DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCES IN THE NEONATE

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ABSTRACT

Digoxin, a steroidal glycoside that inhibits Na^+/K^+-ATPase, is the most commonly prescribed cardiac medication in North America. Blood levels of this drug are routinely measured to reduce the risks of toxicity. Reports questioning the specificity of antisera used in radioimmunoassays for serum digoxin measurements began to appear after 1975[1] when plasma from patients with renal failure, not on glycoside therapy, showed false-positive digoxin levels. Since then, digoxin-like immunoreactive substances (DLIS) have been found in sera from patients with hepatic failure, hypertension, pre-eclampsia, in amniotic fluid and cord blood. Some of the highest values for DLIS have been detected in premature infants, where levels have often exceeded the therapeutic range (0.2-2.0 µg/L) for digoxin. Cord blood has been identified as a rich source of DLIS.

Dahl *et al*[2] were the first to suggest that a circulating saluretic substance "endoxin", may cause hypertension in salt sensitive rats. Gruber *et al*[3] reported on the existence of digoxin-like factor(s) in the plasma of volume-expanded dogs. Plasma from these dogs inhibited Na^+/K^+ATPase activity. A number of other studies have supported the concept that such digoxin-like factors may be of etiological significance in hypertension[4]. In view of these observations, a study was undertaken to isolate and fractionate DLIS from mixed cord blood and determine whether or not any of this digoxin-like material possessed Na^+/K^+-ATPase inhibitory properties.

Cord blood collected in the Grace Hospital Maternity Unit (Vancouver, BC), was pooled and DLIS extracted using C_{18}-Sep Paks. Extracts were

resolved by high performance liquid chromatography (HPLC) into several fractions containing digoxin equivalent immunoactivity as measured by radioimmunoassay (RIA). A number of steroids and bile acids (dehydroepiandrosterone-sulfate, cortisone, cortisol, deoxycortisone, Δ^4androstenedione, progesterone and glycochenodeoxycholic acid) cross-reacted with digoxin antisera and had HPLC retention times similar to DLIS-containing fractions.

The ability of HPLC generated DLIS positive cord blood fractions to inhibit Na^+/K^+-ATPase activity was determined in three different assay systems; red cell ^{86}Rb uptake canine kidney-Na^+/K^+-ATPase and red cell membrane-Na^+/K^+-ATPase. At least six fractions contained DLIS and inhibited Na^+/K^+-ATPase activity. Inhibition varied with the assay system used but none of the fractions inhibited ^{86}Rb uptake by erythocytes. One fraction (which eluted at 29 minutes) contained progesterone; 72% of the inhibitory activity present in this fraction was attributable to this steroid. Another inhibitory fraction co-eluted with dehydroepiandrosterone-sulfate (DHEAS-S). The only fractions found to inhibit both the red cell membrane and canine kidney Na^+/K^+-ATPase enzymes eluted at 7 and 29 minutes.

In summary, a number of digoxin-like immunoreactive substances were isolated from cord blood by HPLC fractionation and found to inhibit Na^+/K^+-ATPase activity. Inhibition varied with the assay system used. There was no apparent correlation between inhibition and digoxin immunoreactivity. Very large quantities (500 mL) of cord blood were extracted to demonstrate these properties. It remains to be determined whether or not DLIS isolated during the perinatal period is of physiological significance.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

III. LIST OF TABLES

IV. LIST OF FIGURES

IV. LIST OF FIGURES (cont'd)

IV. LIST OF FIGURES (cont'd)

V. LIST OF ABBREVIATIONS

AG-AB	Antigen-Antibody Complex
ATP	Adenosine Triphosphate
CN-Br	Cyanogen-Bromide
CSF	Cerebral Spinal Fluid
CV	Coefficient of Variation
DEAE	Diethylaminoethyl
DHEAS-S	Dehydroepiandrosterone-Sulphate
DLIS	Digoxin-Like Immunoreactive Substances
ECG	Electrocardiogram
g	Gravitational Units
G-6-PD	Glucose-6-Phosphate Dehydrogenase
Hct	Hematocrit
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
IgG	Immunoglobulin-Gamma
Na^+/K^+-ATPase	Sodium Potassium Adenosine Triphosphatase
PBS	Phosphate Buffered Saline
Pi	Inorganic Phosphorus
RBC	Red Blood Cell
RIA	Radioimmunoassay
RPM	Revolutions per Minute
SD	Standard Deviation
UV	Ultraviolet

LIST OF ABBREVIATIONS (cont'd)

$[^{3}H]$	Tritium, Radioactive Isotope of Hydrogen
$[^{125}I]$	Radioactive Isotope of Iodine
$[^{32}P]$	Radioactive Isotope of Phosphorus
$[^{86}Rb]$	Radioactive Isotope of Rubidium
MeV	Millielectron Volts
Ci	Curie
mCi	Millicurie
µCi	Microcurie
MBq	Mega Bequerel
γ	Gamma
Δ	Delta
$>$	Greater than
$<$	Less than
mOs	Milliosmoles
M	Molar
mM	Millimolar
mmol	Millimole
µmol	Micromole
L	Litre
dL	Decilitre
mL	Millilitre
µL	Microlitre
mg	Milligram

LIST OF ABBREVIATIONS (cont'd)

µg	Microgram
ng	Nanogram
pg	Picogram
cm	Centimetre
mm	Millimetre
nm	Nanometre
µm	Micron, Micrometre

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VII. INTRODUCTION

Digoxin is a glycoside used primarily for the treatment of congestive heart failure and arrhythmias. It is the most commonly prescribed cardiac medication in North America. Although used for many years, the risks of toxicity remain exceedingly high (18-30%)[5]. In order to minimize these risks, blood levels of digoxin are frequently measured to ensure they remain within the narrow therapeutic range (0.8-2.0 µg/L) for this drug.

The earliest attempt to measure digoxin in biological fluids was undertaken by Friedman et al[6] with a "Duck Embryo Bioassay". This method used concentrated urine and was unable to detect levels below 250 µg/L. In 1953 Schatzman[7] reported that low concentrations of cardiac glycosides inhibited erythrocyte uptake of radiolabelled potassium. The observation of Love and Burch[8] that erythrocytes could not distinguish potassium from rubidium, was utilized by Lowenstein[9] when he developed an assay that measured glycosides by their inhibition of the uptake of ^{86}Rb by red blood cells. A few years later, Smith et al[10,11] developed a radioimmunoassay to measure digoxin in serum. Since then, numerous immunological procedures[1] which use various digoxin-specific antibodies and radiolabelled tracers have been developed.

The first reports that questioned the specificity of digoxin antisera appeared in 1975, seven years after the introduction of digoxin

radioimmunoassays. Belpaire et al[1] reported false-positive digoxin results with a number of assay procedures that used plasma from patients in renal failure who were not on glycoside therapy. Since this observation, a variety of clinical conditions[1,12,13,14,15,] associated with increased blood levels of digoxin-like immuno-reactive substances have been identified.

In 1982 a newborn with cardiac arrhythmia was transferred to the Intensive Care Nursery at the Vancouver General Hospital. Although there was no record of digoxin therapy, a serum digoxin level was requested in order to rule out toxicity as a cause of the arrhythmia. A serum level of 1.1 µg/L (therapeutic range; 0.8-2.0 µg/L) was found. It was confirmed that the baby had not received digoxin. This false-positive result prompted additional investigations. Serum digoxin levels were measured in all untreated infants[16] in the nursery and the data showed that neonates, not on glycoside therapy, had an endogenous substance(s) in their serum which cross-reacted with antisera to digoxin in seven different RIA methods[17]. This material was termed "Digoxin-Like Immunoreactive Substance" (DLIS)[16]. Further examinations in the perinatal period[17,18,19,20,] demonstrated that this substance peaks 4 to 6 days after birth, then slowly decreases to non-detectable levels by 6 months of age[21]. The peak value of DLIS observed in these neonates showed a direct negative correlation to their gestational age and birth weight[16]. Since this report, DLIS has also been described in association with renal insufficiency[19,22]

hepatic failure[15] and pregnancy[24,25]. The highest levels of DLIS reported to date have been found in sera of premature infants.

A number of studies[2,3,4] have identified a substance which circulates in the plasma of salt-sensitive and volume-expanded animals which may cause hypertension. This material has also been noted, in some cases, to cross-react with specific antisera to digoxin and/or inhibit Na^+/K^+-ATPase activity[25,26,27,28,29]. The cardiac glycosides react at specific tissue receptors and inhibit Na^+/K^+-ATPase[30,31]. If this characteristic is shared by DLIS, it could be the putative "Natriuretic Factor". The existence of tissue receptors able to react with both endogenous and exogenous substances is not unheard of. An analogous situation occurs where brain tissue receptors for the endogenous substance "endorphin", also react with the drug morphine[32].

The aim of my project was to isolate DLIS from cord blood with C_{18} Sep Paks, fractionate it by high performance liquid chromatography, and determine the ability of immunoreactive fractions to inhibit Na^+/K^+-ATPase activity. Various steroids and bile acids found naturally which may be related to DLIS or the natriuretic factor, were examined for HPLC retention times, digoxin immunoreactivity and Na^+/K^+-ATPase inhibitory activity.

VIII. HISTORICAL BACKGROUND

A. Digoxin

A-1 Early Use

The ancient Egyptians frequently used plant products as medicines[30]. "Squill", from the dried bulb of the sea onion, was used by the Romans as a diuretic, heart tonic, and rat poison[30]. In 1250, Foxglove was prescribed as a herbal remedy to treat anything from epilepsy to skin ulcers. William Withering published "An Account of the Foxglove, and Some of its Medical Uses: With Practical Remarks on Dropsy and Other Diseases" in 1785. This plant extract was noted to have power over the motion of the heart[5,30]. The cardiac effects of Strophanthus were described by Sir Thomas Fraser in 1890 while studying arrow poisons in Africa. These inotropic drugs (cardiac glycosides) were obtained from various plants and are named according to their botanical origin; digitalis (Digitalis purpurea), digoxin (Digitalis lanata-Foxglove) and ouabain (Strophanthus gratus).

A-2 Physiological Activity of Digoxin

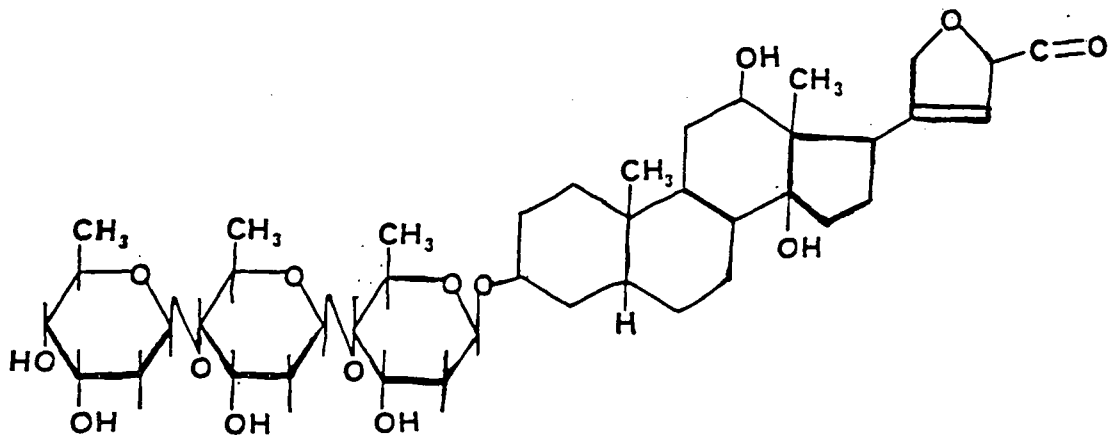
Early in the 20th century, glycosides began to be used specifically in the treatment of "dropsy" (edema) and atrial fibrillation. A diminished cardiac output reduces the renal blood flow and glomerular filtration rate, this causes

retention of salts and fluid. An increase in hydrostatic pressure in the capillaries during heart failure retards the rate of re-absorption of extracellular fluid[30]. When the capillary pressure exceeds the osmotic pressure of plasma proteins, edema results.

Digoxin, a cardiac glycoside has inotropic and chronotropic properties. It inhibits the sarcolemmal transmembrane Na^+/K^+-ATPase pump causing loss of potassium ions and accumulation of intracellular sodium. This stimulates Na^+-Ca^{++} exchange[33]. Troponin, which normally prevents the combination of movable actin filaments with stationary myosin filaments, combines with the calcium ions. This allows the formation of an actin-myosin complex which increases the force and velocity of myocardial systolic contractions[31]. A strong contraction by the heart muscle alters its conduction velocity, and increases the stroke volume and cardiac output. Systolic emptying diminishes the interthoracic blood volume, this reduces the pulmonary arterial and central venous pressures, which decreases the edema.

A-3 Pharmacology of Digoxin

The pharmacological activity of cardiac glycosides resides in their characteristic structure. They are composed of a



D I G O X I N

cyclopentanoperhydrophenanthrene steroid nucleus[5,30,34] with an alpha/beta unsaturated five or six membered lactone ring (aglycone) at position C^{17}. The steroid is coupled to one or more sugar moieties at the C^{3} hydroxyl group. Digitalis glycosides are chemically related to sterols, bile acids, sex and adrenocortical hormones. Metabolism of the drug involves reduction of the lactone ring to its dihydro-form then stepwise removal of sugar molecules to reduce its cardioactivity.

A-4 Complications of Digoxin Therapy

Anorexia, nausea and vomiting are the earliest manifestations of digoxin toxicity. These may be accompanied by gastrointestinal pain, diarrhea, diuresis, neuralgic pain with blurred vision, sinus arrhythmias, ventricular tachycardia, cold sweats, slowing of the heart rate and convulsions. High levels of digoxin affect the electrophysiological properties of the heart[30]. The increased contractile force consumes oxygen and produces a relative hypoxia[35]. A decrease in the intracellular negativity increases the excitability of ventricular Purkinje fibres. These fibres gradually depolarize to cause decreased conductivity, ventricular fibrillation and cardiac arrest. Frequent causes of digoxin toxicity are: hypokalemia, hypomagnesemia, hypercalcemia, hypoxia, and variations in drug bio-availability because of

intestinal absorption and metabolism or renal insufficiency with decreased clearance. Toxicity can be treated with phenytoin and lidocaine to suppress ventricular tachycardia, propranolol to depress excitability or potassium to compete with the glycoside for Na^+/K^+-ATPase binding sites.

An accurate method of monitoring digoxin therapy decreases the possibility of toxicity. Unfortunately, blood levels of the drug do not always correlate well with clinical signs of toxicity[36]. Digoxin takes 6-8 hours to equilibrate in tissue. The best time to assess levels of the drug is immediately prior to the next dose. With the advent of immunologic methods of analysis, specific binding sites with high affinity for radiolabelled glycosides have been identified in tissues other than the heart[37]. This suggests that digoxin may affect many organs in the body.

B. Digoxin-Like Substances

Evidence that an endogenous material could enhance cardiac contractility by its interaction with membrane-bound ouabain-sensitive Na^+/K^+-ATPase was first postulated by Rein[37] in 1942. He suggested that the liver produced a substance which caused a digitalis-like effect on the heart.

B-1 Isolation of DLIS

Gonick *et al*[26] and Gruber *et al*[38] isolated a low

molecular weight extract from rat plasma by Sephadex chromatography that could inhibit Na^+/K^+-ATPase activity, affect sodium transport, and increase vascular tone. Fishman[39] isolated a fraction from bovine hypothalamus which displaced [^{3}H]ouabain and inhibited ^{86}rubidium uptake by human erythrocytes. These observations suggested the existance of an endogenous substance with digoxin-like properties. Several investigators have attempted, but failed, to define the exact nature of this substance[3,40,41].

B-2 Immunoreactivity of DLIS

Gruber et al[3] isolated a methanol-soluble material from dog plasma which was resistant to both acid hydrolysis and the action of proteolytic enzymes. This substance was able to displace [^{3}H]-ouabain from cell membranes and cross-reacted with antibodies specific to digoxin. De Wardener and Clarkson[28] isolated two immunoreactive fractions from the plasma of volume-expanded rats. Their findings, and those of Valdes et al[42] suggested the presence of a heat- resistant biological substance with a molecular weight of less than 1,000 daltons that is covalently bound to a larger (30,000 daltons) heat-sensitive precursor.

Digoxin-like immunoreactive substances (DLIS) are reported to be neutral, water-soluble materials[24,25,28,42] primarily

bound to protein for transport. It has been suggested that an increase in the free-to-bound ratio may have clinical importance[19,42].

B-3 Clinical Significance of DLIS

Serum digoxin is routinely measured by immunoassays. Cross-reactivity has been noted with many of the drug's metabolites as well as some unrelated steroids and bile acids. Soldin and colleagues[5,34] looked at a number of dihydro-derivatives of digoxin and steroid metabolites formed in the intestine which demonstrated immunoreactivity to digoxin antisera. They also showed that injection of epinephrine and hydrocortisone into normal human subjects increased blood levels of DLIS occurred.

Significant concentrations of DLIS have been detected in patients with renal failure[1,22], hypertension[24,25], pregnant women in their third trimester[23], amniotic fluid[42], placental homogenates[41], umbilical cord specimens[43], plasma from neonates[16,18] and normal individuals after salt loading[24,40,44]. DLIS values measured by digoxin RIA doubled in response to a high salt diet[44]. There appears to be a relationship between these clinical states of expanded fluid volume and this raises the possibility that DLIS may have a role in the regulation of

fluid homeostasis. DLIS is increased in patients with essential hypertension and normotensive subjects with a family history of this disorder[5]. Gault et al[40] examined DLIS levels in normal adults (30-60 pg/mL) and hypertensive subjects (20-140 pg/mL). Salt loading doubled these values. Plasma from newborn infants has levels of DLIS that are 50 times higher than those seen in adults[16,17,18,19,20,21].

Valdes and colleagues[18,42] noted increased levels of DLIS during the toxemia of pregnancy. Their study of amniotic fluid, umbilical cord blood, and serum from newborn infants, showed that digoxin-immunoreactive material was produced endogenously by the infant prior to, as well as after birth.

B-4 DLIS in the Neonate

DLIS starts to rise in amniotic fluid after 33 weeks gestation to levels higher than those seen in either the umbilical vein or mother's serum[16]. An assay of samples from the umbilical vein and artery did not reveal significant differences. This indicates a lack of specific DLIS flow between maternal and fetal circulation. Pudek et al[16,17,20] measured DLIS in cord blood and serum from 25 premature infants. They reported levels of DLIS from 0.3 to 4.1 µg/L. Highest levels were seen in the more premature infants. There was a significant negative correlation with

gestational age and birth weight but no relationship to Apgar score, medication, or levels of dehydroepiandrosterone-sulfate (DHEAS-S), an indicator of fetal adrenal-cortical activity[16,17]. Pudek et al[20] also measured the cross-reactivity of DLIS with different digoxin antibodies from seven RIA kits. DLIS levels in neonatal serum were consistent and reached a peak value 4 to 6 days after birth. The cross-reactivity varied between assay systems and with altered assay conditions, to suggest non-specificity in the binding of DLIS to the antibody.

Hyponatremia is frequently observed in early neonatal life[45]. Increased sodium excretion in the premature infant creates a negative sodium balance which correlates positively with levels of DLIS and negatively with gestational age[16,17]. This suggests that perhaps DLIS has a role in the control of sodium and water homeostasis in this population.

C. Hypertension and Natriuresis

C-1 Hypertension

The etiology of "essential hypertension" is unknown. Starling[46] suggested that there may be a genetic defect in the kidney's ability to handle sodium in essential hypertension. Borst et al[46] showed that transplantation of kidneys from hypertensive rat strains (Milan and Okamoto

spontaneous or Dahl salt-sensitive) into normotensive rats leads to hypertension. The reverse was also true[4,24,46]. Curtis et al[47] and Guidi et al[48] noted the development of secondary hypertension after bilateral nephrectomy or renal failure due to nephrosclerosis. De Wardener and MacGregor[4] hypothesized that essential hypertension involves an inherited or acquired renal defect in sodium transport. This defect is aggravated by a high salt diet which results in a rise in extracellular fluid volume. According to their theory, this rise in interstitial fluid volume either stimulates the release of a natriuretic hormone or prevents the secretion of an anti-natriuretic factor from the hypothalamus[49].

