Specific Binding of Cardiac Glycoside Drugs and Endogenous Digitalis-like Substances to Particulate Membrane Fractions from Human Placenta

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We studied the characteristics of binding of cardiac gylco-sides to particulate membrane fractions from human placenta, to demonstrate that placental tissue is a suitable source of receptors for digitalis drugs. Moreover, we performed preliminary experiments with ^{125}I-labeled digoxin and placental particulates to develop a radioreceptor assay for measurement of endogenous substances with activity similar to cardiac glycoside drugs (EDLS). Placental membrane fractions were incubated with [^{3}H]ouabain (10 nmol/L) or ^{125}I-labeled digoxin (50 pmol/L). With both ligands, binding followed a pseudo-first-order reaction kinetics and was saturable. Scatchard analysis revealed a single class of sites [for ouabain, $K_D = 20.2 \pm 5.8$ nmol/L (mean $\pm$ SEM), $B_{max} = 3.1 \pm 0.9$ nmol per gram of protein; for digoxin, $K_D = 29.7 \pm 1.9$ nmol/L, $B_{max} = 24.3 \pm 1.1$ nmol per gram of protein]. As expected, digoxin was less potent than ouabain in displacing both tracers from digitalis drugs receptors; progesterone, cortisone, digitoxose, furosemide, bumetanide, and propranolol had no or little effect. Specific ^{125}I-labeled digoxin binding was competitively inhibited by plasma and (or) urine extracts from newborns, adults, pregnant women, and patients with renal insufficiency. Inhibition of binding and volume of plasma and urine assayed were linearly related. These findings support the hypothesis that cardiac glycosides and EDLS can interact with the human placenta and suggest placental tissue to be a suitable source of receptors for cardiac glycosides.

The presence of an endogenous substance (or some endogenous factors) with digitalis-like immunoreactivity in biological fluids of humans and experimental animals was well confirmed (for review see references 1 and 2). Digoxin-like immunoreactivity was detectable in plasma (or serum) of normal adult subjects, whereas concentrations were significantly higher in pregnant women, newborns, patients with renal impairment, and in some patients with arterial hypertension (1, 2). Some authors have postulated that an endogenous cardiac-glycoside-like factor might play a role in regulating fluid volume and in the pathogenesis of some forms of essential hypertension (2, 3) and of eclampsia in pregnancy (2, 4–6). It was suggested (2) that digoxin-like immunoreactive substance(s) could also inhibit the membrane Na$^+$/K$^+$ pump by binding to the specific cardiac glycoside receptor (Na$^+$/K$^+$-dependent ATPase; EC 3.6.1.37). Therefore, these endogenous factors could have both the immunoreactive and biological properties of cardiac glycoside drugs.

At present, there is no agreement on the best procedure(s) for detecting and measuring this (these) endogenous substance(s) (2, 7). Use of antiserum to digoxin in immunological methods for assay of digoxin-like immuno-reactivity can give some erroneous findings and difficulties in interpretation of results (2, 7). Specific and nonspecific interferences probably are both present in all digoxin immunoassays, and the ratio between these two forms of interferences probably varies greatly with different immunological methods, and also in the same system when different lots of antiserum are used or the assay procedures (incubation time or temperature) are changed (2, 7).

The non-immunological procedures (radioreceptor assay, measurement of the inhibition of ion transport across the membrane, or of Na$^+$/K$^+$-ATPase activity) are considered more specific for the measurement of digitalis-like substances, but are generally cumbersome, time consuming, and less sensitive than immunological methods (2, 7).

Recently, a radioreceptor assay has been described for measurement of digoxin in which [^{3}H]digoxin is used as tracer and Na$^+$/K$^+$-ATPase from autopsied human heart as receptors (8). However, autopsy material is not easily available, and membranes prepared from autopsy tissues may be affected by a general loss of receptors and unpredictable changes of binding properties (9).

Whitsett and Wallick (10) reported the presence of specific receptors for [^{3}H]ouabain in human placenta. Therefore, we studied the binding characteristics of cardiac glycosides to membrane preparations from human placenta to demonstrate that placental tissue may be a suitable source of digitalis drugs receptors. Moreover, ^{125}I-labeled tracers generally improve the practicability and the sensitivity of RIAs and radioreceptor assays, so we performed preliminary experiments with ^{125}I-labeled digoxin and placental particulates, to develop a radioreceptor assay for measurement of endogenous substances with activity similar to that of cardiac glycoside drugs.

