shows evidence of sev-
eral naturally occurring DLIF species consistent with the
deglycosylated congeners of DLIF. This may indicate
in vivo
metabolism of bovine adrenocortical DLIF with SSA (Fig. 2, panel B)
resulted in immunoreactive fractions with identical retention
times as those of the digoxin standards or the digoxin treated
with the same acid. Based on the known sequential removal of
sugars by acid hydrolysis (26) and the similarity in the reten-
tion times observed for the new DLIF fractions and those of the
digoxin congeners (digoxigenin, mono-, and bis-digitoxosides),
we named the DLIF fractions eluting at 15, 19, 23, and 25 min
as DLIF-genin, DLIF-bis, DLIF-mono, and DLIF, respectively.
In addition, note the additional immunoreactive DLIFs appear-
ing in fractions corresponding to less polar products of digoxin.

Acid Hydrolysis of Human Serum DLIF—DLIF was ex-
tracted from human serum (subject not taking digoxin) as
described under “Experimental Procedures.” The initial HPLC
data shows a small peak of endogenous DLIF as seen in Fig. 3A
(open bar) with a retention time (25 min) similar to that of
digoxin or of adrenocortical DLIF. When similar serum was
 treated with SSA (final concentration 1%) for 60 s, the major
part of the 25-min fraction was converted to products with
retention times similar to those after acid exposure of digoxin
or of DLIF from bovine adrenal cortex, including the less polar
product at 31 min (Fig. 3A, solid bar). Fig. 3B shows HPLC
analyses of serum drawn from three human subjects (out of 22
human subjects not taking digoxin) but processed without acid
treatment. The HPLC elution pattern shows evidence of sev-
eral naturally occurring DLIF species consistent with the
deglycosylated congeners of DLIF. This may indicate in vivo
metabolism of DLIF by the cleavage of the sugar moieties
attached at the C-3 position of the steroid nucleus (deglycosy-
lation pathway). The remaining 19 subjects showed only one
DLIF component at fraction 25, suggesting possible inter-indi-
vidual differences in metabolism of DLIF.
Chromatographic Mobility Study—Physical properties related to molecular polarity and solubility can be distinguished by HPLC analysis. Fig. 4, panel A, demonstrates the baseline resolving capacity of the HPLC system used to separate various deglycosylated DLIF congeners. After DLIF and its related deglycosylated congeners were purified and separated by HPLC chromatography, they were mixed and re-chromatographed on HPLC using the gradient described in Fig. 4, panel A. Absorbance at 216 nm is used to monitor elution of the various species and demonstrate the capability of isolating the individual deglycosylated congeners of DLIF by HPLC. Pure DLIF and digoxin were mixed and co-injected on HPLC using an isocratic 25% CH₃CN mobile phase. The two molecules separated by a minimum of 2–3 min and absorbance was monitored at 220 or 216 nm (Fig. 4, panel B). Similarly, the genin compounds (aglycone without sugar molecules) of both parent compounds were clearly separated by 1.5–2.0 min on an isocratic 15% CH₃CN mobile phase (Fig. 4, panel C). Interestingly, the elution order of the genin compounds (DLIF-genin and dig-genin) were reversed from the parent compounds (DLIF and dig). Similar separation results to the parent molecules (DLIF and digoxin) were obtained when DLIF-bis and DLIF-mono were mixed with their counterparts (dig-bis and dig-mono) on selected various isocratic mobile phase (25 and 20% CH₃CN mobile phase, respectively) (data not shown).

Spectrophotometric Analysis of DLIF Congeners—Fig. 5 shows the absorbance spectra of the DLIF, DLIF-genin, digoxin, and digoxigenin. The results show that DLIF congeners (like the parent DLIF) have the same characteristic absorbance maxima at 216 nm compared to that of digoxin and its congeners at 220 nm (see Table I). The absorbance spectra of digoxin, digoxigenin, and cortisone are included for comparison. The absorbance at 250 nm is a characteristic feature of steroids. The less polar species (digoxin-31) has a uv shift with an absorbance maximum at approximately 196 nm.

Molar Immunoreactivity of DLIF and Its Congeners—We investigated the relative molar immunoreactivity based on displacement of ¹²⁵I-labeled digoxin by purified DLIF and its deglycosylated congeners from the antiserum to digoxin. We applied the assumption of comparable molar absorbivities of DLIF (at 216 nm) and digoxin (at 220 nm) to calculate the picomole of actual DLIF congeners per pmol of digoxin equivalent immunoreactivity. Note that deglycosylated DLIF derivatives all show a similar immunoreactive potency (approximately 1000-fold less than digoxin) identical to their parent compound, DLIF. On the other hand, DLIF-31 is approximately 4,000-fold less immunoreactive than is digoxin (Fig. 6 and Table I).