C-2 The Natriuretic Factor

For over 100 years, laboratories have searched for an endogenous natriuretic factor. In 1885, Ringer studied the perfusion of a frog heart with his salt solution and demonstrated a hypodynamic condition which could be reversed by an injection of serum[49]. The first experimental evidence for the existence of a natriuretic factor was credited to de Wardener[27,28,29]. In 1961, while working with volume-expanded rats, he demonstrated that an extract from kidney tissue could act as a natriuretic hormone and regulate sodium excretion. Plasma from these rats, or from those on a high salt diet, when injected into a second set of

animals, caused a prolonged rise in sodium excretion.

Many investigators have attempted to purify this "natriuretic hormone" from several sources including serum, urine, and several tissues (kidney, cerebral cortex, hypothalamus)[26,50,51,52]. Whole blood extracts have yielded a substance that, when injected into the jugular vein of rats, induced natriuresis[27,38]. After twenty years of investigation the site of production and nature of this factor are still unknown.

C-3 Physiological Function of the Natriuretic Factor

Gonick et al[50,53] have suggested that similar to ouabain, natriuresis occurs by inhibition of membrane-bound Na^+/K^+-ATPase. This inhibition increases intracellular sodium and depolarizes the cell membrane. In vascular smooth muscle, this rise also increases the level of intracellular calcium[4,54,55,56,57] leading to increased vascular tone and peripheral vascular resistance. This results in vasoconstriction and an elevation in blood pressure [24,37,52,58]. Calcium antagonists (nifedipine or verapamil) can reduce blood pressure by blocking the vasoconstriction of smooth muscle[4,57,58].

Plasma from patients with essential hypertension has been

shown to contain a factor which inhibits Na^+/K^+-ATPase activity. Hypertensive patients also have increased cellular cation concentrations[4,55,57,59,60]. Normal lymphocytes, when incubated in plasma from hypertensive individuals, have increased intracellular sodium and calcium levels relative to control values. Urine from these patients, when purified by Sephadex chromatography[61], could be resolved into four fractions, two of which exhibited natriuretic properties. The larger (10,000 daltons) did not react with antibodies to digoxin and was suggested to be a precursor of the smaller (< 1,000 dalton) immunoreactive fraction.

C-4 Na^+/K^+-ATPase

The kidney has an abundant supply of Na^+/K^+-ATPase (40 million sites per cell) as estimated by [^{3}H]-ouabain binding[62], while the erythrocyte has only 500 sites per cell. This enzyme exists in an asymmetric orientation within the cell membrane. It is 10.5 - 12.5 nm in length and has a 5 nm extension at the cytoplasmic surface of the lipid bilayer for binding ATP. Ouabain interacts with a 1-3 nm receptor at the extracellular matrix. Crystal analysis has provided an insight into the molecular shape and diameter of this protein[62]. Each lipid-embedded alpha/beta subunit of the enzyme binds one ATP molecule. Phosphorylation of the alpha subunit occurs as the enzyme hydrolyses ATP. Inhibition of

Na^+/K^+-ATPase by cardiac glycosides can reduce ATP hydrolysis by 75%. The Na^+/K^+-ATPase pump functions to maintain electrochemical gradients that regulate ion transport as well as the movement of nutrients, metabolites and organic acids. By regulating cytoplasmic sodium, it indirectly controls the Na^+–Ca^{++} co-transport system associated with the actin-myosin cytoskeleton. The sodium to potassium ratio within the cell controls the synthesis and/or degradation of this enzyme[62]. Aldosterone augments sodium transport across the kidney cell membrane by inducing an increase in the pump rate[62]. There is a loss in the number of enzyme sites per cell following adrenalectomy[62].

C-5 Membrane Sensitivity

The composition of membrane lipid plays an important role in the sensitivity of Na^+/K^+-ATPase to glycosides. The proportion of saturated to unsaturated fatty acids influences the acyl chain membrane characteristics for a species and determines its susceptibility to glycosides[63]. Eighty percent of the membrane phospholipid is phosphatidylcholine and phosphatidylethanolamine, the remainder is diphosphatidylglycerol, sphingomyelin, phosphatidylserine and phosphatidylinositol[63]. Differences in the proportion of long chain polyunsaturated docosahexaenoic acid are seen. The more insensitive an animal is to ouabain (rat, mouse), the

higher the level of this fatty acid in the membrane. More sensitive species (human, dog, cat) have lower levels. The integrity of the lipid component is essential for enzyme activity. It is easily inactivated with detergents or organic solvents[33].

C-6 Inhibitors of Na^+/K^+-ATPase

A number of substances are known to inhibit Na^+/K^+-ATPase activity[27,41] some of these are: bradykinin, prostaglandins, vasopressin, oxytocin, parathormone, calcitonin, thyroid hormone, vanadium and catecholamines. Gruber et al[3] purified a substance from the plasma of volume-expanded dogs that was able to inhibit Na^+/K^+-ATPase activity. When injected into rats, the material caused natriuresis.

C-7 Is DLIS the Natriuretic Factor?

A factor seen in hypertension has not as yet been proven to be the putative "Natriuretic Hormone". To fulfill all criteria, it should be able to inhibit Na^+/K^+-ATPase, stimulate G-6-PD (glucose-6-phosphate dehydrogenase) activity, displace [^{3}H]-ouabain from membrane receptors, cause natriuresis when injected into an animal, limit transepithelial sodium transport and cross-react immunologically with antibodies to

cardiac glycosides (digoxin). No natural material has yet been identified that satisfies all of these criteria.

The steroid nature of cardiac glycosides suggests that the endogenous factor "endoxin" may be a natural steroid ligand for the adenosine triphosphatase receptor. Chlormadinone-acetate (7-chloro-6-dehydro-17-alpha-acetoxyprogesterone) a synthetic derivative of 17-alpha hydroxyprogesterone[37], satisfies most of these criteria and provides a potential clue to the chemical nature of the factor. Its endogenous analogue has not been found. Elevated blood pressure induced by deoxycorticosterone-acetate can be reversed with the administration of digoxin antibodies[19,37,64,65]. However, the antibodies have no effect on adult Milan rats that express spontaneous genetic hypertension[64,65].

Efforts to establish the bioactivity of DLIS have been limited by problems with its isolation and purification. Soldin[5] reported that extracts from placenta and brain reacted with antibodies to digoxin, were able to inhibit Na^+/K^+ATPase activity and could cause natriuresis. Many investigators [43,44,66,67] have reported on factors that could mimic the effect of ouabain on net sodium efflux and ^{86}rubidium influx across cell membranes. Diamandis et al[43], examined

digoxin immunoreactive substances from cord and maternal serum. They processed this material by HPLC and identified fractions that could both react with digoxin antibodies and interfere with ^{86}Rb uptake by erythrocytes. There are a number of steroids present in cord blood[43]. Cortisone and progesterone are both immunoreactive with digoxin antisera and able to inhibit Na^+/K^+-ATPase. The presence of an endogenous "Natriuretic Factor" which may in increased quantities inhibit Na^+/K^+-ATPase at glycoside receptor sites could play a major role in the pathogenesis of essential hypertension. No one has yet isolated a substance from animal tissue that fulfills all criteria necessary to be this factor.

IX. MATERIALS AND METHODS

A. Digoxin Radioimmunoassay

In 1969[10] a simple radioimmunoassay for serum digoxin was first introduced for routine therapeutic monitoring of the drug. In commercially available kits, endogenous ligand competes with a fixed amount of ^{125}I-digoxin for a limited number of binding sites on the antibody. With competitive binding assays, it is assumed that the affinity constants for endogenous ligand, exogenous tracer and antisera are equal[68]. The formation of an antigen-antibody complex is dependent on the mass action equation. This assumes a single combining site for the antigen with its antibody. After the antigen-antibody complex has formed, the bound phase is separated from free antigen.

A-1 Manual Method

The Digi-Tab kit (Nuclear Medical Laboratories, Irving, TX, 75061) was used in all experiments. This commercial kit uses rabbit-derived antibodies directed against the steroid portion of digoxin. It combines the rationale of a competitive protein-binding assay with the specificity of immunologic reactions and sensitivity of radioisotope methodology.

The specimen (0.05 mL) is added to 0.40 mL ^{125}I-tracer (digoxin-^{125}I in 0.05 M propionic acid-NaOH buffer pH 8.0,

specific kit concentration 0.11 µCi/mL, 0.05 µCi/test). The contents of the tubes are mixed. Subsequently, digoxin antiserum (0.40 mL) is added and the tubes incubated for 30 minutes at room temperature. A charcoal tablet (135 mg) is then added to each tube. After dispersion of the tablet, the contents of the tubes are mixed for 5 seconds. The charcoal is then pelletted at room temperature by centrifugation (2,000 X g) for 20 minutes. The supernatant is discarded, the tubes are drained and the radioactivity present in the charcoal adsorbant determined using a Iso-Data 20/20 gamma counter, (Iso-Data Inc., Rolling Meadows, IL, 60008). Five digoxin serum standards (0 µg/L, 0.5 µg/1, 1.5 µg/L, 3.0 µg/L, 5.0 µg/L) are run in duplicate with each assay. A standard curve is constructed by plotting digoxin concentration against percent free antigen adsorbed by the charcoal in each tube. Percent free antigen is determined by dividing the radioactivity in each tube by the total radioactivity originally added. Previous studies in this laboratory have indicated that antisera lot number DB-157[17] was the most cross-reactive with DLIS of seven different antisera tested. Therefore DB-157 was selected for use throughout all of these studies.

A-2 Automation of the Manual Digoxin Method

A Tecan Liquid Handling sampler 500 System (Terochem

Laboratories Ltd., P.O. Box 8188, Station "F", Edmonton, Alberta, T4H 4P1) was used to automated serum digoxin analyses. The five standard digoxin solutions, tracer (^{125}I-digoxin), antibody, controls and unknown samples are appropriately loaded into the primary sampling racks. Duplicate assay tubes for total count, non-specific binding, standards, controls and samples are loaded into the secondary rack. The assay method is programmed into the instrument so that appropriate volumes from each sample and reagent are dispensed into the correct tube. Test tubes are transferred to a separate rack after mixing. The method is then continued as outlined for the Manual Assay.

A-3 Factors Influencing the Digoxin Assay

Assays for serum digoxin were developed to give a low degree of cross-reactivity with substances which have a similar ligand[69]. Previous studies in this laboratory[17,20] have indicated that DLIS cross-reactivity in the routine RIA method for digoxin is very much influenced by assay conditions. DLIS cross-reactivity can be reduced by increasing the temperature and time of incubation to 18 hours, or increased by doubling the antiserum concentration and incubating for 15 minutes at room temperature. An inadequate amount of protein in the digoxin assay mixture is also known to increase DLIS values; therefore, all DLIS fractions were

routinely reconstituted in DLIS-free serum. DLIS-free sera were prepared by passing pooled serum through a C_{18} Sep-Pak and subsequently treating the eluate with activated charcoal (5 mg/mL) for 30 minutes at room temperature. This treatment eliminates all detectable DLIS from the serum.

It has previously been suggested that DLIS may be steroidal in nature[5,34,70]. The cross-reactivity of a number of common steroids and bile acids to antisera DB-157 in the Digi-tab RIA was therefore examined. Compounds were dissolved in 100% ethanol [10 mM], then diluted in DLIS-free serum to final concentrations of 0.1 mM for steroids and 2.0 mM for bile acids. Digoxin-like immunoreactivity of eight steroids and nine bile acids using the RIA method as outlined in A-1 are listed in Tables I and II of the Results section. The routine digoxin assay method was unaffected by alcohol concentrations up to 50%.

B. Source and Isolation of DLIS

In 1983 Pudek et al[16] reported levels of serum digoxin in neonates not on digoxin therapy. It was suggested from these results, and those of others[18,43], that there was an endogenous digoxin-like substance(s) in the circulation of newborns which cross-reacted with digoxin-specific antibodies. Stored sera from 25 premature and full-term infants which showed DLIS values that

ranged from 0.3 to 4.1 µg/L were separated into four pools by concentration of DLIS: less than 1 µg/L, 1-2 µg/L, 2-3 µg/L, and greater than 4 µg/L. These pools were subsequently used in preliminary extraction studies designed to determine optimal conditions for recovery of DLIS. Mixed cord blood (500 mL) was also collected at the time of delivery in the low-risk delivery unit at Grace Hospital (Vancouver, British Columbia). The samples were collected over a two month period and stored at -20°C until used.

B-1 Solvent Extraction of DLIS

Dahl and colleagues[2,37] suggested that the most likely candidate for a physiologically active endogenous digitalis-like substance would be a progesterone-like steroid. It was felt that substances which demonstrated immunoreactivity with specific antibodies to digoxin might be similar in structure to this steroid; therefore, methods used to isolate steroids were examined. A series of studies were undertaken to determine the effect of different solvents on the recovery of DLIS and digoxin from serum.

Patient serum samples (1 mL) that contained digoxin, DLIS, or normal serum spiked with known concentrations of digoxin were treated with each of the following solvents (2.5 mL): absolute

ethanol, chloroform, methylene dichloride[12], and absolute methanol. After mixing, the tubes were incubated at room temperature for 1 hour then centrifuged for 15 minutes at 6,000 X g. The supernatants were removed and evaporated to dryness under nitrogen in a 56°C water bath. Extracts were reconstituted in 1 mL phosphate-buffered saline (PBS, pH 7.4) and shaken at room temperature for 18 hours. Digoxin equivalents were then determined by diluting each extract with an equal volume of DLIS-free serum and measuring the recovery of material by RIA as outlined previously (see Section A-1).

B-2 Immuno-Extraction of DLIS

Substances that cross-react with specific antibodies to digoxin are present in serum from neonates and cord blood[13,16,18,43]. This property could conceivably be utilized to isolate DLIS from serum, by use of an immunoadsorbant column which would specifically bind the material.

The isolation and concentration of rabbit immunoglobulin from digoxin antisera DB-157 was carried out by standard salting-out procedures[71]. Ammonium sulfate was added to antisera (10 mL) to a final concentration of 29%. The solution was mixed, incubated for 30 minutes at 60°C and

cooled. The precipitate was recovered by filtration (Whatman No. 1 filter paper), washed with 31% ammonium sulfate and the filtrate recovered. Ammonium sulphate is then added to a 48% concentration, the filtrate mixed for 1 hour at room temperature, and the supernatant recovered by centrifugation. Ammonium sulfate was added to saturation and the solution left at $4^{o}C$ overnight. The precipitate was recovered, dissolved in 0.85% saline to the original volume of antisera used, and dialyzed (standard cellulose dialysis tubing) against 2000 mL saline for 24 hours at $4^{o}C$.

Isolated immunoglobulin was subsequently purified by DEAE-52 anion exchange chromatography (Diethylaminoethyl cellulose, Whatman, Terochem Laboratories, Edmonton, Alberta, T6H 9Z9). The immunoglobulins were eluted with a linear phosphate gradient (0.02 M - 0.1 M KH_2PO_4, pH 7.4). The eluate was monitored at 280 nM with a Pye Unicam SP8-400 UV/VIS Spectrophotometer (Phillips, Cambridge, England, CB1-2PX) and fractions (3.0 mL/min) collected with a Fractomette Alpha 200 (Buchler Instruments, Inc., Fort Lee, NJ, 07024). Fractions were appropriately pooled and concentrated using Sartorius collodian bags (BDH Chemicals, Vancouver, BC, V5T 1E8). The protein concentration was determined[72] and immuno-reactivity monitored by Ouchterlony double immunodiffusion (Titan IV - 9009, Helena Laboratories, Beaumont, TX, 77704-0752) against anti-rabbit immunoglobulin produced in

swine (Cedarlane Laboratories Ltd., London, Ontario, N6C 4R3). Immunoglobulin from normal rabbit serum was isolated and purified simultaneously by the same procedure to monitor recovery and to assess non-specific binding of digoxin.

After isolation, the purified soluble antibody (1 mL) was bound to cyanogen bromide activated Sepharose-4B (1 gm)[73,74], (Pharmacia Biotechnology International, Uppsala, Sweden, S-751-82) with coupling buffer (0.1 M $NaHCO_3$ - 0.5 M NaCl pH 7.3). The sample was continuously rotated overnight at 4°C. The resin was recovered, activated in ethanolamine (1 M, pH 8), and then washed 6 times with an acid-base cycle of buffers (0.1 M sodium acetate pH 4 followed by 0.1 M sodium borate pH 8).

The activity of this immunoadsorbent was determined by reacting 1.0 mL ^{125}I-digoxin tracer (specific concentration 0.11 µCi/mL) from the NML Digi-tab kit, with 0.20 mL Cn-Br-anti-digoxin Sepharose (200 mg/mL). In order to monitor non-specific binding of digoxin, duplicate experiments with CN-Br Sepharose-4B-bound normal rabbit IgG were run simultaneously. After 24 hours incubation at 25°C, the resin was recovered by centrifugation, washed once with 1 mL saline, and the amount of radiolabelled digoxin adsorbed to the resin determined on a gamma counter.

Cord serum (1mL) that contained 2.6 µg/L DLIS was added to

CN-Br Sepharose-4B immunoadsorbent (0.2 mL) and incubated at 25°C for 24 hours. The serum was then separated from the resin by centrifugation and the DLIS content which remained in the supernatant determined by RIA. The binding of DLIS to the resin was calculated.

B-3 Sep-Pak Extraction of DLIS

Sep-Pak cartridges are used for the isolation of a narrow range of compounds based on their polarity. C_{18} cartridges are washed with a polar solvent such as water to eliminate high polarity compounds first. Compounds of lower polarity are then specifically eluted with non-polar solvents such as methanol.

Sep-Pak C_{18} cartridges were activated prior to use by washing with absolute methanol (2 mL) and distilled water (5 mL). Serum (5 mL) was loaded onto the column which was subsequently washed twice with distilled water (2 mL). DLIS was then eluted with methanol (2 mL). The methanol eluate was either concentrated or taken to dryness at 60°C. DLIS recovery was monitored throughout.

Valdes et al[19,23,42] state that 90% of DLIS in serum is tightly bound to protein. This fraction could be released by heating serum, and the amount of measurable DLIS

enhanced[75]. Cord blood serum was boiled for 5 minutes and the coagulated protein removed by centrifugation. DLIS levels in the supernatant were measured and compared to the levels found prior to heating.

Throughout the literature, a number of different buffers have been mentioned for use in functional assays. In order to determine which would be the most appropriate for use throughout our experiments, the following study was carried out. Sep-Paks were used to extract 6.0 mL of cord blood which contained 5.52 µg/L DLIS. DLIS was eluted with methanol (2.5 mL) and divided into five equal (0.5 mL) aliquots. These were evaporated under nitrogen at 56°C. Each extract was then reconstituted in a different buffer: 1.0 mL 50 mM Tris-HCl (pH 7.4) containing 5% human serum albumin (HSA), 0.5 mL Tris-HCl with 0.5 mL DLIS-free serum, 1.0 mL 0.05 M phosphate ($NaHPO_4$)-buffered saline pH 7.4 - PBS with 5% HSA, 0.5 mL PBS with 0.5 mL DLIS-free serum, and 1.0 mL DLIS-free serum. Samples were shaken overnight at room temperature. The recovery of DLIS was monitored for each buffer (see Results, Section B).

For the isolation of DLIS from cord blood (500 mL), 5 x 5 mL aliquots of serum were extracted with one Sep-Pak. All methanol extracts were pooled (200 mL) and concentrated to a 5 mL volume (100 fold) under vacuum at 60°C. Particulate

matter was removed by Millipore filtration (0.22 μm, Water's Scientific, Mississauga, Ontario, L4V 1M5), prior to HPLC injection. An aliquot (0.05 mL) of this concentrate was dried, reconstituted in 1.0 mL 50 mM Tris-HCl (pH 7.4) and diluted 1:1 with DLIS-free serum for analysis of DLIS recovery.

C. High Performance Liquid Chromatography

In high pressure liquid partition chromatography, separation is based on molecular polarity. The resolution of the column is dependent on the interaction between a solute and the stationary phase, the most common of which is silica gel having polar hydroxyl groups on its surface. In reverse phase chromatography, these surface groups have been masked or bonded with non polar compounds; octadecylsilane (ODS-C_{18}) imparts this characteristic to the stationary phase which then preferentially attracts non-polar components from the mobile phase.

Initially, liquid chromatography was used to purify DLIS[26], but procedures have evolved with the invention of advanced technologies. HPLC has been used for a number of years in the isolation of steroids and bile acids. The separation of digoxin from its metabolites[76] by this procedure suggested it could be of value in the characterization of DLIS[43,77].