Materials and Methods

Preparation of placental membranes. Particulate membrane fractions from human placenta were prepared as described by Schocken et al. (11). Term placentas, obtained with consent immediately after delivery, were trimmed free of adherent membranes, minced, and homogenized with a Kinematica GmbH Polytron PT 10–35 in 2.5 volumes of ice-cold homogenization buffer (per liter, 250 mmol of sucrose, 1 mmol of MgCl$_2$, and 5 mmol of Tris hydrochloride, pH 7.4, at 4 °C) by means of three consecutive 10-s bursts at maximum speed. The homogenates were filtered through two layers of cheesecloth and subjected to differential centrifugation at 4 °C. After centrifugation for 10 min at 2000 × g, the supernates were centrifuged at 39 000 × g for 10 min. The resulting pellets were resuspended by gentle homogenization at 4 °C in 50 mmol/L Tris hydrochloride buffer, pH 7.4, washed, and recentrifuged twice under the same conditions. The final pellets were resuspended in the same buffer at a protein concentration of 8–12 g/L and kept frozen at −70 °C until used (for as long as two months). Protein was quantified with the BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL)

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with bovine serum albumin (Cohn Fraction V; Sigma Chemical Co., St. Louis, MO) as the standard.

Receptor binding assay. The technique for measuring [³H]ouabain binding was a modification of that described by Lichtstein and Samuelov (*12*). The incubation mixture consisted of 200 μL of placental membranes (50 μg of protein), 50 μL of [³H]ouabain (10 nmol/L; NEN Du Pont, Boston, MA; specific radioactivity 25 kCi/mol), 10 μL of 100 mmol/L ATP (vanadium-free, Sigma Chemical Co.), and 50 μL of competing ligands at various concentrations in a total volume of 500 μL of incubation buffer (per liter: 0.5 mmol of EDTA, 80 mmol of NaCl, 4 mmol of MgSO₄, and 50 mmol of Tris hydrochloride, pH 7.4, at 37 °C). [³H]Ouabain and all other ligands were freshly prepared in incubation buffer. We terminated the reaction by adding 4 mL of 50 mmol/L Tris hydrochloride buffer, pH 7.4, at 37 °C, followed by rapid filtration through GF/C glass-fiber filters (Whatman Inc., Clifton, NJ). Each filter was washed three times with an additional 4 mL of the same buffer, and the radioactivity bound to the filter was counted by standard liquid-scintillant technique at about 45% efficiency. Specific binding of [³H]ouabain was defined as the difference between radioactivity bound in the absence or presence during incubation of ouabain, 0.1 mmol/L. The filter blank accounted for most of the usual nonspecific binding.

For ¹²⁵I-labeled digoxin binding, 200 μL of placental membranes (150 μg of protein) and 50 μL of ¹²⁵I-labeled digoxin (0.5 nmol/L; NEN Du Pont; specific radioactivity 2200 kCi/mol) were incubated in polypropylene tubes at 37 °C, with or without 100 μL of digoxin solutions (from 10 nmol/L to 10 μmol/L) or sample extracts in a total volume of 500 μL of incubation buffer (per liter: 5 mmol of MgCl₂, 5 mmol of Na₂HPO₄, and 50 mmol of Tris hydrochloride, pH 7.4, at 37 °C). After incubation, 400 μL of the incubation mixture was transferred to pre-chilled microtubes and promptly centrifuged in a Beckman Microfuge B for 2.5 min at 4 °C. Membrane pellets were washed with 400 μL of 50 mmol/L Tris hydrochloride buffer, pH 7.4, at 4 °C and counted in a gamma spectrometer at about 75% efficiency. We chose centrifugation because a large amount of ¹²⁵I-labeled digoxin was nonspecifically bound to glass-fiber filters, thus obscuring specific receptor binding of the ligand. A 10 mmol/L stock solution of digoxin was prepared in dimethyl sulfoxide, which did not affect the maximal specific binding of ¹²⁵I-labeled digoxin to the membranes. Subsequent dilutions were prepared in incubation buffer. Specific binding of ¹²⁵I-labeled digoxin was defined as the difference between radioactivity bound in the absence or presence, during incubation, of digoxin, 10 μmol/L.

Each binding assay was performed in duplicate and repeated at least three times. Binding data were expressed as mean ± SEM. The values measured by the radioreceptor assay were expressed as digoxin equivalents (nmol per liter of serum or urine). We used a Macintosh SE personal computer to analyze receptor radioligand curves and results. The precision profile of the radioreceptor assay was calculated by previously described computer programs (*13, 14*).