Kinetics of DLIF Species Formation—To study the kinetics of formation of the individual DLIF congeners, we compared the time course for production of both DLIF and digoxin congeners by acid hydrolysis. Fig. 7 shows the production of the deglyco-
compete with a unit molar concentration of digoxin, with its antibody. Abs/true ratio for the standard compound (digoxin). This ratio represents the molar concentration of DLIF (or any of its congeners) needed to demonstrate the existence of several naturally occurring deglycosylated factors. We have recently identified the existence of a naturally occurring reduced lactone ring form of DLIF (or any of its congeners) by microsomal cytochrome P450 mediated activity (9). In this study, we demonstrated the existence of several naturally occurring deglycosylated species of DLIF in human serum. We show that these derivatives can also be produced in vitro by acid-induced deglycosylation of DLIF isolated from bovine adrenal cortex. The various DLIF species (DLIF-genin, DLIF-mono, DLIF-bis, and DLIF, by analogy to the cardenolidic counterparts, digoxin and its deglycosylated congeners) correspond to the factor with no sugars (aglycone), one sugar, two sugars, and three sugars (DLIF), respectively. These findings provide a working model for characterizing two important structural epitopes found on these endogenous factors and also establish a basis for characterizing metabolic pathways of synthesis of endogenous DLIF in mammals.

We used several techniques, each specific for probing structural differences on different portions of the cardiac glycoside-like molecules. Reverse-phase HPLC is sensitive to modifications affecting molecular polarity and solubility; UV-spectral analysis is sensitive to structural features of or in close proximity to the lactone ring; kinetics of deglycosylation of the sugars detects differences in sugar stoichiometry and their binding to the aglycone; and, immunoreactivity is sensitive to molecular modifications near the C- and D-rings of the sterol and the lactone-ring portion of DLIF or digoxin.

By optimizing HPLC elution conditions we demonstrate the ability to separate DLIF from digoxin and their respective aglycones from each other, including two here-to-for unknown DLIF species. The HPLC elution pattern of deglycosylated adrenocortical DLIF was identical to that of digoxin using the linear elution gradient (Fig. 2, B and C). The DLIF fractions eluted in fractions correspond to the standards of digoxin and its deglycosylated congeners (Fig. 2A). A similar chromatographic pattern was observed after acid-deglycosylation of DLIF isolated from human serum (Fig. 3A) and, in fact, the immunoreactive fragments were found naturally in human serum (3 of 22 subjects) without acid treatment (Fig. 3B). These results are consistent with our recent hypothesis suggesting molecular similarity between DLIF extracted from bovine adrenal cortex and that from human serum (27).

Additional HPLC studies using mobile phases clearly showed relative mobility shifts when comparing DLIF and digoxin (Fig. 4B) and also for their respective aglycones (Fig. 4C). Interestingly, the mobility of DLIF-genin was shifted toward increased polarity relative to digoxigenin when compared to the opposite relative mobilities of the parent compounds. The genin compounds are aglycone sugar-free molecules (see Fig. 1), hence, these findings are the first reported observation indicating that structural differences between DLIF and digoxin.

The "true" concentration of DLIF and its related components could also be estimated by multiplying the measured concentration by the molar immunoreactivity ratio of each compound (Table I).

**Table I**

| Compound       | Molar immunoreactivity |
|----------------|------------------------|
|                | Mean | Range |
| DLIF           | ND   | n=5  |
| DLIF-bis       | 3.3×10^4 | 2.8-3.8×10^4 |
| DLIF-mono      | 3.7×10^4 | 3.1-3.9×10^4 |
| DLIF-genin     | 4.3×10^4 | 3.9-4.6×10^4 |
| Fraction 31    | 1.2×10^4 | 1.0-1.3×10^4 |
| Digoxin        | 3.3×10^4 | 2.7-3.9×10^4 |
| DLIF-genin     | 3.7×10^4 | 3.1-3.9×10^4 |