A Varian 5000 HPLC (Varian Instrument Corp., Walnut Creek, CA,

94958) with a dual reservoir single pump delivery system, U.V. visible detector (210 nm) and Varian 9176 dual pen recorder was used throughout for all HPLC fractionations. A gradient-elution system was established to optimize the separation of digoxin and its metabolites. Subsequently, this system was used to fractionate steroids and bile acids known to cross-react with digoxin-specific antibodies. Hydrocortisone, cortisone, progesterone, dehydroepiandrosterone-sulfate, Δ^4-androstenedione, deoxycorticosterone, 17-alpha hydroxyprogesterone, 21-deoxycortisone, taurocholic, glycocholic, glycodeoxycholic, taurochenodeoxycolic, taurodeoxycholic, glycochenodeoxycholic, deoxycholic, chenodeoxycholic, dehydrocholic and cholic acids were dissolved in absolute methanol to a final concentration of 0.1 mM. Aliquots (0.5 mL) of these standards were injected onto the HPLC column and retention times recorded (see Results).

C-1 HPLC Isolation of DLIS

DLIS Sep-Pak concentrate (1.0 mL) was fractionated into 50 aliquots with a Magnum-9 fully capped ODS-3 (25 cm x 9 mm, 10 µm particle) C_{18} - reverse phase preparatory column (Whatman, Terochem Laboratories, Edmonton, Alberta, T6H 4P1). Compounds were eluted with a deionized water-acetonitrile (10-100%) linear gradient, which flowed at a rate of 6.2 mL/minute. Fractions were collected with a Foxy 8025 fraction collector (Isco, Lincoln, Nebraska, 68505). Internal

standardization of each run was achieved by tagging DLIS extracts with the following radiolabelled steroids: ^{3}H-DHEAS (specific activity - 10.5 Ci/mmol, concentration - 168 mCi/mL), ^{3}H digoxin (specific activity 15.5 Ci/mmol, concentration - 248 mCi/mL) and ^{3}H progesterone (specific activity - 45.6 Ci/mmol, concentration - 730 mCi/mL). Retention times and recoveries of the internal standards were monitored by counting the radioactive counts in 0.5 mL aliquots of each fraction. Between injections, the HPLC system was washed twice by injecting methanol and eluting with the gradient. This procedure ensured that there was no carry-over of DLIS between runs. The individual fractions from each DLIS injection were pooled and an aliquot (0.5 mL) removed for DLIS determination. The remainder of the pool was divided into two equal aliquots. The extracts were then dried under vacuum at 60^{o}C with a Haake-Buchler Vortex evaporator (American Hospital Supply, Richmond, BC, V6X 1X5). Each fraction was reconstituted by shaking overnight in 1.0 mL of buffer for determination of Na^{+}/K^{+}-ATPase inhibitory properties in the assay systems as outlined below.

D. Sodium/Potassium-Adenosine Triphosphatase Inhibition Assays

Membrane-bound Na^{+}/K^{+}-ATPase regulates ion transport across cell membranes. Cardiac glycosides are thought to inhibit this enzyme[31,33]. To be of physiological significance, DLIS

should also inhibit Na^+/K^+-ATPase activity. In order to monitor the Na^+/K^+-ATPase inhibitory properties of the isolated DLIS fractions, two assay systems were developed. The first monitors the transport of K^+ ions by intact cells. The second measures the ability of a semi-purified Na^+/K^+-ATPase enzyme preparation to hydrolyze ATP.

D-1 Rubidium Uptake by Erythrocytes

Erythrocytes are a readily available source of cellular Na^+/K^+-ATPase. The method of Lowenstein and Corrill[9,12] has been adopted, with some modifications, for the measurement of Na^+/K^+-ATPase activity. Through the action of the Na^+/K^+-ATPase pump, erythrocytes incorporate radiolabelled rubidium (^{86}Rb) in direct competition with potassium. Red blood cells are incubated in a buffer which contains a fixed concentration of $^{86}Rb^+$, cells are recovered by centrifugation and the incorporation of the isotope is measured. Inhibition of Na^+/K^+-ATPase activity would decrease the uptake of $^{86}Rb^+$.

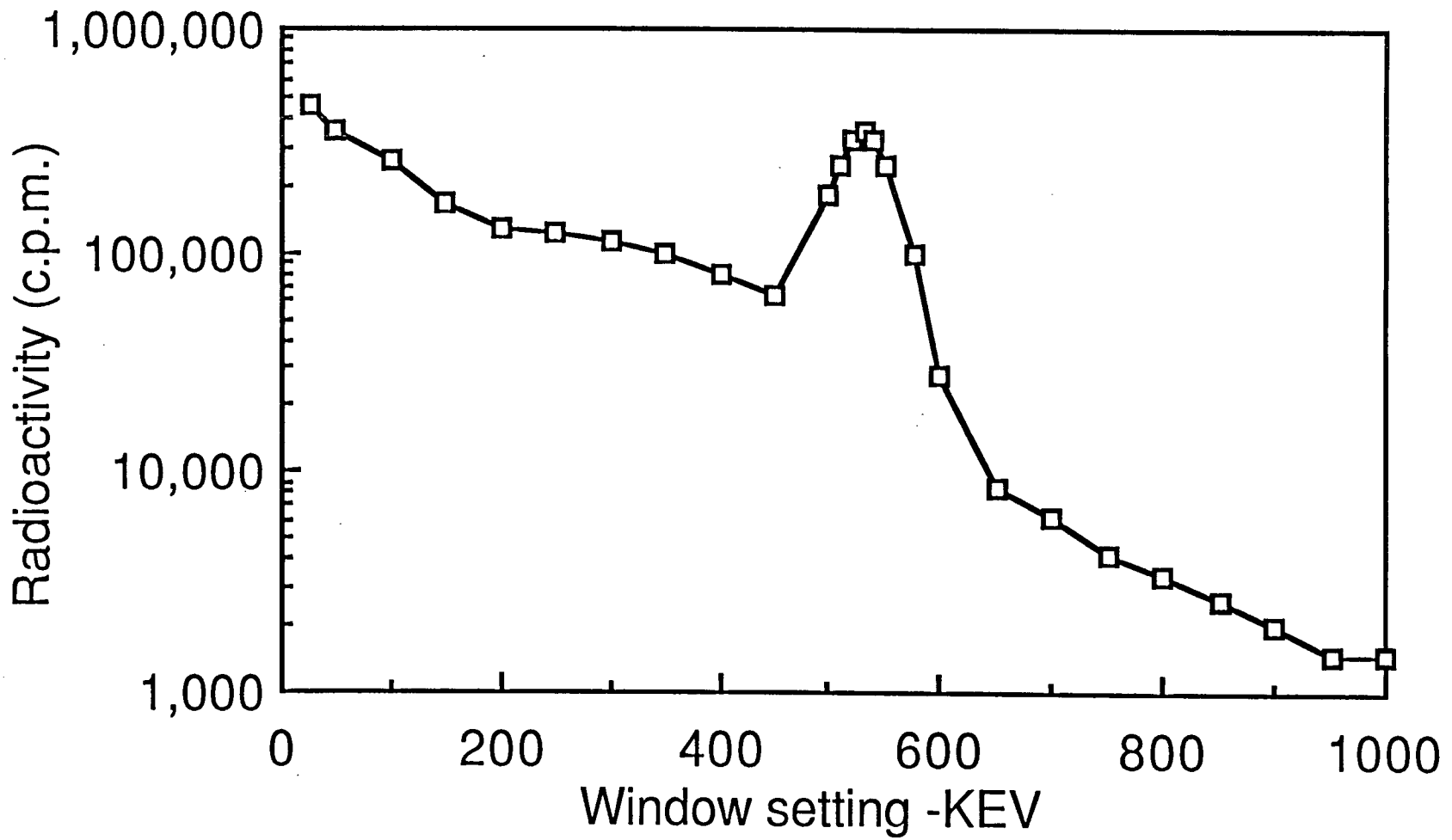
Freshly drawn group O - D positive erythrocytes were separated from CPD-plasma by centrifugation at 2,000 x g. The red cells were washed three times with normal saline and subsequently resuspended in 50 mM Tris-HCl buffer (pH 7.4) which contained 1 mM Na_2EDTA, 5.2 mM $MgCl_2.6\ H_2O$ and 85 mg/dL glucose to

a final hematocrit of 45%. An aliquot of this erythrocyte suspension (0.60 mL) was added to 0.40 mL of sample. After 1 hour pre-incubation at 37°C, 1.0 mL radiolabelled ^{86}Rb was added (specific activity - 2.06 mCi/mg, concentration - 3 µCi/mL, Amersham Corporation, Arlington Heights, IL, 60005). The contents of the tubes were mixed and shaken for 3 hours at 37°C in a water bath (Haake-SWB 20, Allied Fisher Scientific, Vancouver, BC, V5Y 1E9). At the conclusion of the incubation, the cells were recovered by centrifugation (1800 x g, 10 minutes) at 4°C. They were subsequently washed three times with ice-cold saline and the radioactivity in the cell pellet determined with a gamma counter (LKB-1272 CliniGamma, Allied Fisher Scientific) on an expanded scale. The rubidium isotope has a half-life of 18.7 days and decays by both Beta (91.2%) and Gamma (8.8%) radiation. It is easier to quantify the energy released from an intact red cell, so the gamma radiation spectrum was utilized (Figure 1). Optimal instrument settings were determined with a rubidium standard by plotting nuclear transformations (1.077 meV) on a double scale with window settings between zero and 1000 KeV.

The effect of various concentrations of digoxin and ouabain on rubidium uptake by erythrocytes was determined. Stock concentrations (10 mM) of glycoside dissolved in 100% ethanol

FIGURE I

Energy profile of gamma radiation released by ^{86}Rubidium.


1,000,000
100,000
10,000
1,000
Radioactivity (c.p.m.)
0
200
400
600
800
1000
Window setting -KEV

were diluted with Tris buffer to final concentrations of 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200 and 250 µg/L. The amount of ^{86}Rb taken up by the red cells was expressed as a percent of the total radioactivity added to each tube. The activity in a "Reagent Blank" which contained buffer, erythrocytes and isotope was taken as maximum ^{86}Rb-uptake.

DLIS isolated from samples of neonatal serum by ethanol precipitation showed variable amounts of red cell hemolysis when tested in this assay. In order to investigate the cause of this hemolysis, a series of studies were conducted to determine if there was any change in the assay conditions throughout the incubation period. The conditions were as previously described. Normal serum (DLIS-free) was treated with 70% ethanol. The ethanolic extract was dried under nitrogen at 56°C and reconstituted in an equal volume of 50 mM Tris buffer (pH 7.4). Fifteen identical samples were carried through the assay procedure. After 10, 30, 60, 90 and 120 minutes of incubation at 37°C, three tubes were removed from the water bath and used to determine pH, pCO_2, pO_2, $\%O_2$ saturation, glucose content and osmolarity. The variability with time in these assay parameters was compared (see Table III Results Section). Although any alterations in assay conditions were not appreciable enough to explain the hemolysis, it could be eliminated if the samples were

pre-filtered with Amicon-100 Centriflo-Membrane cones (Amicon Corp., Lexington, MA, 02173). This was an unsuitable alternative to the procedure because of severe loss of serum volume.

D-1a The Effect of Potassium on ^{86}Rb Uptake

Most systems for that measure Na^+/K^+-ATPase activity utilize a 50 mM Tris buffer (pH 7.4) which also contains 100 mM NaCl and 20 mM KCl. Although potassium is required for optimal enzyme activity, it can also compete directly with rubidium in this assay system. Such a competition will directly affect the assay results. Therefore a study was conducted to examine the effect of various concentrations of potassium (0.1, 0.5, 1.0, 2.0, 5.0, 10 and 20 mM) on the activity of erythrocyte Na^+/K^+-ATPase. The conditions of the assay were as outlined previously with the exception that the potassium content of the assay mixture was varied accordingly.

D-1b The Effect of Steroids and Bile Acids on ^{86}Rb Uptake

A number of steroids and bile acids which cross-react with digoxin antisera DB-157 have been identified. A study was therefore undertaken to examine the effects of these steroids and bile acids on erythrocyte

Na^+/K^+-ATPase activity. Stock concentrations (10 mM) of the following steroids and bile acids - Δ^4-androstenedione, deoxycorticosterone, 21-deoxycortisone, hydrocortisone, progesterone, 17-alpha hydroxyprogesterone, cortisone, glycochenodeoxycholic, taurochenodeoxycholic, taurocholic, glycocholic, glycodeoxycholic, taurodeoxycholic, deoxycholic, chenodeoxycholic and dehydrocholic acids - were prepared in absolute methanol. These stock standards were then diluted with 50 mM Tris buffer (pH 7.4) to final concentrations of 0.05, 0.10 and 0.2 mM steroid and 0.1, 0.5, 1.0, and 2.0 mM bile acid prior to use in the assay.

All bile acids examined caused severe hemolysis at these concentrations. To determine if this hemolysis was a function of concentration or incubation time, further studies with glycochenodeoxycholic acid (the bile acid most immunoreactive to digoxin antisera) were carried out. Twenty-eight assay tubes were set up; four sets of seven contained identical final concentrations of bile acid (0.1, 0.5, 1.0, and 2.0 mM). These tubes were carried through the ^{86}Rb uptake assay and every thirty minutes one tube from each concentration set was examined. At the completion of the assay, it was apparent that hemolysis was more evident the higher

the concentration of glycochenodeoxycholic acid and occurred within the first 30 minutes. In order to determine if there was a relationship between the ability of glycochenodeoxycholic acid to inhibit Na^+/K^+-ATPase activity and cause hemolysis, a series of assays were conducted in which lower concentrations of bile acid were used (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM). Hemolysis was determined by measuring the optical density at 415 nm of the supernatant recovered from each tube after a 3 hour incubation at 37°C. Total hemolysis was determined by lysing the red cell suspension (0.6 mL) with 1.4 mL of distilled water. Inhibition of ^{86}Rb uptake was compared to the percent hemolysis in each tube.

Steroids and bile acids often require the presence of alcohol for complete dissolution. A study was conducted to examine the effect of ethanol in the buffer, on the ^{86}Rb uptake assay. The conditions of the assay were as outlined previously with the exception that ethanol content of the assay mixture (0.5, 1, 2, 3, 4 and 5%) was varied accordingly.

D-1c <u>The effect of DLIS on ^{86}Rb Uptake</u>

DLIS extracts from cord blood were fractionated by HPLC

as outlined earlier. The fractions were evaporated and reconstituted in 50 mM Tris buffer (pH 7.4) containing 85 mg/dL glucose. The ability of these reconstituted fractions to inhibit erythrocyte Na^+/K^+-ATPase activity was examined. Cord blood is known to contain a number of steroids. Earlier studies indicated alcohol was necessary to ensure adequate solubilization of DLIS extracts; however, because it causes hemolysis of red cells in the ^{86}Rb uptake assay it was not incorporated into the buffer.

D-2 Canine Kidney Na^+/K^+-ATPase Assay

There are two types of adenosine triphosphatase activity within cell membranes. Both types require magnesium (Mg^{++}) to hydrolyze high energy phosphate groups from adenosine triphosphate (ATP). One enzyme requires sodium and potassium for its stimulation, utilizes energy from ATP for cation transport, and is inhibited by cardiac glycosides such as ouabain or digoxin. The other enzyme, Mg^{++}-ATPase, does not require the presence of sodium and potassium and is ouabain insensitive. The role of this latter enzyme is not known. Assay systems that measure Na^+/K^+-ATPase activity[33,44,50,66,77,78] typically monitor the rate of ATP hydrolysis in the presence or absence of ouabain. One

such assay was established for use in the present studies.

The assay mixture consisted of 0.05 mL canine kidney Na^+/K^+-ATPase dissolved in 20 mM Tris-HCl, pH 7.4 (0.6 mg/mL, 1.35 units/mg, 0.8 units/mL, Sigma Chemical Company, St. Louis, MO, 63178 added to 1.0 mL of a Tris buffer (50 mM Tris, 100 mM NaCl, 20 mM KCl, 5.2 mM, $MgCl_2.6H_2O$, 1 mM Na_2EGTA, pH 7.4). DLIS extracts and standards were routinely pre-incubated for 1 hour at 37°C prior to initiation of the reaction by adding 0.05 mL 40 mM ATP containing adenosine 5' [gamma ^{32}P] triphosphate (specific activity - 1.25 µCi/mmol, concentration - 0.02 µCi/test, Amersham Corporation, Arlington Heights, IL, 60005) at 20 second intervals to each tube. Exactly 5, 10, and 15 minutes after the addition of ATP, the reaction is stopped by withdrawing 0.2 mL from each tube and adding it to 0.2 mL ice-cold 4N H_2SO_4. Subsequently, 0.2 mL of 5% ammonium molybdate is added and the contents of each tube brought up to 1.0 mL with the addition of 0.4 mL distilled water. The reaction mixture (1.0 mL) is then extracted with 2.0 mL of a benzene-isobutanol (1:1) solution, the phases separated by centrifugation (1800 x g at 4°C), and the organic phase recovered. A portion of the organic phase (1.0 mL) is added to 10 mL distilled water and the radioactivity determined by Cerenkov counting in a liquid scintillation counter (LS 9000

Beckman Instruments Inc., Palo Alto, CA, 94304). The percent ATP hydrolyzed in one hour is then calculated and enzyme activity expressed as μmoles of inorganic phosphate released per hour per mg protein. The assay control tube is taken as 100% Na^+/K^+-ATPase activity. The background tube contains all components of the assay mixture except the enzyme. This tube is used to monitor non-specific hydrolysis of ATP. Sodium-potassium-independent activity is calculated from the hydrolysis of adenosine [γ^{32}P] triphosphate in the absence of sodium and potassium.

D-2a The Effect of Glycosides on Canine-Kidney-ATPase Activity

Once the conditions of this assay had been optimized, a study was conducted to examine the inhibitory effects of ouabain and digoxin in this system. Stock standards of glycosides were prepared in absolute methanol and diluted with 50 mM Tris buffer (pH 7.4) to final concentrations of 0.01, 0.1, 1.0, 10 and 100 mmol/L. The assay was carried out as previously outlined.

D2-b The Effect of Steroids on Canine-Kidney-ATPase Activity

A study was undertaken to examine the effects of those steroids which cross-react with digoxin-specific antisera on canine-kidney-Na^+/K^+-ATPase activity. Progesterone, Δ^4-androstenedione, deoxycorticosterone,

21-deoxycortisone, cortisone, hydrocortisone and 17-alpha -hydroxyprogesterone were dissolved (10 mM) in absolute methanol and then diluted in 50 mM Tris buffer (pH 7.4) to final concentrations of 0.05, 0.15, and 0.2 mmol/L.

D-2c The Effect of DLIS on Canine-Kidney-ATPase Activity

DLIS extracts from cord blood were fractionated by HPLC as outlined earlier. The fractions were reconstituted in 50 mM Tris buffer (pH 7.4) and shaken overnight. Subsequently the effect of these fractions on both the ^{86}Rb uptake and canine-kidney Na^+/K^+-ATPase assay systems was examined. The results from these studies were compared to the content of DLIS in each fraction as measured by RIA with DB-157 antisera.

Previous studies with this assay system indicated that alcohol at 1% concentration had no effect on enzyme activity. Complete solubilization of DLIS from HPLC fractions is ensured if alcohol is included in the reconstitution buffer. This modification is therefore used in the preparation of HPLC extracts for measurement of their enzyme activity by the canine-kidney Na^+/K^+-ATPase assay.

D-3 Erythrocyte Membrane Na^+/K^+-ATPase Assay

These studies indicated that some HPLC cord blood fractions inhibited the canine kidney assay system, yet had no effect on the human erythrocyte ^{86}Rb uptake assay. The effects of cardiac glycosides on Na^+/K^+-ATPase activity are known to vary with enzyme source[63]. It was therefore decided to assess the inhibitory activity of these HPLC fractions with a human erythrocyte membrane preparation as the source of Na^+/K^+-ATPase. It was felt that this preparation may be more sensitive to the inhibitory effects of these fractions than was the intact cell.

D-3a Preparation of Erythrocyte Membranes

Red cell membranes were prepared according to the method of Godin et al[79]. One unit (250 mL) of whole blood was diluted with three volumes of ice cold saline and centrifuged at 1200 x g for 5 minutes (4^oC). The plasma and buffy coat were removed. The red cells were recovered, and washed for 10 minutes each, with decreasing concentrations of ice-cold sodium chloride: 0.08 M, 0.06 M, 0.04 M, 0.02 M, and 0.009 M, at pH 7.4. After each wash, cells were recovered by centrifugation at 12,000 x g for 10 minutes at 4^oC. During the last wash, cells were maintained at pH 7.4. By this method red cells were gently hemolyzed and the isolated

membranes resuspended in 10 mM Tris buffer (pH 7.4). They were then quick-frozen in a dry ice-acetone bath and stored at -80°C until utilized.