Samples. Plasma pools from 44 normal subjects, from six uremic patients, and from more than 50 pregnant women in the first, second, and third trimesters of pregnancy; one pooled specimen of serum from some newborns (cord blood); and two urine pools from some newborns (first or second postnatal day) and from normal adult subjects were prepared and stored at −20 °C until assayed with the radiore-

ceptor assay.

Before the analysis, each plasma (serum) or urine sample was extracted on fresh SepPak C18 cartridges (Waters Associates) as previously described (*15*). A 2-mL sample was applied to each cartridge that had been previously activated with 4 mL of methanol followed by 20 mL of distilled water. After we washed the resin with 40 mL of distilled water, each cartridge was eluted with 4 mL of methanol. The extracts were evaporated under reduced pressure, the dry residues were redissolved in 250 μL of incubation buffer, and 100 μL of this solution were then used in the radioreceptor assay.

Extraction of urine or plasma (serum) samples with SepPak C18 cartridges avoided interferences from ions (such as K⁺) (*15*), proteins, or fatty acids. More than 99.5% of the albumin or total proteins in the sera, and more than 99% of the free fatty acids were eliminated by the washings of the resin. Moreover, even though steroid hormones are surely present in the extracts, we found that progesterone or cortisone, at least, did not significantly inhibit ¹²⁵I-labeled digoxin binding (see *Results,* Figure 5a). The extraction procedure, moreover, allowed an eightfold concentration of samples.

Results

[³H]Ouabain binding to placental membranes. The binding of [³H]ouabain to particulate membrane fractions from human placenta was time-, protein-, and ligand-concentration dependent. At 10 nmol of [³H]ouabain per liter and 0.1 mg of protein per milliliter, respectively, specific binding reached a steady-state of 1.53% within 2 h and remained constant until 5 h. During this time, nonspecific binding was less than 2% of the total binding.

The binding of [³H]ouabain to placental membranes was a saturable process (Figure 1a). Scatchard analysis (*16*) of the specific binding data, as shown in Figure 1b, revealed a single class of binding sites with a K_D value of 20.2 ± 5.8 nmol/L and a binding capacity of 3.1 ± 0.9 nmol per gram of protein. Binding of [³H]ouabain was competitively inhibited by unlabeled ouabain and digoxin, with 50% inhibition at concentrations of about 10 nmol/L and 30 nmol/L, respectively (Figure 2). In contrast, furosemide, (±)-propranolol, and (−)-norepinephrine had no effect in concentrations up to 10 mmol/L (Figure 2). The dissociation constant for the interaction of digoxin with [³H]ouabain binding sites, as calculated by the equation of Cheng and Prusoff (*17*), was 22.3 ± 3.5 nmol/L.

Specific ouabain binding to human placental membranes was inhibited by a serum extract of umbilical cord blood (Figure 3). Binding inhibition, up to a concentration of 1 nmol of ouabain per liter, was about 45% (Figure 3). A change in affinity, rather than in the receptor number, seems to account for the observed change in [³H]ouabain binding. In contrast, plasma extracts from blood donors had no significant effect on the specific binding of [³H]ouabain to human placental membranes (data not shown).

¹²⁵I-labeled digoxin binding to placental membranes. The time course of total, specific, and nonspecific binding of ¹²⁵I-labeled digoxin (50 pmol/L) to human placental particulates (300 μg/mL) at 37 °C and pH 7.4 is shown in Figure 4. Specific binding reached a plateau (26.5% ± 2.6%) within 4 h and remained constant for 2 h more. Nonspecific binding averaged 4.6% ± 1.0%. The time required to obtain steady-state decreased with increasing ¹²⁵I-labeled digoxin or protein concentration.
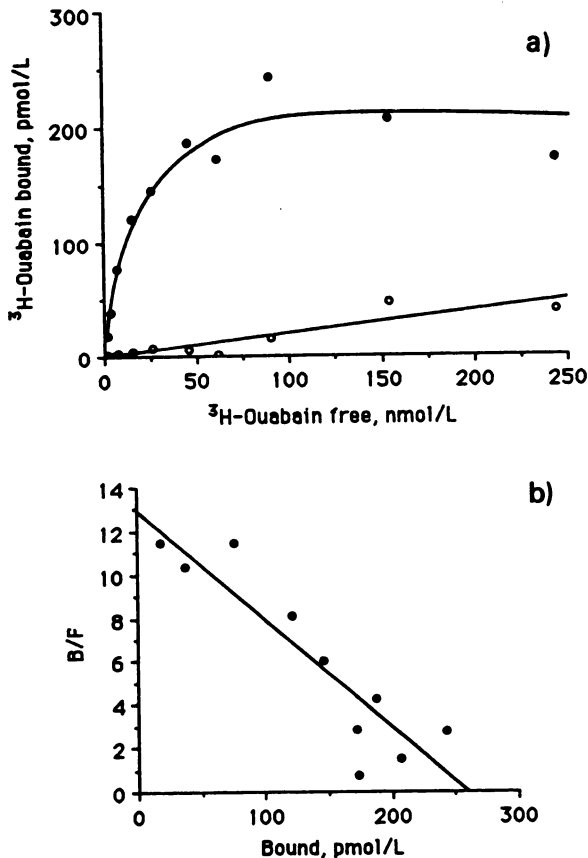
Fig. 1. a. Saturation curve of [³H]ouabain to human placental membranes: ●, specific binding; ○, nonspecific binding
The data represent the mean of four experiments conducted in duplicate
b. Scatchard plot of the specific binding data from Fig. 1a
B/F = bound [³H]ouabain/free [³H]ouabain