a Absorbance was measured at λmax for each compound (DLIF, DLIF-bis, DLIF-mono, and DLIF-genin, 216 nm; digoxin, 220 nm; fraction 31, 196 nm). Similar molar absorptivity is assumed for DLIF and its congeners as a result of incubating DLIF or digoxin with 7.6% SSA. The reaction was terminated (by neutralization at zero time (no acid) plus 9 time points over the course of 24 h. After each neutralization at the times indicated the contents of the reaction were processed through Sep-Pak and then fractionated by HPLC chromatography. Having identified the HPLC fractions above (Fig. 2), we constructed a fractional molar distribution versus time graph (Fig. 7). This graph provides the relative amount of each species present in the reaction mixture at each time point. Note that acid hydrolysis of both digoxin and DLIF produces a less polar immunoreactive product (F-31). To confirm that digoxigenin (and DLIF-genin by analogy) was being converted to fraction 31, we incubated digoxigenin with 1% SSA at 22 °C and monitored the formation of fraction 31 by HPLC. Fig. 8 shows the disappearance of digoxigenin (monitored by absorbance at 220 nm) and the simultaneous appearance of fraction 31 (monitored at 196 nm).

**DISCUSSION**

Since the original discovery of DLIF in mammals (1, 2) little has been learned about the metabolism of these endogenous steroid-like factors. We have recently identified the existence of a naturally occurring reduced lactone ring form of digoxin-like immunoreactive factor (dihydro-DLIF, analogous to that of dihydrotroglide) and showed that dihydrotroglide is converted to a digoxin-like immunoreactive compound by microsomal cytochrome p450 mediated activity (9). In this study, we demonstrate the existence of several naturally occurring deglycosylated congeners of DLIF as a result of incubating DLIF or digoxin with 7.6% SSA. The reaction was terminated (by neutralization) at zero time (no acid) plus 9 time points over the course of 24 h. After each neutralization at the times indicated the contents of the reaction were processed through Sep-Pak and then fractionated by HPLC chromatography. Having identified the HPLC fractions above (Fig. 2), we constructed a fractional molar distribution versus time graph (Fig. 7). This graph provides the relative amount of each species present in the reaction mixture at each time point. Note that acid hydrolysis of both digoxin and DLIF produces a less polar immunoreactive product (F-31). To confirm that digoxigenin (and DLIF-genin by analogy) was being converted to fraction 31, we incubated digoxigenin with 1% SSA at 22 °C and monitored the formation of fraction 31 by HPLC. Fig. 8 shows the disappearance of digoxigenin (monitored by absorbance at 220 nm) and the simultaneous appearance of fraction 31 (monitored at 196 nm).

**Fig. 6.** Competitive displacement (cross-reactivity) of 125I-digoxin from polyclonal digoxin-specific antiserum by DLIF and its congeners. Digoxin as a standard is included for comparison. The concentration of DLIF and its congeners were determined by multiplying the measured concentration by the molar immunoreactivity of DLIF and its deglycosylated congeners with digoxin.

**Table 1**

| Compound       | Molar immunoreactivity |
|----------------|------------------------|
|                | Mean | Range |
| DLIF           | ND   | n=5  |
| DLIF-bis       | 3.3×10^4 | 2.8-3.8×10^4 |
| DLIF-mono      | 3.7×10^4 | 3.1-3.9×10^4 |
| DLIF-genin     | 4.3×10^4 | 3.9-4.6×10^4 |
| Fraction 31    | 1.2×10^4 | 1.0-1.3×10^4 |
| Digoxin        | 3.3×10^4 | 2.7-3.9×10^4 |
| DLIF-genin     | 3.7×10^4 | 3.1-3.9×10^4 |

a Absorbance was measured at λmax for each compound (DLIF, DLIF-bis, DLIF-mono, and DLIF-genin, 216 nm; digoxin, 220 nm; fraction 31, 196 nm). Similar molar absorptivity is assumed for DLIF and its congeners.

b Molar immunoreactivity ratio of DLIF and its related congeners was calculated by dividing the Abs/true ratio of each compound by the Abs/true ratio for the standard compound (digoxin). This ratio represents the molar concentration of DLIF (or any of its congeners) needed to compete with a unit molar concentration of digoxin, with its antibody.

d ND, not determined.
digoxin in the aglycone portions of these compounds. Both the chromatographic mobility shifts and the immunoreactive potency differences observed for the two genin compounds of DLIF and digoxin (Table I and Fig. 6) suggests structural differences in or near the lactone ring for the aglycones. This is supported by the spectral analysis showing a uv shift in the maximum absorbance for DLIF (216 nm) compared to digoxin (220 nm) (Fig. 5). However, the kinetics of acid-induced deglycosylation studied by monitoring the rate of formation of different DLIF species showed that DLIF deglycosylation is considerably faster (approximately 8-fold) than that observed for digoxin (see Fig. 7). This is an indication that the sugars on DLIF are different than those on digoxin and thus in the strength of the bond to each other in the linear linkage and/or to the DLIF-aglycone at position C-3 (Fig. 1).