D-3b Na^+/K^+-ATPase Assay

A modification of the canine-kidney-ATPase assay was employed in the assessment of erythrocyte membrane enzyme activity. The assay conditions were optimized for measurement of Na^+/K^+-ATPase activity. Erythrocyte membranes (1.0 mL), having a protein concentration of 2-4 mg per mL[72], were added at 20 second intervals to 1.85 mL of an assay mixture which consisted of; 89 mM Tris (pH 7.4), 4.9 mM $MgCl_2.6H_2O$, 0.16 mM Na_2EGTA, 0.13 M NaCl, and 0.03 M KCl. Test tubes were shaken at 37°C in a water bath. Exactly 15 minutes after the addition of enzyme, 0.15 mL of a 40 mM ATP solution containing adenosine-5 [γ^{32}-P] triphosphate (specific activity 1.25 µCi/mmol) was added to each tube. Incubation was continued for 15, 30, 60 and 90 minute intervals at which time 0.2 mL of the assay mixture was withdrawn and added to 0.2 mL ice cold 4N H_2SO_4. The remainder of the method paralleled that for the canine-kidney-ATPase assay (see methods D-2). The radioactivity in the organic phase represents the amount of inorganic [^{32}P]

phosphate released by Na^+/K^+-ATPase. It is measured by Cerenkov counting in a liquid scintillation counter and expressed as the percent ATP hydrolyzed (μmoles) in 1 hour by 1 mg of membrane protein.

The lipid content of human red cells is different from that of canine kidney cells[63]. This may account for the modifications necessary to achieve maximum activity of the erythrocyte membrane enzyme. A number of studies examined variables in the assay conditions outlined previously. The activity of Na^+/K^+-ATPase as a function of protein concentration was determined by varying the volume of erythrocyte membrane added to the assay mixture (0.2, 0.5, 1.0 and 1.5 mL). The effect of various preliminary incubation times at 37°C (5, 15, 30 and 60 minutes) were examined because of the considerable loss in membrane-ATPase activity seen if a pre-incubation time of 60 minutes as in the canine-kidney assay was used. The linearity of erythrocyte membrane Na^+/K^+-ATPase hydrolysis of ATP was measured by the release of inorganic phosphate after 5, 15, 30, 60 and 90 minutes. In order to optimize the Na^+/K^+-ATPase assay characteristics, 1.0 mL of red cell membranes (2-4 mg/mL protein) were pre-incubated for 15 minutes prior to the addition of ATP, and the

amount of inorganic phosphate released after 90 minutes measured.

D-3c The Effect of Glycosides on Erythrocyte Membrane-ATPase Activity

Digoxin and ouabain were dissolved in absolute methanol (10 mM) then diluted in the assay mixture outlined previously (D-3b) to final concentrations of 10^{-11}M to 10^{-4}M. The effect of these concentrations of cardiac glycosides on the erythrocyte membrane assay was determined.

D-3d The Effect of Steroids on Erythrocyte Membrane-ATPase Activity

Progesterone and Δ^4androstenedione are two steroids which cross-react with digoxin-specific antisera, and inhibit Na^+/K^+-ATPase as measured by both the ^{86}Rb uptake and canine-kidney-ATPase assay systems. These compounds, as well as DHEAS and glycochenodeoxycholic acid, were dissolved in absolute methanol (10 mM). They were then diluted in the Tris assay mixture (D-3b) to final concentrations of 0.05, 0.10, 0.15 and 0.20 mM to determine their effect on Na^+/K^+-ATPase activity as measured by the erythrocyte membrane-ATPase assay.

D-3e The Effect of DLIS on Erythrocyte Membrane-ATPase Activity

DLIS extracts from cord blood were fractionated by HPLC as outlined earlier. The fractions were reconstituted in 89 mM Tris reaction mixture (pH 7.4, D-3b) which contained 1% ethanol for complete solubilization of the extract, and shaken overnight. The ability of these fractions to inhibit the canine-kidney and erythrocyte membrane Na^+/K^+-ATPase assay systems were compared with regard to their estimated DLIS content.

X. RESULTS OF LABORATORY INVESTIGATION

A. Digoxin Radioimmunoassay

The radioimmunoassay used for the measurement of digoxin and digoxin-like immunoreactive substance(s) was carried out as outlined in "Materials and Methods" (A-1). The precision of this assay in our laboratory was determined with the use of quality control samples which contained three different levels of digoxin. The inter-assay coefficient of variation for Level 1 (0.6 $\pm$ 0.1 µg/L) was 14.1%, Level II (2.0 $\pm$ 0.2 µg/L) 6.4%, and Level III (2.9 $\pm$ 0.2 µg/L) 5.9%. The "Tecan" automated system inter-assay precision for the different levels was similar.

The immunoreactivity of some common steroids and bile acids to digoxin antisera DB-157 was studied because of their structural similarities to digoxin (see Tables I and II). The relative cross-reactivities of the steroids listed in decreasing order were: progesterone, deoxycortocosterone, 21-deoxycortisone, 17-alpha hydroxyprogesterone, Δ^4-androstenedione, cortisone, cortisol and dehydroepiandrosterone-sulfate. Glycochenodeoxycholic acid was the highest cross-reacting bile acid studied (see Table 2). It should be noted that the concentrations of steroids and bile acids[92] required to show cross-reactivity were much higher than those seen in normal plasma (steroids; 0.4 nmol/L - 800 µmol/L, and bile acids; 0.79 $\pm$ 0.8 µmol/L).

TABLE I

HPLC RETENTION TIMES AND DIGOXIN

IMMUNOREACTIVITY OF EIGHT KNOWN STEROIDS

STEROID	RETENTION TIME (min)	CROSS-REACTIVITY* (µg/L digoxin equivalents)
Progesterone	28.8	4.0
Deoxycorticosterone	21.8	3.25
21-Deoxycortisone	22.7	2.50
17 alpha Hydroxyprogesterone	25.5	1.80
Δ^4-Androstenedione	22.4	1.61
Cortisone	20.6	1.01
Cortisol	20.4	0.70
Dehydroepiandrosterone-sulfate	15.5	0.40

*All steroids were at 0.1 mM concentration. Cross-reactivity studies were performed with the NML-Digoxin radioimmunoassay kits, antisera lot DB-157.

TABLE II

HPLC RETENTION TIMES AND DIGOXIN

IMMUNOREACTIVITY OF NINE BILE ACIDS

BILE ACID	RETENTION TIME (min)	CROSS-REACTIVITY* (µg/L, Digoxin Equivalents)
Glycochenodeoxycholic	18.4	1.91
Chenodeoxycholic	26.0	1.32
Taurochenodeoxycholic	18.0	1.10
Deoxycholic	26.4	1.03
Glycodeoxycholic	18.2	0.63
Taurodeoxycholic	18.2	0.40
Taurocholic	17.2	0.18
Glycocholic	17.2	0.14
Dehydrocholic	21.2	0.14

*All bile acid were at 2.0 mM concentration. Cross-reactivity studies were performed with the NML-digoxin radioimmunoassay kit, antisera lot DB-157.

B. ISOLATION OF DLIS

B-1 Solvent Extraction of DLIS

Studies were undertaken to determine the effect of solvent extraction on the recovery of digoxin and DLIS from serum. Pooled serum from neonates which contained known concentrations of DLIS (0.7 and 1.4 µg/L digoxin equivalents), and serum from patients receiving digoxin (1.1 and 3.5 µg/L) were treated with different organic solvents. When serum was treated with ethanol at a final concentration of 70%, the DLIS recovery was 0.62 µg/L (89%), and 1.34 µg/L (96%) respectively. The recoveries of digoxin were 0.52 µg/L (47%) and 1.32 µg/L (38%) respectively. Recoveries seen with absolute methanol at a final concentration of 70% were as follows: 0.60 µg/L (86%) and 1.28 µg/L (91%) for DLIS, and 0.60 µg/L (55%) and 1.24 µg/L (40%), respectively, for digoxin. Treatment with methanol was the more efficient solvent for extraction of digoxin from serum. Normal serum spiked with digoxin to a final concentration of 10 µg/L and then treated with either ethanol or methanol to a final concentration of 70% resulted in recoveries of 7.8 µg/L and 8.9 µg/L, respectively. These findings are in contrast to the low recoveries found with serum obtained from patients on digoxin therapy.

B-2 Immunoextraction of DLIS

The possibility of using of specific immunoadsorbent columns

for large-scale isolation and purification of DLIS was examined. Purified rabbit immunoglobulin specific for digoxin was bound to CN-Br activated Sepharose-4B. Radiolabelled ^{125}I-digoxin (0.1 µCi) was added to 40 mg of the resin. After 24 hours, the resin was washed as outlined in "Materials and Methods" (B-2), and the amount of bound digoxin determined. Eighty-three percent of the radiolabelled digoxin was bound to the resin. Only 3% was non-specifically bound to the control immunoadsorbent. It was difficult to elute ^{125}I-digoxin from the column. Elution with 0.1 M glycine-HCl buffer (pH 2), a standard technique for dissociating antigen-antibody complexes[74], released only 10% of the bound label. Absolute methanol eluted all of the radiolabelled digoxin from the affinity column. However, this procedure resulted in destruction of the digoxin binding capacity of the column. If the immunoadsorbent was washed immediately after methanol treatment with the coupling buffer (Materials and Methods, B-2) its digoxin binding capacity was maintained.

The specific anti-digoxin immunoadsorbent absorbed only 20% of DLIS from serum. In contrast to digoxin, DLIS could easily be eluted from the resin with a phosphate-saline buffer (pH 3). The binding and elution characteristics of DLIS suggests that it has a lower affinity than digoxin for the digoxin-specific antibody. This approach for the purification of DLIS was abandoned because of expense and low yield.

B-3 Sep-Pak Extraction of DLIS

The use of C_{18} Sep-Pak cartridges proved to be the most efficient method for preliminary purification and concentration of DLIS from large volumes of cord blood (500 mL). This method gave a mean recovery of DLIS of 80 $\pm$ 4% (N=5, SD=4%). The polar eluate (water-wash) contained 6% of the DLIS and 14% remained in the serum.

The technique of boiling serum for 5 minutes prior to its application on C_{18} Sep-Paks, has often been used to increase the yield of DLIS[19,23,42,80,81]. This procedure when applied to cord blood did not enhance the recovery of DLIS in my hands.

B-4 Reconstitution of DLIS Extracts

Five different buffer systems were checked for their efficiency in the reconstitution of DLIS following evaporation of Sep-Pak methanol extract. Cord serum (6 mL) that contained 5.52 ng of DLIS (0.92 µg/L), as determined by digoxin RIA, was applied to a Sep-Pak column and eluted with methanol (2.5 mL) as outlined in "Materials and Methods" (B-3). The extract was divided into five aliquots (0.5 mL) each of which was evaporated to dryness then reconstituted in a different buffer. DLIS recoveries were determined and expressed relative to theoretical values. Reconstitution in 0.5 mL 50 mM Tris buffer (pH 7.4) yielded

1.05 µg/L DLIS (95% recovery), in 1.0 mL Tris buffer which contained 5% human serum albumin 0.71 µg/L DLIS (64% recovery), 0.5 mL 0.05 M phosphate buffered saline (pH 7.4) yielded 1.16 µg/L (105% recovery), 1.0 mL phosphate buffered saline which contained 5% human serum albumin 1.06 µg/L DLIS (96% recovery) and in 1.0 mL DLIS-free serum 1.19 µg/L (108% recovery). The radioimmunoassay for digoxin was established for serum analysis and requires the presence of protein in dilution buffers to eliminate non-specific reactions. Prior to the determination of DLIS in samples reconstituted with protein free buffers, 0.5 mL DLIS free serum was added (1:1). The digoxin equivalent activity recovered in these samples was corrected for the dilution effect. Buffers that contained human serum albumin gave a background of 1.33 µg/L cross-reactivity in the assay; therefore, this quantity was subtracted from the values obtained when extracts were reconstituted in its presence. Tris buffer is used in most Na^+/K^+-ATPase assays described in the literature. It was therefore used for reconstitution of extracts, and DLIS-free serum was added (1:1) prior to the analysis of DLIS by digoxin radioimmunoassay.

C. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

An HPLC system was established to optimize the separation of digoxin and its metabolites with reverse phase chromatography (see Materials and Methods). The retention times of eight steroids and nine bile acids by this method are listed in Tables I and II (page 51).

C-1 HPLC Fractionation of Cord Sera and Analysis of DLIS

Cord serum (500 mL) was extracted with C_{18} Sep-Paks, the methanol eluates pooled and concentrated by evaporation; then, the resulting fraction was separated by injection onto an HPLC column. Radiolabelled internal standards were routinely used to monitor column reproducibility and recovery during cord serum fractionation. Digoxin (^{3}H), progesterone (^{3}H), and dehydroepiandrosterone-sulfate (^{3}H), which elute at 20.2, 28.8 and 15.5 minutes, respectively, demonstrated greater than 90% recovery. The elution profile was monitored at 210 nM (Figure 2). Digoxin-like immunoreactivity was measured by the cross-reactivity to antisera DB-157 in each of the fifty eluted fractions. The profile seen in cord blood is shown in Figure 3. It was impossible to estimate accurately DLIS recovery in this procedure because of the non-linear cross-reactivity expressed by DLIS with the digoxin antisera.

A number of DLIS-containing peaks eluted between 15 and 24 minutes. Many steroids and bile acids also have retention times in this area. A large peak of immunoreactivity elutes at 29 minutes. This area was examined with a radioimmunoassay for progesterone (retention time - 28.8 minutes). The concentration of progesterone recovered, when spiked into digoxin free serum, accounted for 72% of the digoxin-like immunoreactivity expressed by this fraction. The other eluted DLIS peaks have yet to be identified.

FIGURE 2

HPLC elution profile of cord blood, monitored at 210 nm wavelength.

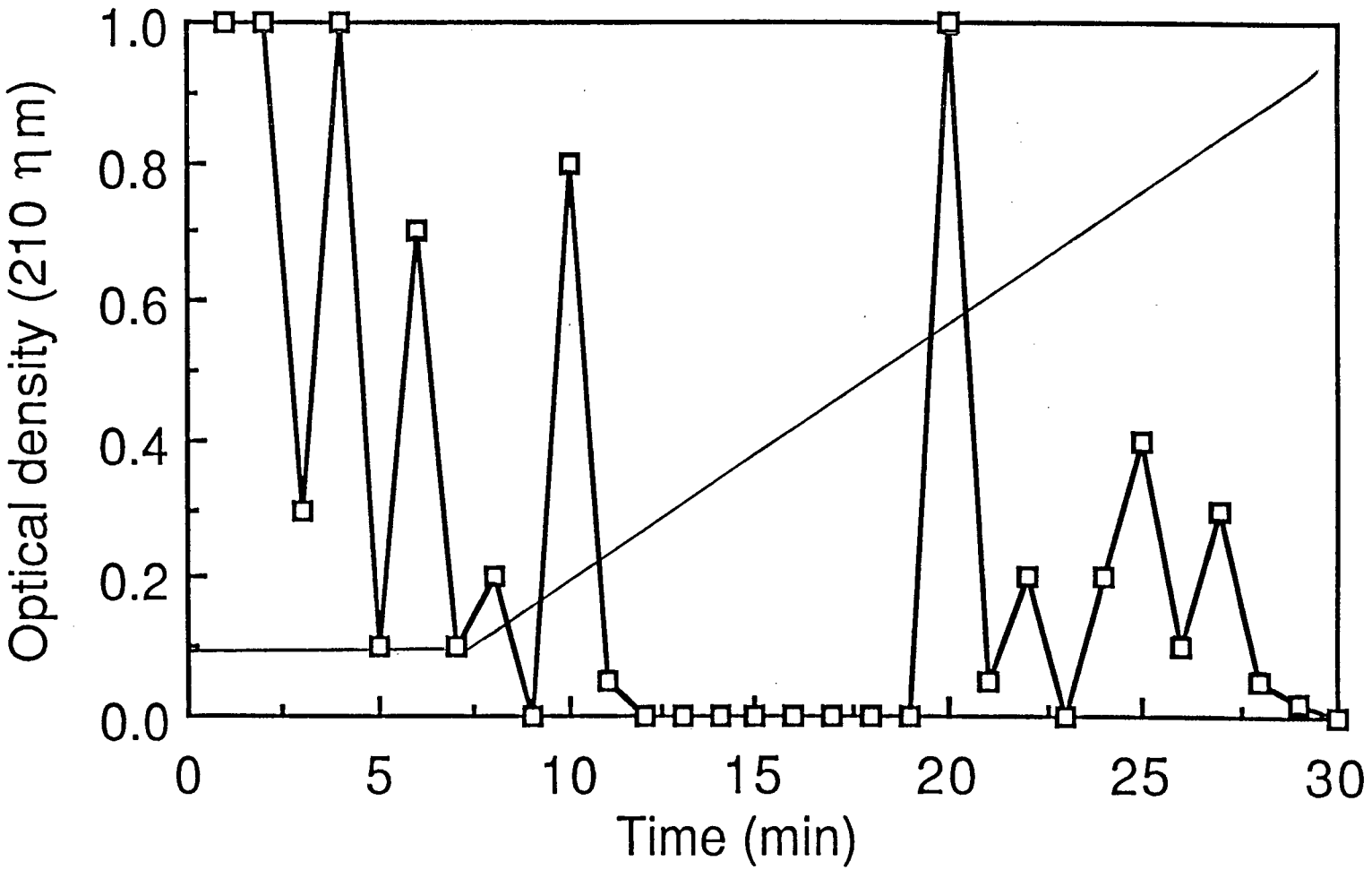
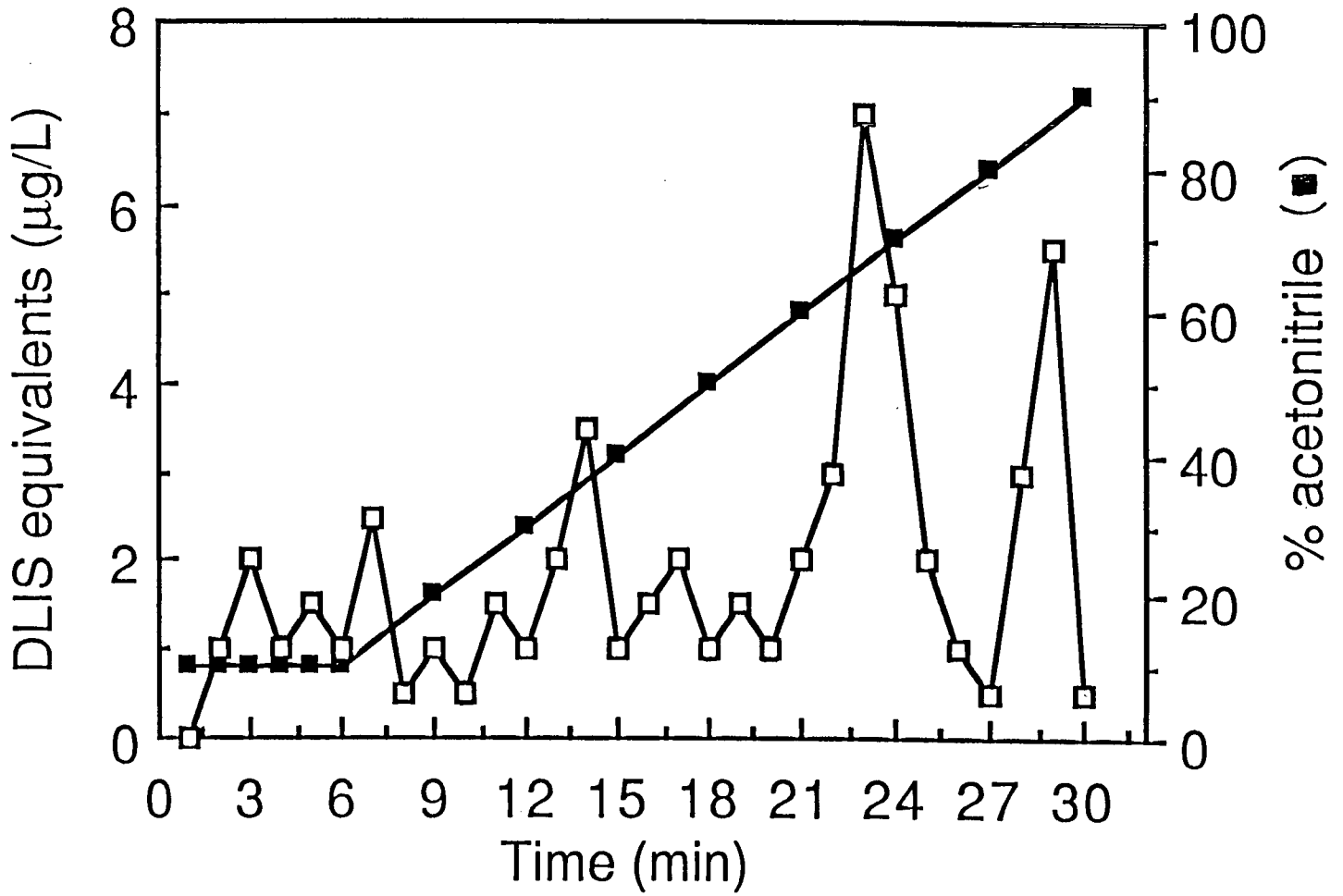

Optical density (210 η m)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time (min)

FIGURE 3

HPLC elution profile of digoxin immunoreactivity in cord blood.