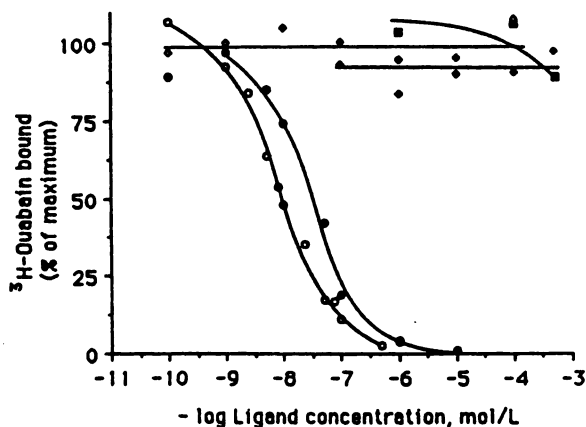
Fig. 2. Inhibition of specific [³H]ouabain binding to human placental membranes by (○) ouabain, (●) digoxin, (◆) furosemide, (◇) (±)propranolol, and (■) norepinephrine

Specific binding increased linearly ($r = 0.99$, n = 6) with increasing protein concentration up to a value of nearly 400 mg/L and became saturated at protein concentrations exceeding 700 mg/L (data not shown). An incubation time of 5 h and a protein concentration of 300 mg/L were therefore routinely used.

To evaluate binding specificity, we studied the ability of several unlabeled ligands to inhibit the binding of ^{125}I-labeled digoxin to placental particulates (Figure 5a). ^{125}I-Labeled digoxin binding was inhibited in a dose-dependent
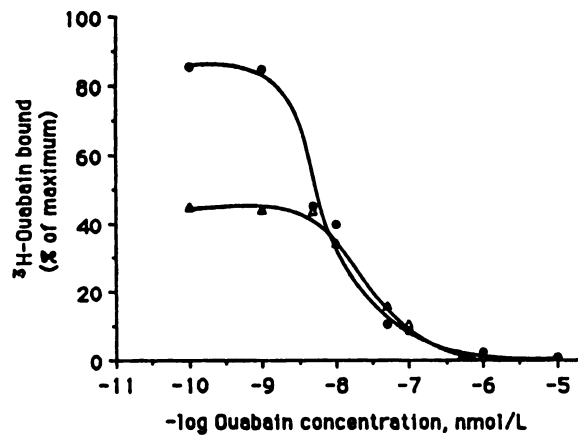

Fig. 3. Inhibition of specific binding of [³H]ouabain to human placental membrane fractions by (●) ouabain and by (△) a serum extract of umbilical cord blood
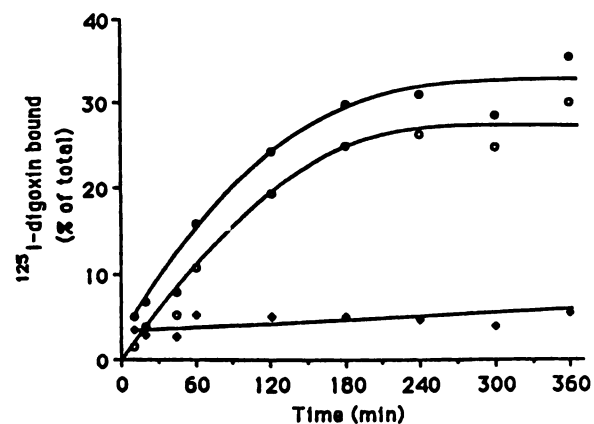