Characterizing the structural features of endogenous digoxin- or ouabain-like counterparts of the cardenolides has been difficult because of their low concentrations in tissues and difficulty in extracting sufficient quantities for detailed structural analysis. For example, initial data suggested that OLF (ouabain-like factor) was structurally identical to ouabain using mass spectroscopy and other related techniques (28, 29). However, more recent studies using an exciton-coupled circular dichroism (CD) technique show that OLF and HIF (an isomer of ouabain from bovine hypothalamus) are structurally different from ouabain (30). The initial suggestion of a structural similarity between OLF and ouabain was based on the similarity in their chromatographic mobility, mass spectral data, and their unit immunoreactivity with antibodies to ouabain. However, in the case of DLIF we now have evidence of chromatographic, spectral, and molar immunoreactivity differences which suggests that DLIF is more structurally distinct from digoxin than OLF is to ouabain. However, sufficient quantities of these compounds will still need to be harvested to fully permit the identification of fine structure.

Of interest is the discovery of a new as yet undefined product of DLIF and of digoxin (fraction 31, less polar by HPLC elution than DLIF or digoxin) produced by acid-induced deglycosylation of these molecules and also existing naturally in vivo in human serum. Our data are consistent with the hypothesis that DLIF-31 (or digoxin-31) is formed preferentially from the genin derivatives (Fig. 8). The far uv shift to 196 nm observed for the DLIF-31 immunoreactive component is, however, consistent with and characteristic of a structural modification or near the lactone ring. A similar uv shift is observed after formation of dihydrodigoxin (196 nm) from digoxin (220 nm) (31) or after modification by a double bond saturation on the A- or B-ring of the steroid (32). However, the presence of a double bond in the A- or B-ring structures of the DLIF aglycone is not suspected because that structural feature is usually associated with a characteristic absorption band at 250 nm (see Fig. 5, e.g. cortisone). DLIF-31, therefore, is a new species and since it is detected naturally in some human sera, may be a metabolic...

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**Fig. 7.** Apparent molar fractional distribution and time course of formation of DLIF and digoxin congeners after incubation with acid. Conditions are as described under “Experimental Procedures.” Panel A shows the relative production of DLIF congeners from DLIF (isolated from bovine adrenal cortex (starting with 10 ng of digoxin equivalent DLIF) after treatment with 7.6% SSA (7.6 g of SSA for each 100-ml homogenate). Panel B shows a comparable fractional distribution profile for digoxin congeners produced (starting with 50 ng of digoxin) under similar experimental conditions. Note: time scales are different and that DLIF deglycosylates more rapidly than digoxin.

**Fig. 8.** Chromatographic analysis of formation of HPLC fraction 31 from digoxigenin. Panel A, digoxigenin (100 μmol/liter) treated with 1% SSA showing the formation of fraction 31. Panel B, one of the chromatograms used to construct the above figure, representing the 2-h incubation of digoxigenin.
The 4-fold decrease in immunoreactivity of DLIF-31 compared to DLIF and relative to digoxin (Fig. 6 and Table I) may explain some of the discrepancies reported in other studies isolating digoxin-like compounds (36). In fact, some of these diglycosylated congeners (generated during isolation procedures and/or those found naturally) may have been identified as different kinds of digoxin-like immunoreactive compounds.

DLIF is considered as one of the most interesting and detrimental interferant to most commonly used digoxin assays. The variable cross-reactivity and the different amounts or ratios of the DLIF congeners in serum may explain the wide variation in detection sensitivities for endogenous DLIF reported by digoxin immunoassays (37). Recognizing the presence of these newly discovered congeners and understanding their relative immunoreactivity with the antibodies used will provide a more accurate means of therapeutic digoxin monitoring for digoxin (38). DLIF is known to exist bound tightly to a 22-36-kDa protein in serum (25, 39). The stoichiometry of DLIF-glycosylation may also affect this protein binding and thus influence the detection of these factors in serum when using different digoxin immunoassays. This may well explain changes in the extent of protein binding of DLIF observed in some clinical conditions, e.g. pregnancy induced hypertension (40) or renal failure (2).