DLIS equivalents (µg/L)
% acetonitrile (■)
Time (min)
0
2
4
6
8
0
20
40
60
80
100
0 3 6 9 12 15 18 21 24 27 30

D. SODIUM/POTASSIUM-ADENOSINE TRIPHOSPHATASE INHIBITION ASSAYS

D-1 Rubidium Uptake by Erythrocytes

In the presence of cardiac glycosides, Na^+/K^+-ATPase activity is inhibited, and this limits the passage of potassium into the cell. Erythrocytes mediate the transport of rubidium in direct competition with potassium. Figures 4 and 5 demonstrate the effects of digoxin and ouabain on ^{86}Rb uptake by the red cell. Fifty percent of rubidium uptake is inhibited by 3.5 nmol/L digoxin and 6 nmol/L ouabain, respectively. The assay is sensitive to 1.0 nmol/L glycoside ($10^{-9}M$) concentrations and maximal inhibition occurs above 100 nmol/L ($10^{-7}M$). The coefficient of variation for the erythrocyte ^{86}Rb uptake assay is 2.4% (n=20).

Hemolysis was a frequent problem when DLIS-containing serum samples that had been treated with ethanol in the ^{86}Rb uptake assay were examined. In order to evaluate the cause of this, changes in assay conditions during the incubation period were examined. Alterations in pH, pCO_2, pO_2, oxygen saturation, osmolality, and glucose consumption were measured at thirty minute intervals. Table III outlines the changes in these parameters. The variations expressed did not seem large enough to account for the red cell hemolysis. The problem may have

FIGURE 4

The effect of increasing concentrations of digoxin on erythrocyte uptake of ^{86}Rubidium.

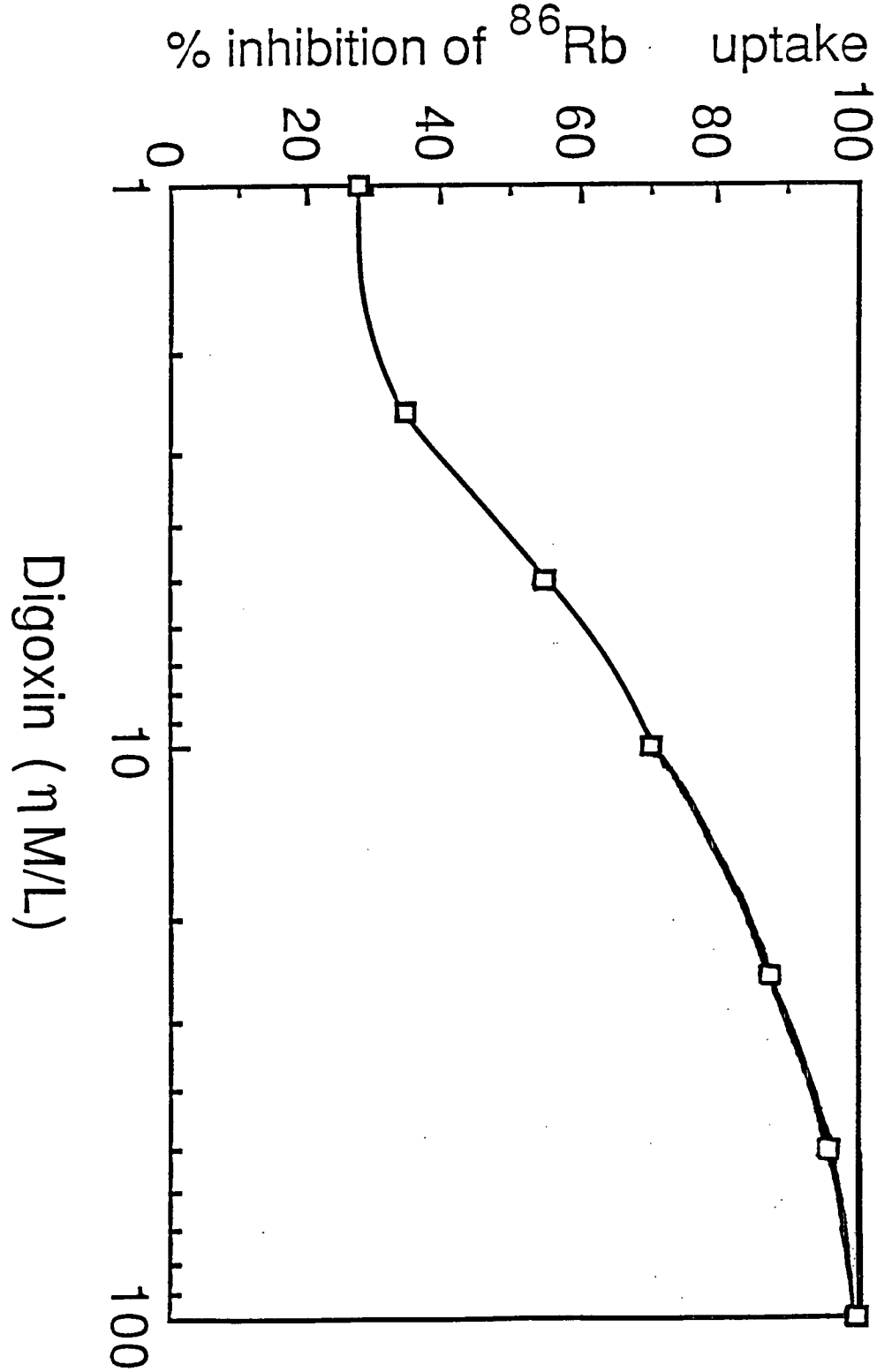

% inhibition of ^{86}Rb uptake
0
20
40
60
80
100
1
10
100
Digoxin (n M/L)

FIGURE 5

The effect of increasing concentrations of ouabain on erythrocyte uptake of ^{86}Rubidium.

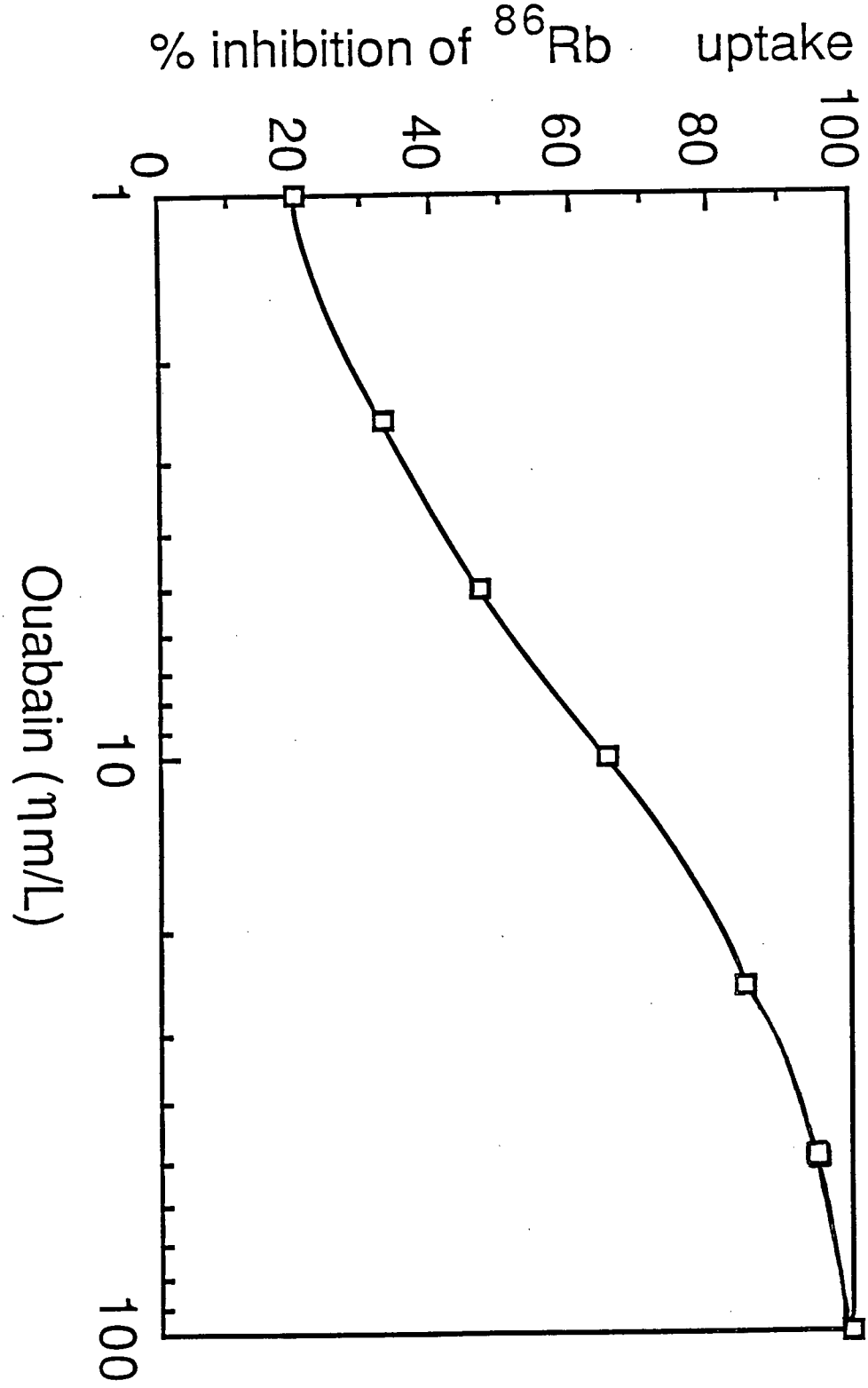

% inhibition of ^{86}Rb uptake
0
20
40
60
80
100
1
10
100
Ouabain (ηm/L)

TABLE III

TIME-DEPENDENT CHANGES IN THE REACTION BUFFER
OF THE ERYTHROCYTE ^{86}RUBIDIUM UPTAKE ASSAY

TIME:	10 min.	30 min.	60 min.	90 min.	120 min.
pH	7.23	7.18	7.16	7.15	7.13
pCO_2 mmHg	5.5	6.2	7.0	8.2	7.3
pO_2 mmHg	47.0	45.0	43.5	39.5	40.5
$\%O_2$ saturation	73.4	68.4	64.0	56.9	57.3
Glucose, mg/dL	785	775	750	725	730
Osmolality, mOsm	388	390	420	441	440

been due to the solvent employed for the recovery of DLIS. Therefore, the sensitivity of the assay to different solvents was examined. When absolute methanol instead of ethanol was used, the hemolysis was eliminated.

Figure 6 demonstrates the effect of methanol treatment on normal serum spiked with various concentrations of digoxin. The ability of this serum and its corresponding extract to inhibit the uptake of rubidium by erythrocytes is compared. More than 80% of digoxin is recovered in the methanol extract of serum, and there is no significant difference in the effect of extracted or non-extracted digoxin spiked serum on rubidium uptake. Treatment of serum samples with methanol can therefore be used in this assay system without causing the interference of hemolysis.

D-1a The Effect of Potassium on ^{86}Rb uptake

Normal serum contains 4.0 $\pm$ 0.3 Meq/L of potassium. In the ^{86}Rb uptake assay there is direct competition between rubidium and potassium for transport into the red cells. Figure 7 demonstrates the effect of potassium on erythrocyte uptake of rubidium. As little as 0.5 mmol/L will inhibit ^{86}Rb uptake by 7%. Concentrations normally present in serum can inhibit uptake by 50%. The presence of potassium actually enhances the activity

FIGURE 6

The effect of plasma spiked with digoxin (□-□) and the methanol extract of digoxin-spiked plasma (■-■), on the inhibition of erythrocyte uptake of ^{86}Rubidium.

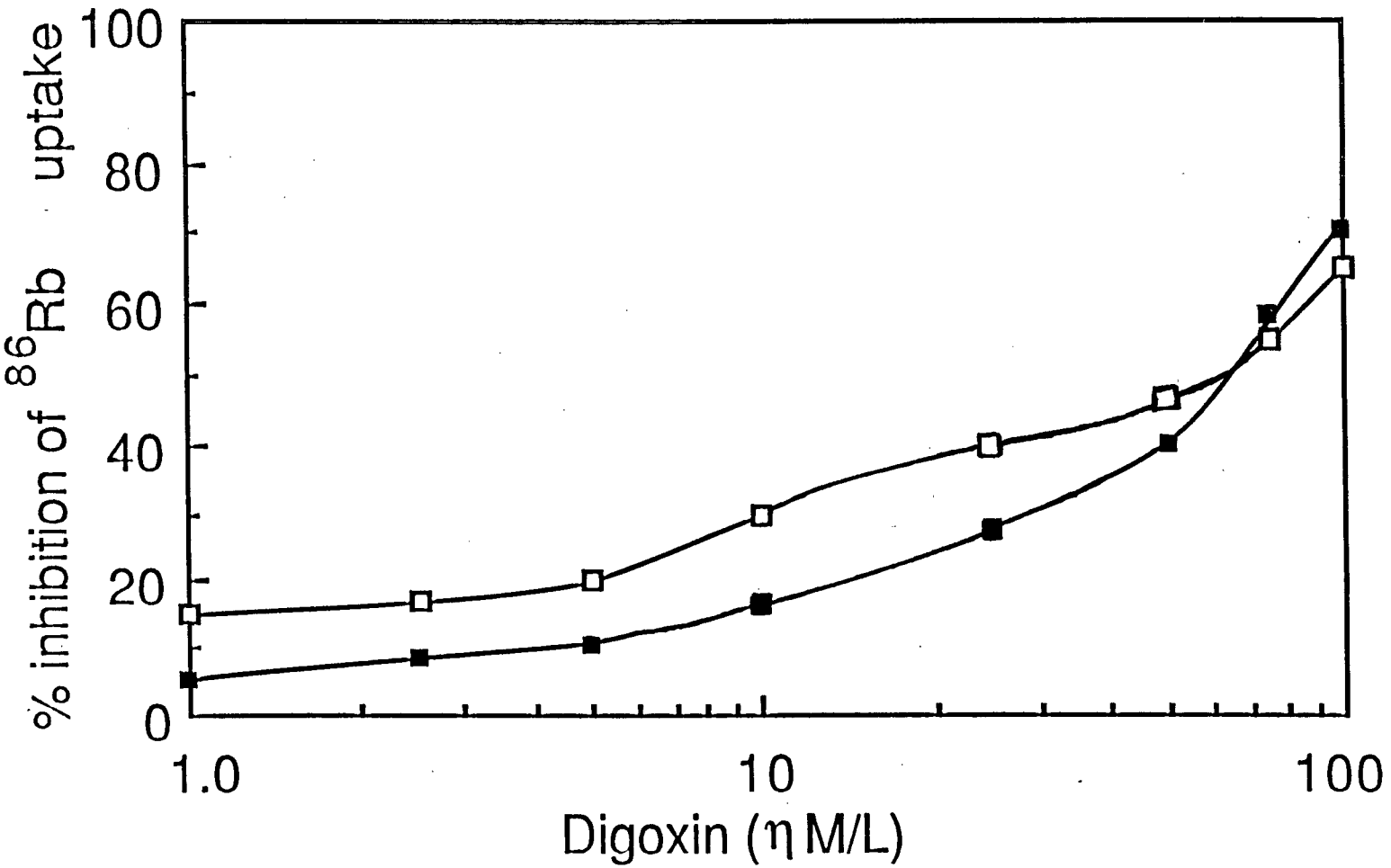
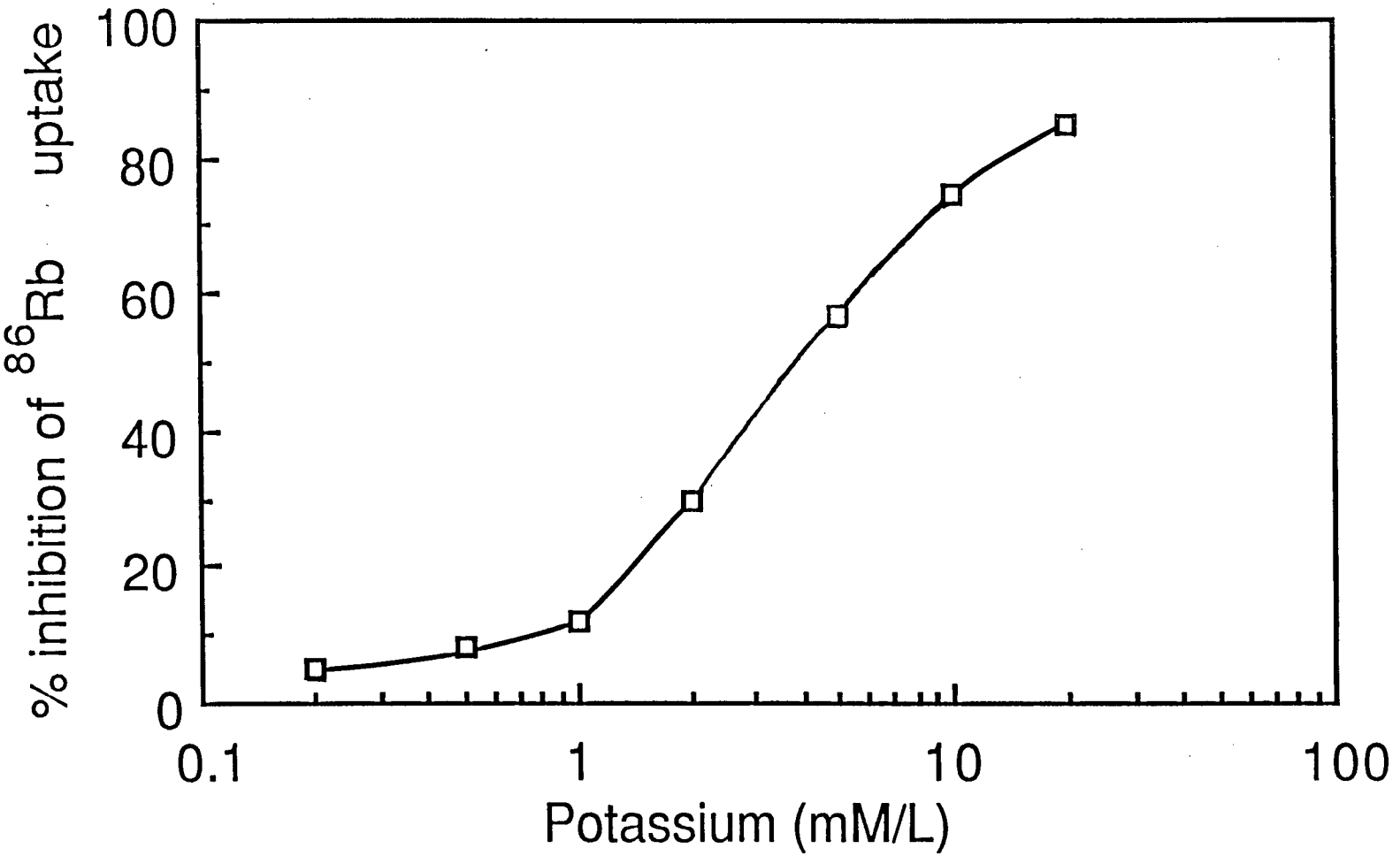

% inhibition of ^{86}Rb uptake
100
80
60
40
20
0
1.0
10
100
Digoxin (η M/L)

FIGURE 7

The effect of increasing concentrations of potassium on the inhibition of erythrocyte uptake of ^{86}Rubidium.