Fig. 4. Time course of total (●), specific (○), and nonspecific (◆) ^{125}I-labeled digoxin binding (50 pmol/L) to human placental membranes (300 g/L) at 37 °C, pH 7.4

manner by ouabain, digoxin, and digoxigenin, one of the cardioactive metabolites of digoxin. As expected, ouabain was more effective than digoxin in inhibiting binding with EC_{50} values (molar concentration for 50% inhibition) of about 7 nmol/L and 45 nmol/L, respectively. The inhibition constant for ^{125}I-labeled digoxin binding to placental membranes by ouabain was 7.2 ± 1.0 nmol/L. Digoxigenin, which was reported to have a lower cardioactivity than digoxin (18), was unexpectedly found to have the same potency in displacing ^{125}I-labeled digoxin from its binding sites. However, the shape of the displacement curve for the two substances was quite different: the curve for digoxigenin was flat and, after transformation into a "pseudo Scatchard plot" (19)—i.e., by plotting percent inhibition of binding vs percent inhibition divided by digoxigenin concentration—curvilinear plots were obtained. In contrast, when the specific digoxin binding data were analyzed according to Scatchard (16), as presented in Figure 5b, a linear plot was obtained, suggesting one class of binding sites with a K_D value of 29.7 ± 1.9 nmol/L and a B_{max} of 24.3 ± 1.1 nmol per gram of protein.

[125]I-Labeled digoxin bound to human placental particulates was not displaced by digitoxose (one of the cardio-inactive metabolites of digoxin) or by steroids such as cortisone and progesterone (Figure 5a). Bumetanide (the specific inhibitor of the cotransport system), furosemide, and (±)-propranolol inhibited binding only at concentra-
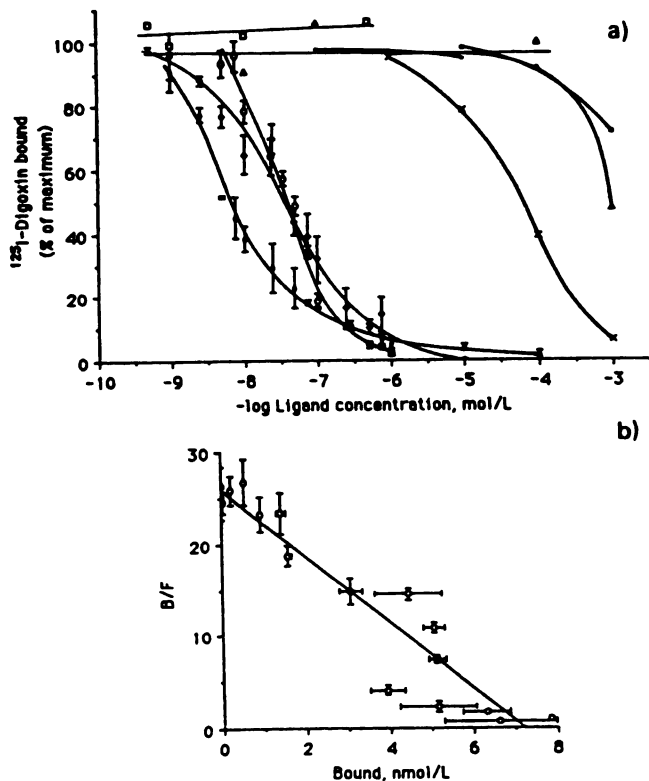
Fig. 5. *a*. Inhibition of specific ^{125}I-labeled digoxin binding to human placental membranes by (o) ouabain, (O) digoxin, (♦) digoxigenin, (□) digitoxose, (+) progesterone, (Δ) cortisone, (■) furosemide, (X) bumetanide, and (▲)(±)-propranolol; *b*. Scatchard plot of specific digoxin binding data
B/F = bound ^{125}I-labeled digoxin /free ^{125}I-labeled digoxin. Mean ± SEM

tions exceeding 100 µmol/L (Figure 5a).