% inhibition of ^{86}Rb uptake
100
80
60
40
20
0
0.1
1
10
100
Potassium (mM/L)

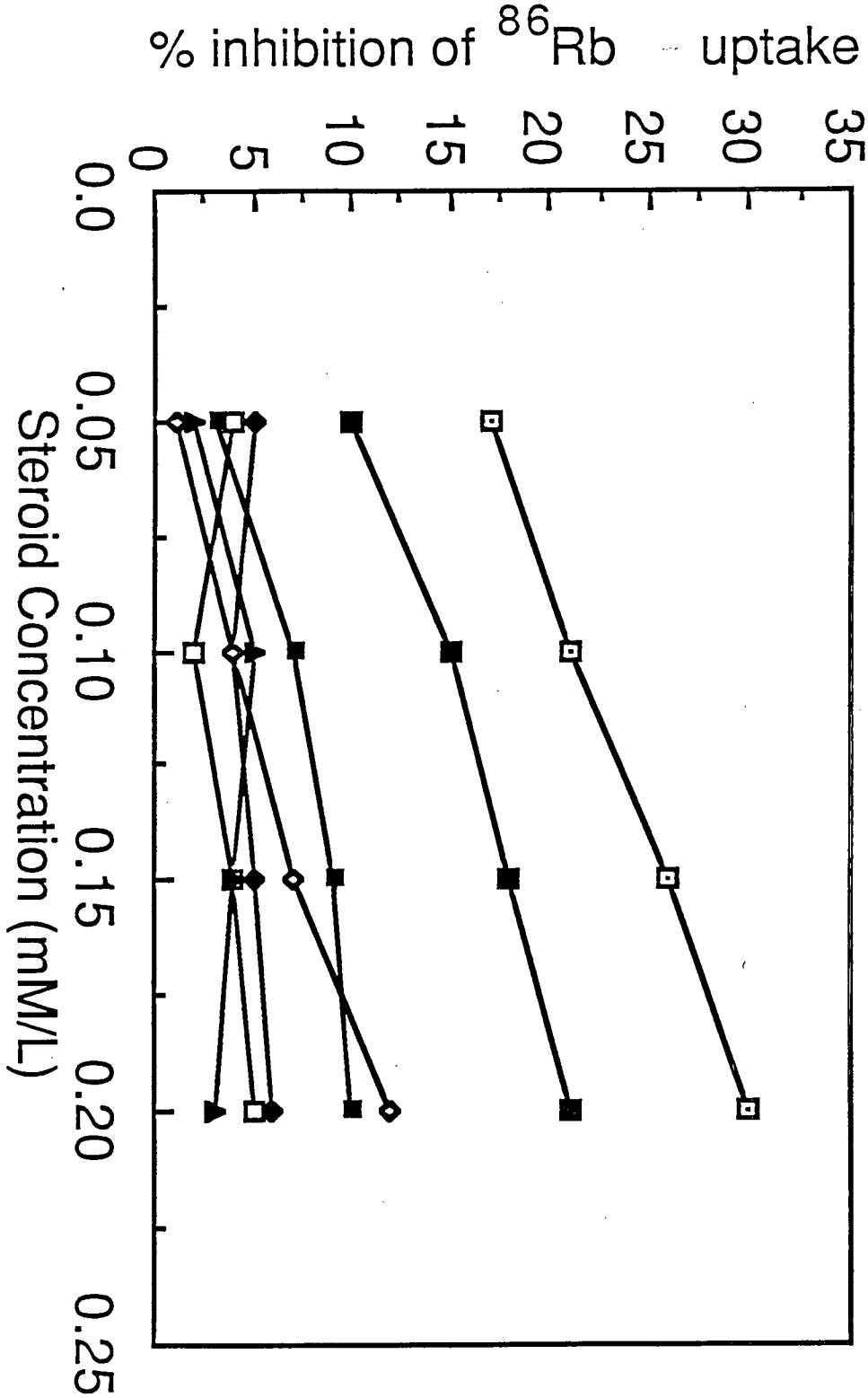
of Na^+/K^+-ATPase[24]; however, its presence in assay buffers or serum samples seriously affects the ability to interpret the results of inhibition studies in the ^{86}Rb uptake assay.

D-1b The Effect of Steroids and Bile Acids on ^{86}Rb Uptake

Many steroids and bile acids cross-react with digoxin antisera. We examined the effect of these steroids on the erythrocyte uptake of rubidium. The data are summarized in Figure 8. Seven steroids were diluted in the assay buffer to final concentrations of 0.05, 0.10, and 0.20 mmol/L. Table I outlines the cross-reactivities of these steroids at 0.1 mmol/L concentration. Δ^4-androstenedione (1.6 µg/L digoxin elquivalents) has the greatest effect on the uptake of rubidium by erythrocytes, it inhibits this function by 22% at 0.1 mmol/L and 30% at 0.2 mmol/L. Progesterone, which demonstrates the highest immunoreactivity (4.0 µg/L digoxin equivalents) with digoxin antisera DB-157, does not significantly affect ^{86}Rb uptake (less than 5% inhibition). Deoxycorticosterone, which shows the second highest level of cross-reactivity (3.25 µg/L), also has the second greatest effect on red cell ^{86}Rb uptake, inhibiting it by 14% at 0.1 mmol/L and 21% at 0.2 mmol/L.

FIGURE 8

The effect of increasing concentrations of seven naturally occurring steroids - Δ^4androstenedione (⊡-⊡), progesterone (◆-◆), deoxycorticosterone (■-■), 21-deoxycortisone (◇-◇), cortisol (▬-▬), 17-α hydroxyprogesterone (□-□), and cortisone (▲-▲) - on the erythrocyte uptake of ^{86}Rubidium.


% inhibition of ^{86}Rb uptake
0
5
10
15
20
25
30
35
0.0
0.05
0.10
0.15
0.20
0.25
Steroid Concentration (mM/L)

All bile acids studied caused hemolysis of the red cells. Since glycochenodeoxycholic acid is the most cross-reactive to digoxin antisera DB-157 (1.91 µg/L digoxin equivalents at 2.0 mmol/L concentration), it was decided to investigate the concentration dependence of hemolysis with this bile acid. Figure 9 demonstrates the effect of various concentrations of glycochenodeoxycholic acid (0.1, 0.5, 1.0, and 2.0 mmol/L), on erythrocyte uptake of rubidium. The uptake of ^{86}Rb and the extent of erythrocyte hemolysis were examined every 30 minutes. With the high concentrations of bile acid, hemolysis was evident within 30 minutes. Figure 10 compares the appearance of hemolysis with the inhibition of ^{86}Rb uptake in concentrations of glycochenodeoxycholic acid up to 0.5 mmol/L after a three hour incubation at $37^{o}C$. Hemolysis and inhibition occur simultaneously at concentrations greater than 0.2 mmol/L bile acid. The inhibition of ^{86}Rb uptake may be secondary to a non-specific detergent effect that bile acids have on erythrocytes which leads to disruption of the membrane and not to a direct specific effect on Na^{+}/K^{+}-ATPase activity.

Ethanol was required to solubilize the steroids and bile

FIGURE 9

The effect of different concentrations of glycochenodeoxycholic acid - 0.1 (⊡-⊡), 0.5 (◆-◆), 1.0 (■-■), and 2.0 mM (◇-◇) - on erythrocyte uptake of ^{86}Rubidium, as expressed relative to duration of incubation time.

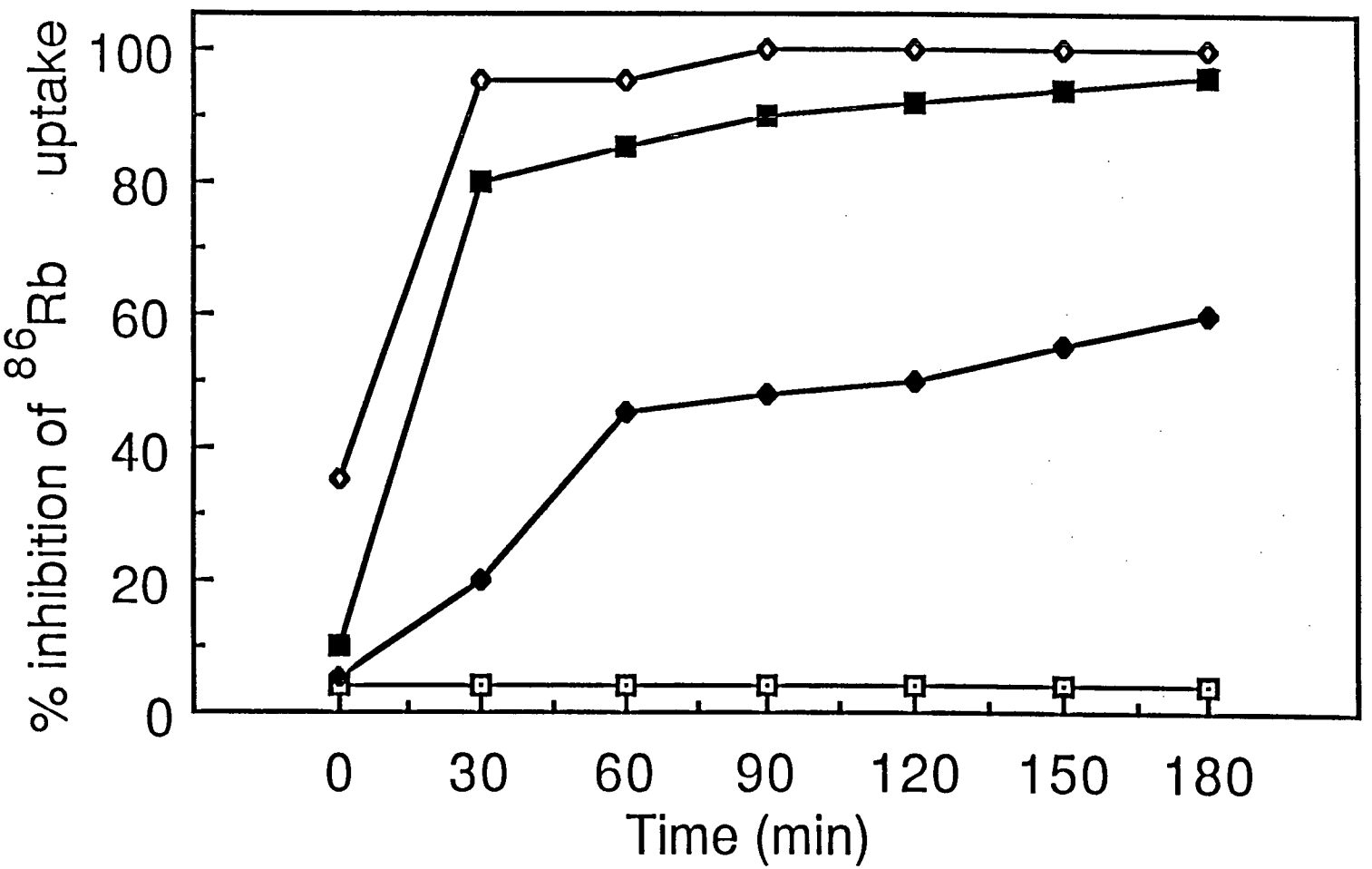
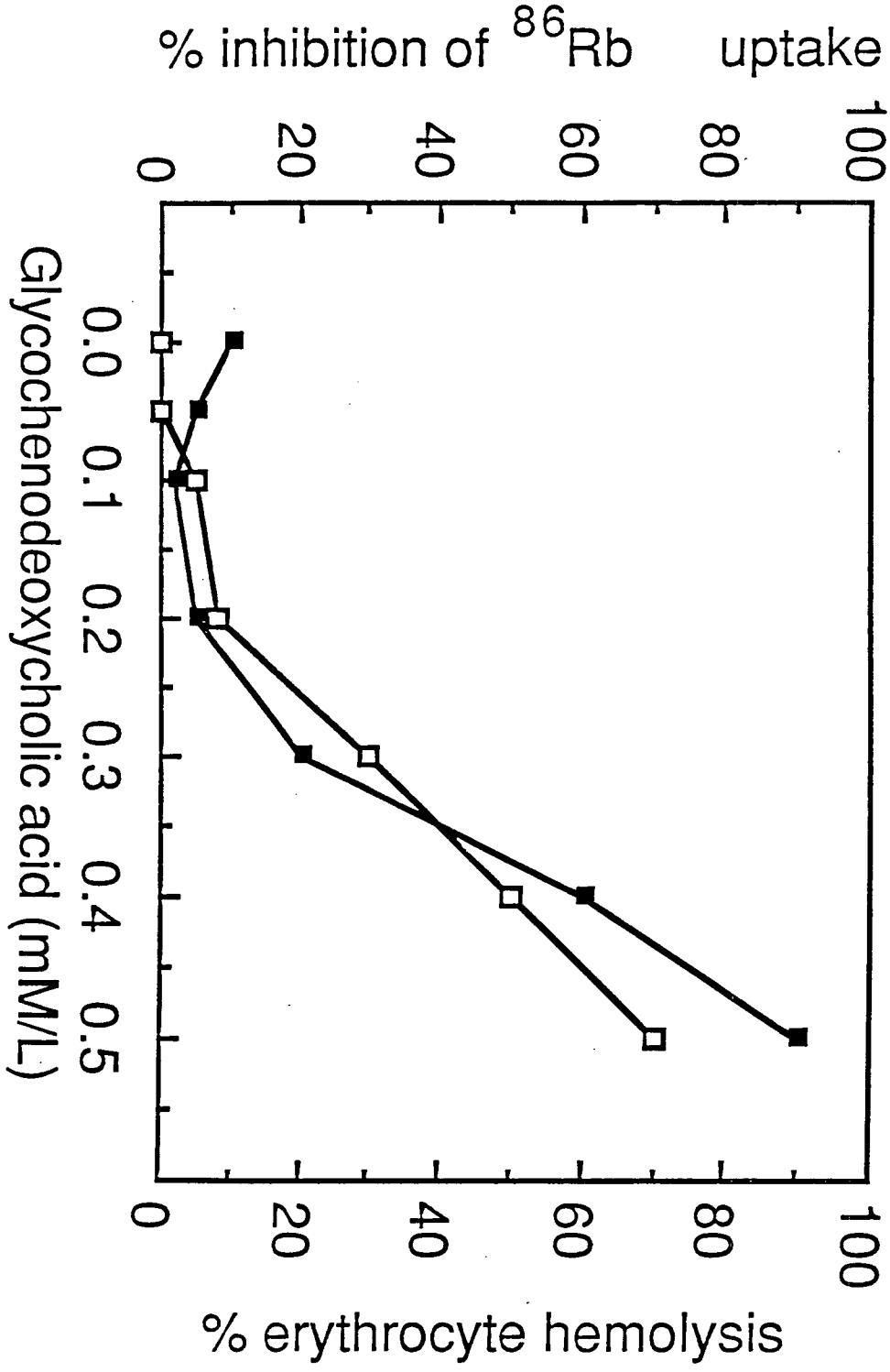

% inhibition of ^{86}Rb uptake
100
80
60
40
20
0
0
30
60
90
120
150
180
Time (min)

FIGURE 10

The effect of increasing concentrations of glycochenodeoxycholic acid on red cell hemolysis (□ - □) and the inhibition of erythrocyte uptake of ^{86}Rubidium (■ - ■).


% inhibition of ^{86}Rb uptake
0
20
40
60
80
100
Glycochenodeoxycholic acid (mM/L)
0.0
0.1
0.2
0.3
0.4
0.5
% erythrocyte hemolysis

acids in the assay system we used. We therefore examined the effect of ethanol on the ^{86}Rb uptake assay. Figure 11 demonstrates the effect of various concentrations of absolute ethanol (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0%) on ^{86}Rb uptake by erythrocytes. Ethanol at a concentration of 0.5% inhibited ^{86}Rb uptake by 10%. Red cells began to hemolyze at 4% alcohol concentrations. Inter-assay reproducibility of inhibition was examined at an ethanol concentration of 1%. Erythrocyte uptake of rubidium was inhibited by 15.5 $\pm$ 0.5% at this concentration with a coefficient of variation of 2.9% (n=10).

D-1c The Effect of Fractions of Cord Serum Separated by HPLC On ^{86}Rb Uptake

Cord serum (500 mL) extracted on C_{18} Sep-Paks and fractionated by HPLC resulted in 50 fractions which were dried by evaporation and then reconstituted in 50 mM Tris buffer (pH 7.4) containing glucose (85 mg/L) as outlined in "Materials and Methods" (D-1). None of the fifty fractions inhibited ^{86}Rb uptake by erythrocytes (Figure 12). This experiment was repeated twice.

D-2 Canine Kidney Na^+/K^+-ATPase Assay

The canine kidney ATPase assay measures the release of

FIGURE 11

The effect of increasing concentrations of ethanol on erythrocyte uptake of ^{86}Rubidium.