Radioreceptor assay using ^{125}I-labeled digoxin. A typical standard curve of the radioreceptor assay for the measurement of endogenous digitalis-like substances, in which ^{125}I-labeled digoxin (50 pmol/L) is used as a tracer and with particulate membrane fractions from human placenta (300 mg/L) as receptors, is shown in Figure 6. The standard-curve concentrations ranged from 0 to 1 µmol of digoxin per
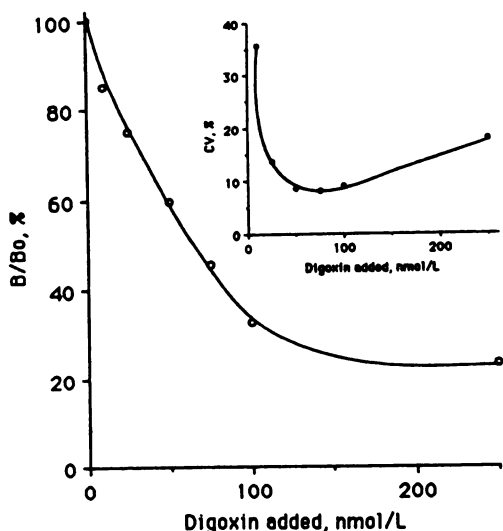

Fig. 6. Typical standard curve and precision profile (*inset*) of the radioreceptor assay

liter. The mean sensitivity (n = 7) of the assay was 5.7 ± 0.9 nmol/L.

Linear relationships with correlation coefficients >0.98 were found between the volumes assayed and the digoxin equivalents measured by the radioreceptor assay in different pooled specimens of plasma (serum) or urine from newborns, adult normal subjects, pregnant women, and uremic patients (data not shown).

Newborns, pregnant women, and uremic patients showed higher values for circulating digitalis-like substances than did normal subjects; moreover, higher values were found for urine of newborns and adult subjects compared with those for plasma (Table 1).

Discussion

Our findings confirm that specific receptors for cardiac glycoside drugs are present in human placenta and indicate that this tissue may be a readily available source of Na^+/K^+-ATPase. In fact, the putative receptor showed high affinity and saturable binding to [3H]ouabain and ^{125}I-labeled digoxin. The affinity of placental Na^+/K^+-ATPase for digitalis drugs was similar to that shown by the enzyme isolated from other tissues [e.g., dog kidney, porcine heart, human heart (8), human erythrocytes (20)], and the maximum number of binding sites was high. As expected, digoxin was less potent than ouabain in displacing tracer from digitalis drugs receptors, whereas digitoxose, progesterone, cortisone, furosemide, bumetanide, and (±)-propranolol had no or little effect.

Multiple sites with high and low affinity for cardiac glycosides have been reported in rat cardiac ventricle (21) and cultured chick heart cells (22), with [3H]ouabain used as a ligand. The presence of multiple forms of binding sites with different affinity to digitalis drugs may affect the sensitivity and specificity of a radioreceptor assay. However, in the present study both [3H]ouabain and ^{125}I-labeled digoxin shared only one class of binding sites for cardiac glycosides in the human placenta, thus confirming [3H]ouabain binding data previously reported by Whitsett and Wallick (10), and indicating that human placenta is a suitable tissue for radioreceptor assay of digitalis drugs.

The ^{125}I-labeled digoxin was found to label digitalis drugs receptors of the human placenta with an affinity similar to that shown by [3H]ouabain, but its use enhanced practicability and sensitivity of the radioreceptor assay of digoxin-like immunoreactive substances.

A linear relationship was found between the volumes assayed and the digoxin equivalents measured by the

Table 1. Digitalis-like Substances Measured by Radioreceptor Assay in Pooled Specimens of Urine and (or) Plasma from Various Groups of Subjects

Subjects	Sample	Digoxin equivalents, nmol/L
Normal adults	Plasma	3.2
	Urine	11.2
Pregnant women		
(I trimester)	Plasma	9.3
(II trimester)	Plasma	17.0
(III trimester)	Plasma	13.5
Newborns (1st–2nd day)	Urine	43.6
	Cord blood	11.2
Uremic patients	Plasma	11.8

[125]I-labeled digoxin radioreceptor assay in different plasma (serum) or urine pools of newborns, adult normal subjects, pregnant women, and uremic patients. As expected (1, 2), pooled specimens of plasma (serum) from newborns, pregnant women, and uremic patients showed higher values for digitalis-like substances in the circulation than did a plasma pool from normal subjects. Moreover, higher values were found for urine of newborns and adult subjects as compared with concentrations in the circulation.

The presence of high-affinity digitalis drugs receptors in human placenta and the high values for endogenous digitalis-like substances in the circulation of mothers and newborns indicate that these endogenous factors may have an important role in the regulation of electrolyte and fluid balance in the feto-placental unit and in the pathogenesis of hypertension and eclampsia in pregnancy, as also suggested by other authors (4-6).

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