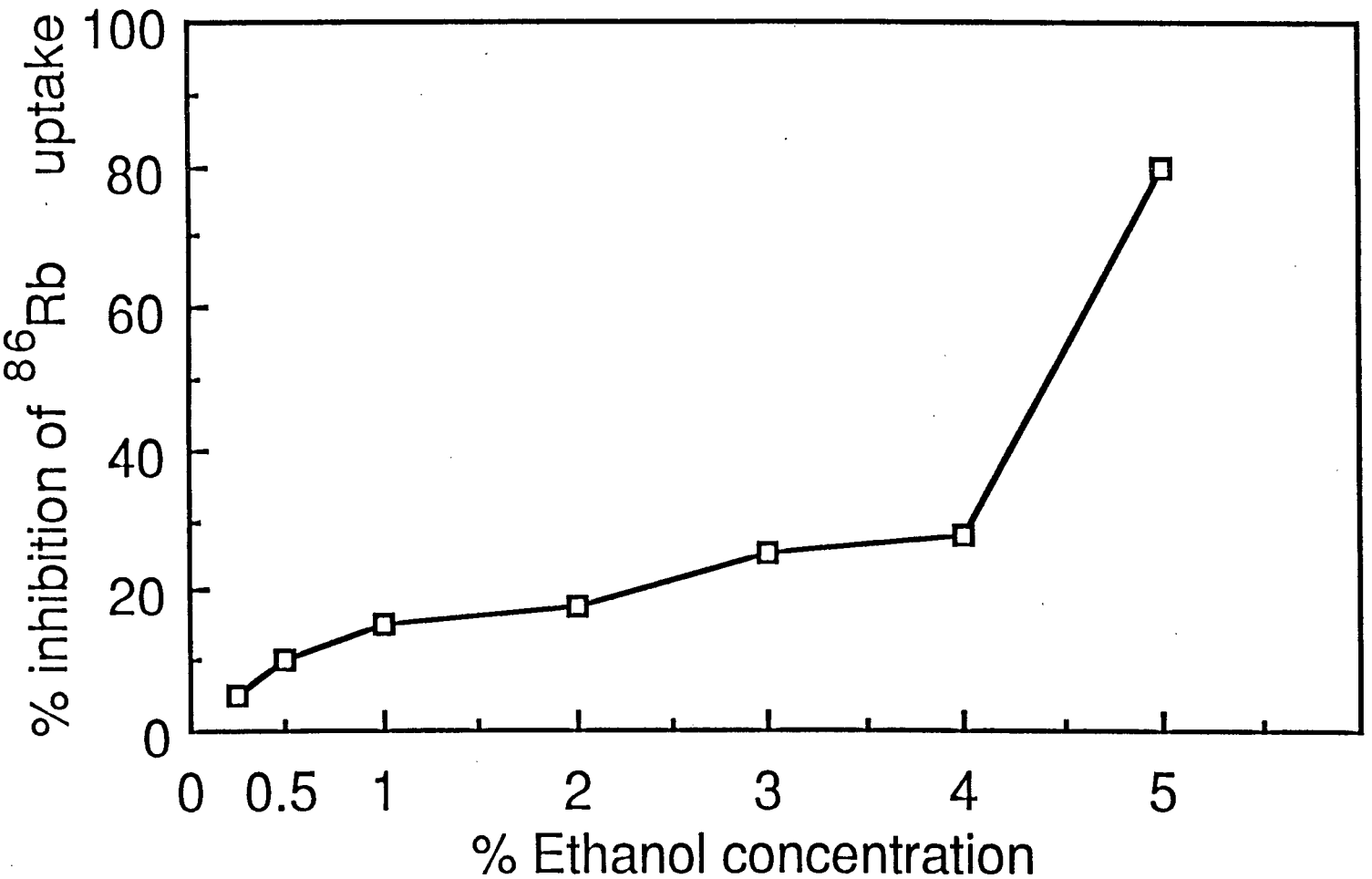
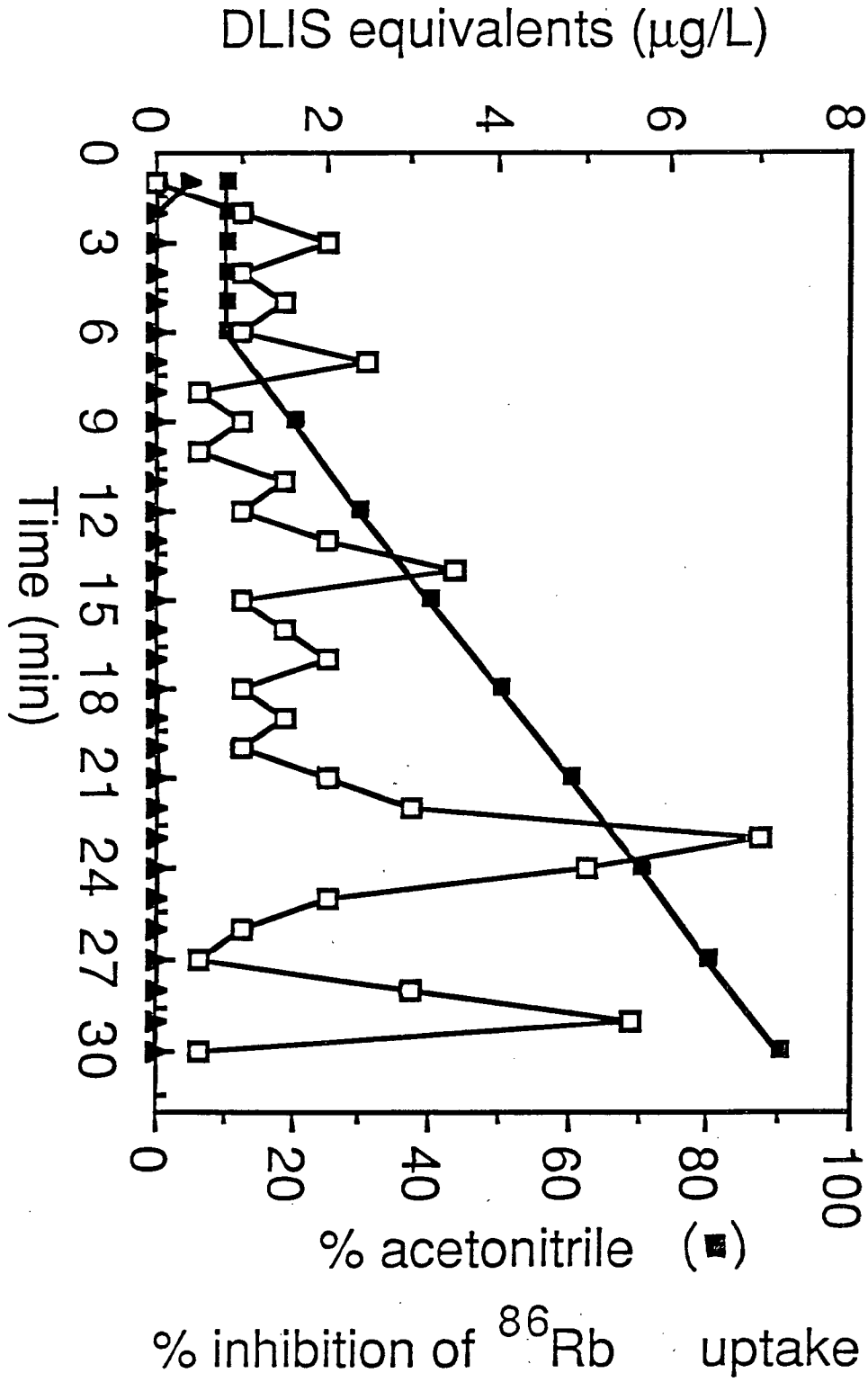

% inhibition of ^{86}Rb uptake
100
80
60
40
20
0
0 0.5 1 2 3 4 5
% Ethanol concentration

FIGURE 12

HPLC elution profile showing the inhibition of ^{86}Rubidium uptake by erythrocytes (▲-▲) and digoxin-like immunoreactivity (□-□), in cord blood.


DLIS equivalents (µg/L)
0
2
4
6
8
0 3 6 9 12 15 18 21 24 27 30
Time (min)
0
20
40
60
80
100
% acetonitrile (■)
% inhibition of ^{86}Rb uptake

^{32}P-labelled inorganic phosphate as adenosine triphosphate (ATP) is hydrolyzed by Na^+/K^+-ATPase. Figures 13 and 14 demonstrate the effect of various concentrations of digoxin and ouabain on enzyme activity. The concentrations of digoxin and ouabain which caused fifty percent inhibition of Na^+/K^+-ATPase were 0.9 µmol/L and 0.6 µmol/L respectively, with a coefficient of variation of 5.8% (n=5). The detection limit of the assay, 0.1 umol/L (10^{-7}M) glycoside, results in 12% enzyme inhibition with maximum inhibition at a concentration of 100 µmol/L (10^{-4}M). The canine kidney Na^+/K^+-ATPase activity in this assay system was 4.8 µM ATP hydrolyzed per mg protein per hour. In the absence of sodium and potassium this activity was reduced by 95%.

D-2a <u>The Effect of Steroids on Canine Kidney Na^+/K^+-ATPase Activity</u>

We examined the ability of those steroids which showed the highest cross-reactivity to digoxin antisera DB-157 (Table 1) to inhibit canine kidney Na^+/K^+-ATPase activity. The results are summarized in Figure 15. Progesterone, Δ^4-androstenedione and deoxy-corticosterone showed the highest degree of inhibition of Na^+/K^+-ATPase activity. At 0.1 mmol/L progesterone, canine kidney ATPase activity was inhibited by greater than 40%. There does not appear to be any correlation

FIGURE 13

The effect of increasing concentrations of digoxin on canine kidney Na^+/K^+-ATPase activity.

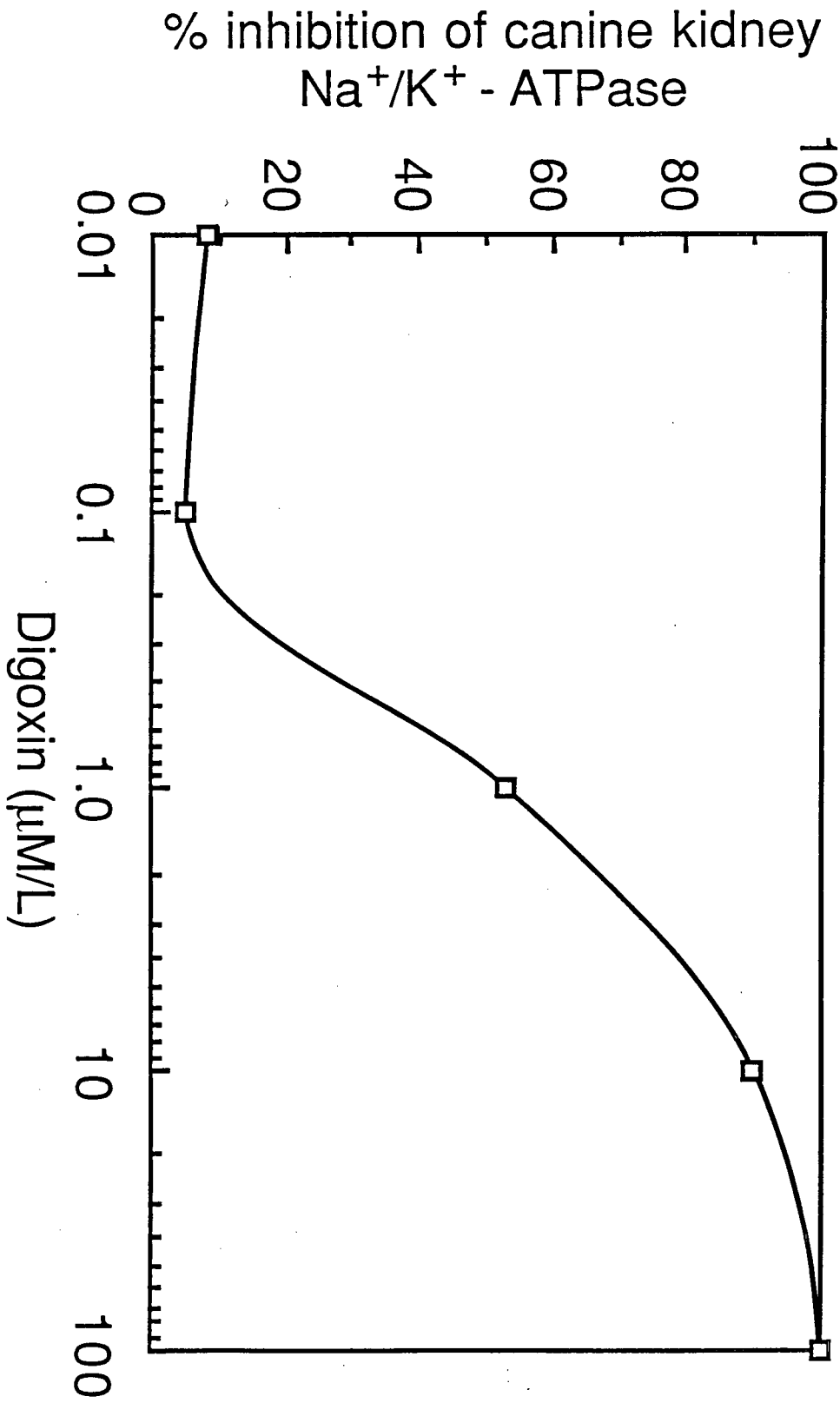

% inhibition of canine kidney
Na+/K+ - ATPase
0
20
40
60
80
100
0.01
0.1
1.0
10
100
Digoxin (μM/L)

FIGURE 14

The effect of increasing concentrations of ouabain on canine-kidney Na^+/K^+-ATPase activity.

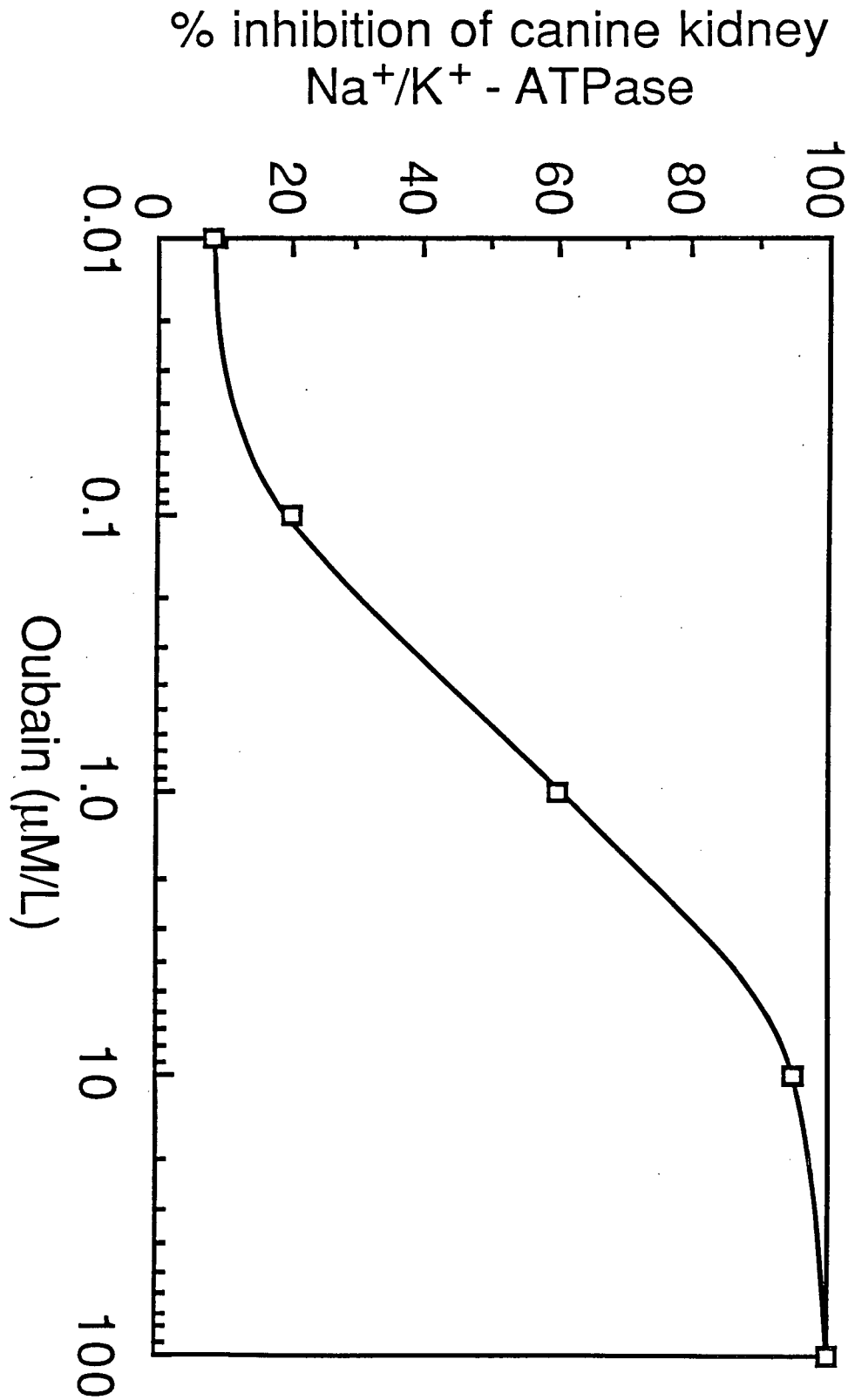
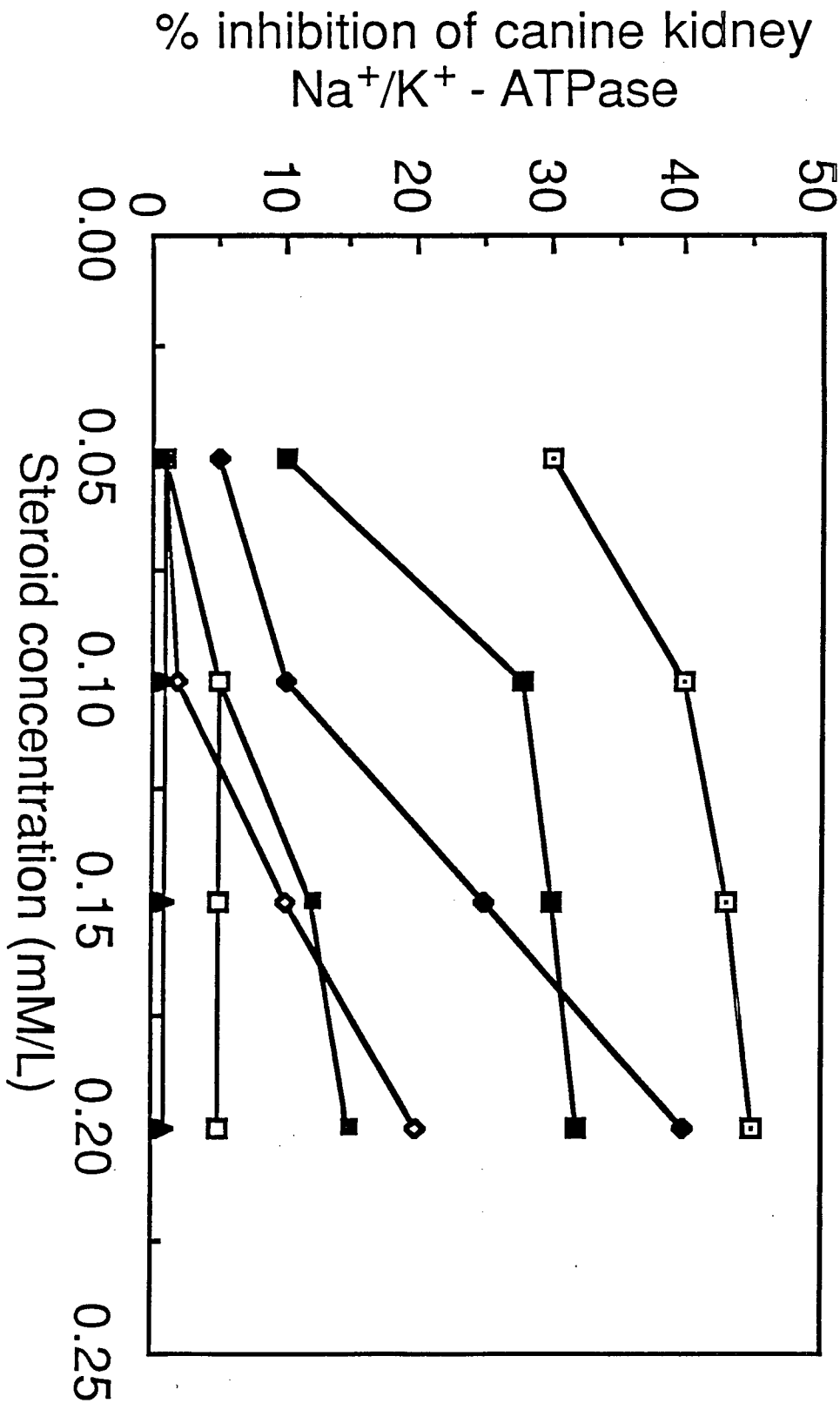

% inhibition of canine kidney
Na+/K+ - ATPase
0
20
40
60
80
100
0.01
0.1
1.0
10
100
Oubain (µM/L)

FIGURE 15

The effect of increasing concentrations of seven naturally occurring steroids - progesterone (⊡ - ⊡), Δ^4-androstenedione (◆ - ◆), deoxycorticosterone (■ - ■), cortisone (◇ - ◇), cortisol (▪ - ▪), 17∝-hydroxyprogesterone (□ - □), and 21-deoxycortisone (▲ - ▲) - on canine-kidney Na^+/K^+-ATPase activity.


% inhibition of canine kidney
Na+/K+ - ATPase
0
10
20
30
40
50
0.00
0.05
0.10
0.15
0.20
0.25
Steroid concentration (mM/L)

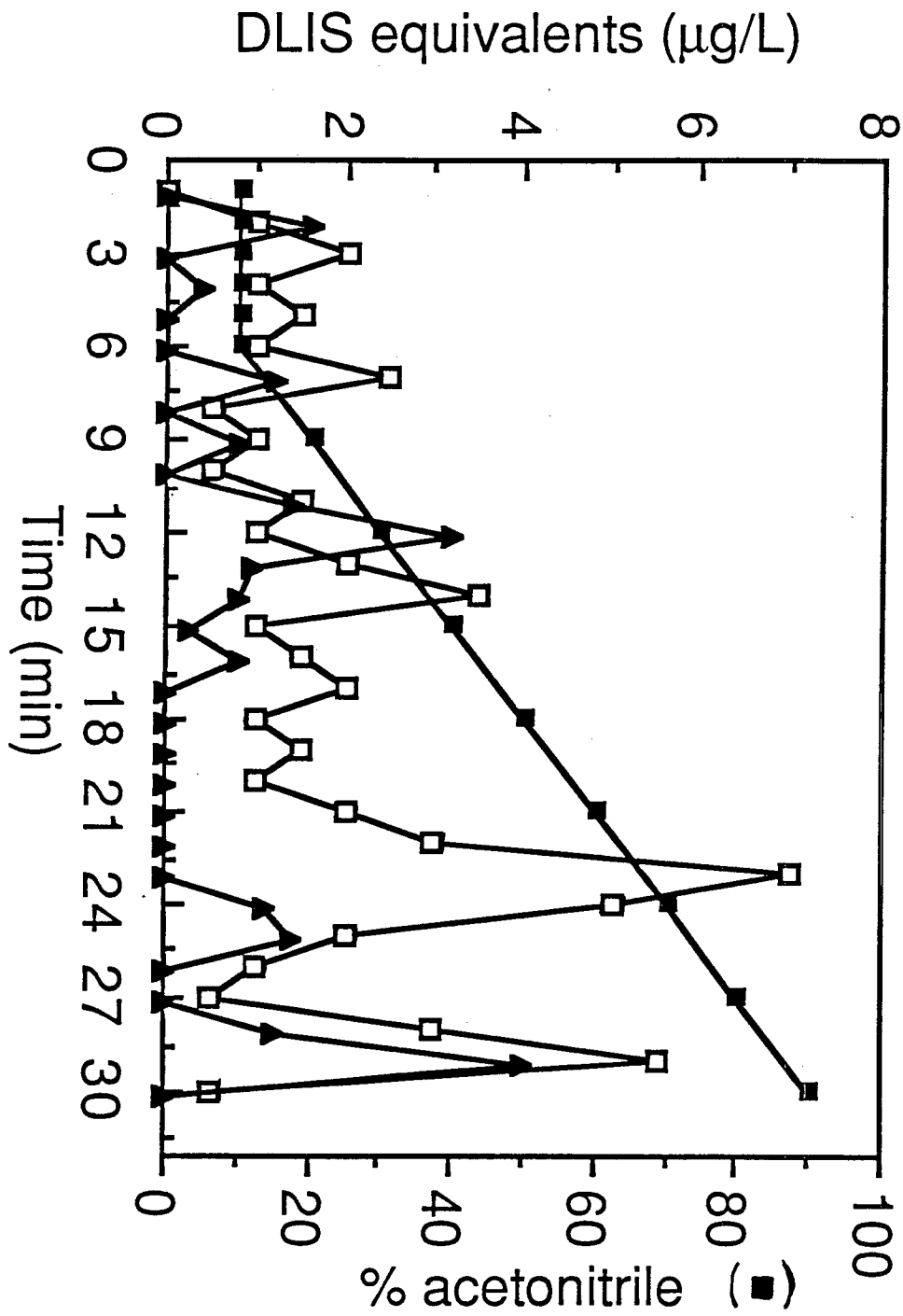
between the inhibitory activities of these steroids on canine kidney Na^+/K^+-ATPase activity, ^{86}Rb uptake by erythrocytes or immunoreactivity to digoxin antisera. The effect of glycochenodeoxycholic acid on this system was also examined (data not shown). Concentrations of 0.5 mmol/L bile acid caused 50% inhibition of canine kidney Na^+/K^+-ATPase activity. Whether this inhibition is due to direct interaction with the enzyme, or to non-specific detergent effects of the bile acid is not known.

D-2b <u>The Effect of HPLC-Fractionated Cord Serum on Canine Kidney Na^+/K^+-ATPase Activity</u>

Cord serum (500 mL) was first processed by passing it through disposable C_{18} Sep-Pak cartridges as outlined in "Materials and Methods". The methanol eluates were concentrated and fractionated by reverse phase HPLC. The fractions (50) were reconstituted in 50 mM Tris buffer (pH 7.4) containing 1% ethanol to ensure complete solubilization of non-polar substances. Figure 16 compares the digoxin-like immunoreactivity in each of these fractions with their ability to inhibit canine kidney Na^+/K^+-ATPase activity. There are many fractions present in cord serum which can inhibit canine kidney Na^+/K^+-ATPase activity by more than 20%. These fractions elute at 3, 7, 12, 16, 25, and 28

FIGURE 16

HPLC elution profile of the canine-kidney Na^+/K^+-ATPase inhibitory activity (▲-▲) and digoxin-like immunoreactivity (□-□) seen in cord blood.



% inhibition of canine kidney Na^+/K^+ - ATPase

minutes. The peak that elutes at 28 minutes has been shown to contain progesterone. None of these fractions had any effect on rubidium uptake by erythrocytes (Figure 12).

The buffers used to reconstitute HPLC fractions for the canine kidney-ATPase or ^{86}Rb uptake assays are slightly different. Glucose is necessary for erythrocyte function, but neither ethanol nor potassium (both present in the canine kidney-ATPase buffer) can be used in the ^{86}Rb uptake assay. Table IV demonstrates DLIS recovery from cord serum fractions separated by HPLC and reconstituted in each of the assay buffers. This experiment was carried out to determine if there was a discrepancy in the resolubilization of DLIS that could account for the different inhibitory results noted between the canine kidney Na^+/K^+-ATPase and ^{86}Rb uptake assays. Except for peak 11, which elutes in fractions 42 - 45, there is no significant difference in the recovery of DLIS with the two buffers used. Ethanol is necessary for complete solubilization of progesterone which elutes in fraction 43.

D-3 Erythrocyte Membrane Na^+/K^+-ATPase Assay

Our studies have indicated that cord serum extracted with C_{18} Sep-Paks and fractionated by reverse phase HPLC contains a

TABLE IV

COMPARISON OF DIGOXIN IMMUNOREACTIVITY FOLLOWING HPLC FRACTIONATION AND RECONSTITUTION IN TWO DIFFERENT BUFFER SYSTEMS EMPLOYED FOR THE ERYTHROCYTE UPTAKE OF ^{86}RUBIDIUM AND CANINE KIDNEY Na^+/K^--ATPase ASSAY METHODS

Peak	Pooled Fraction	Digoxin Equivalents (µg/L) Canine Kidney ATPase Buffer	Digoxin Equivalents (µg/L) RBC ^{86}Rb-Uptake Assay Buffer
1	1- 3	1.65	1.65
2	4- 6	0.45	0.45
3	7-10	1.50	1.55
4	11-14	1.90	2.0
5	15-18	0.90	0.70
6	19-23	0.80	0.85
7	24-28	0.55	0.80
8	29-32	0.45	0.55
9	33-37	1.0	0.9
10	38-41	0.20	0.30
11	42-45	4.1	1.9
12	46-50	0.20	0.50

number of DLIS fractions. Many of these fractions inhibit canine kidney Na^+/K^+-ATPase activity, but none of them affect the uptake of rubidium by red cells. This discrepancy may be due either to the source of Na^+/K^+-ATPase examined or the nature of the assay system employed. Erythrocyte membranes were isolated and prepared[79] so that their enzyme activity could be measured in a manner similar to the canine kidney-ATPase assay, to attempt to answer this question.

The effects of digoxin and ouabain on erythrocyte membrane Na^+/K^+-ATPase activity as measured by the rate of $[^{32}P]$-ATP hydrolysis are demonstrated in Figures 17 and 18. The inhibition curves were essentially identical for both cardiac glycosides. This method is sensitive to 1.0 nmol/L (10^{-9}M) concentrations which inhibits Na^+/K^+-ATPase activity by 10% with a coefficient of variation of 3% (n=5). Fifty percent of the enzyme activity is inhibited at 55 nmol/L digoxin and ouabain, with maximum inhibition occurring at 1.0 µmol/L (10^{-6}M) glycoside concentration. Enzyme activity in the absence of inhibitors is expressed as the hydrolysis of 0.05 µM ATP per mg membrane protein per hour. In the absence of sodium and potassium, 94% of this activity is eliminated.

D-3a <u>The Effect of Steroids and Bile Acids on Erythrocyte Membrane Na^+/K^+-ATPase Activity</u>

A summary of the effects of glycochenodeoxycholic acid

FIGURE 17

The effect of increasing concentrations of digoxin on the Na^+/K^+-ATPase activity of isolated erythrocyte membranes.

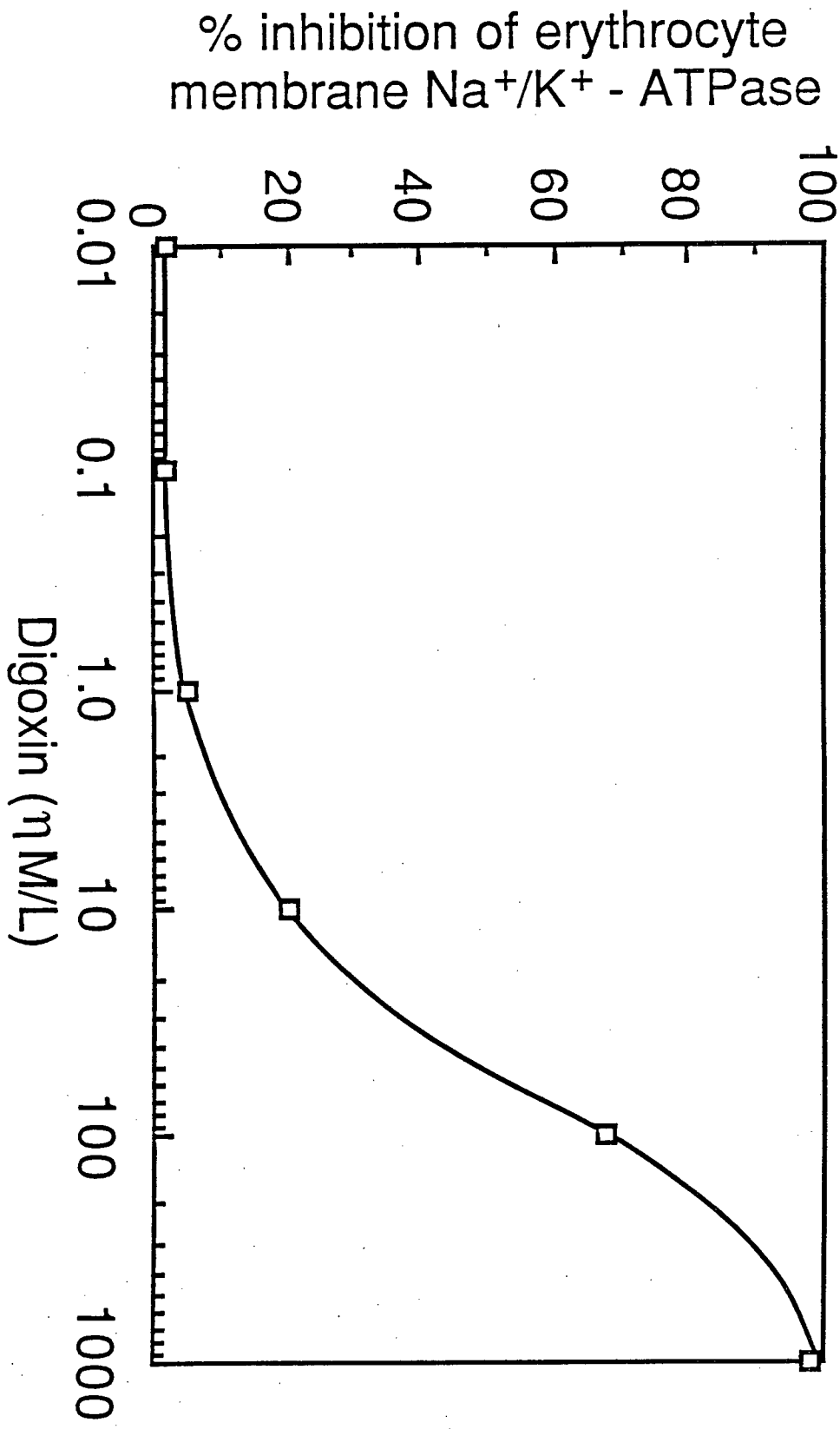
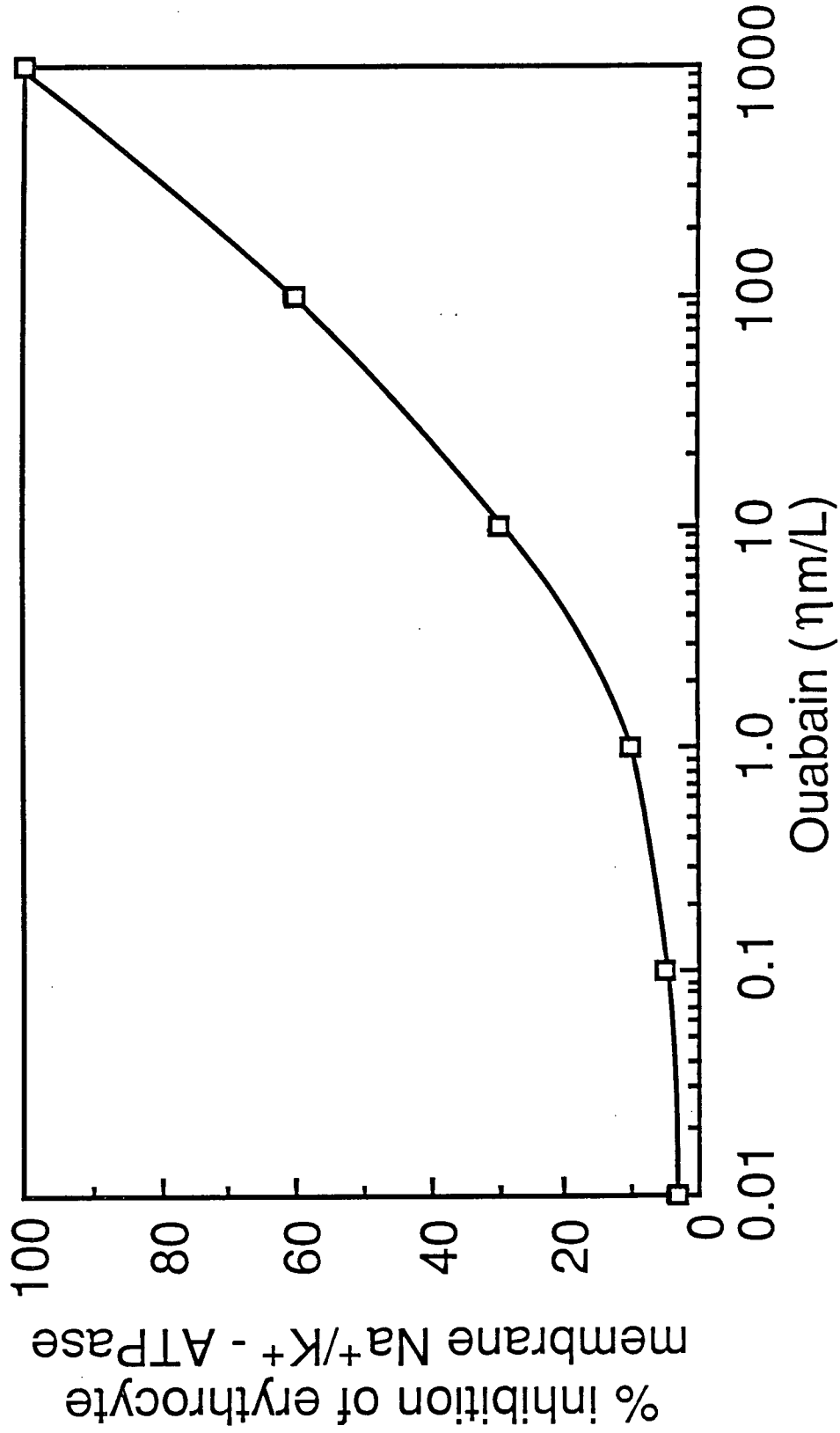

% inhibition of erythrocyte membrane Na+/K+ - ATPase
0
20
40
60
80
100
0.01
0.1
1.0
10
100
1000
Digoxin (n M/L)

FIGURE 18

The effect of increasing concentrations of ouabain on the Na^+/K^+-ATPase activity of isolated erythrocyte membranes.


% inhibition of erythrocyte membrane Na+/K+ - ATPase
100
80
60
40
20
0
0.01
0.1
1.0
10
100
1000
Ouabain (ηm/L)

(2.0 mM) and eight immunoreactive steroids (0.1 mM) on the four assay systems studied (radioimmunoassay for digoxin ^{86}Rb uptake by erythrocytes, canine kidney Na^+/K^+-ATPase and erythrocyte membrane Na^+/K^+-ATPase assays) is shown in Table V. There is no significant correlation between these systems. Progesterone, the most immunoreactive to digoxin antisera DB-157, is a strong inhibitor of canine kidney Na^+/K^+-ATPase but has only a mild effect on the erythrocyte enzyme. Δ^4-Androstenedione affects the red cells, but has less than half the cross-reactivity and only minimally inhibits canine kidney ATPase activity.

These two steroids, progesterone and Δ^4-androstenedione, were examined for their ability to inhibit the Na^+/K^+-ATPase activity of isolated erythrocyte membranes. Figure 19 demonstrates the effect of varying concentrations of these steroids. At 0.1 mmol/L progesterone and Δ^4-androstenedione can inhibit the enzyme activity by 10 and 12%, respectively. Δ^4-Androstenedione has a similar effect on canine kidney Na^+/K^+-ATPase activity at this concentration, but progesterone is four times as active (Figure 15). At 0.2 mmol/L steroid the inhibitory activity of Δ^4-androstenedione remains the same, whereas that of progesterone

TABLE V

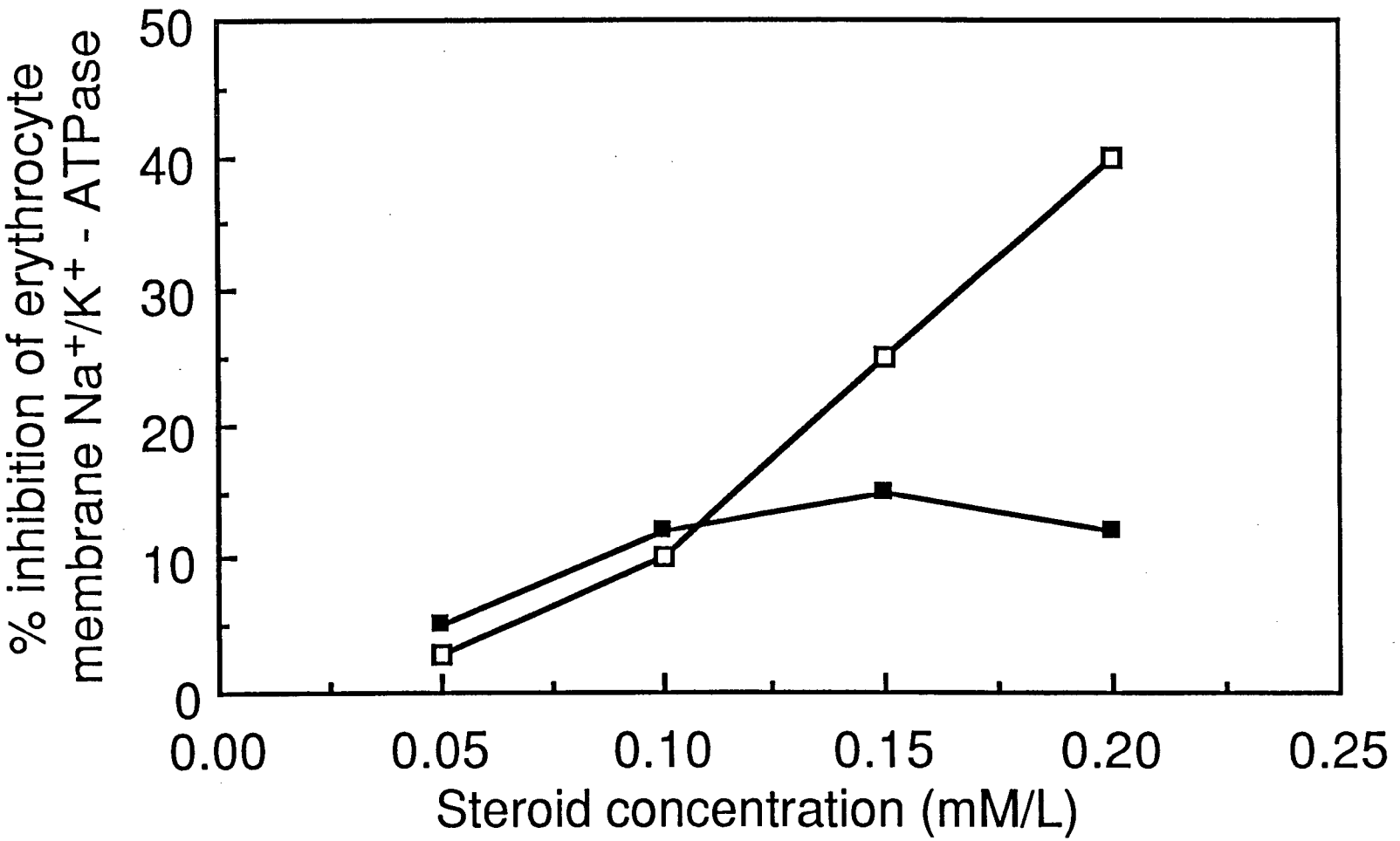
COMPARISON OF DIGOXIN IMMUNOREACTIVITY AND Na^+/K^+-ATPASE INHIBITORY ACTIVITY AS MEASURED BY THREE DIFFERENT METHODS FOR SEVERAL STEROIDS AND GLYCOCHENODEOXYCHOLIC ACID

Steroid or Bile Acid*	Cross Reactivity µg/L/Digoxin Equivalents	% Inhibition ^{86}Rb Uptake	% Inhibition Canine Kidney ATPase	% Inhibition Erythrocyte Membrane ATPase
Progesterone	4.0	3	40	10
Deoxycorticosterone	3.2	14	30	10
21-Deoxycortisone	2.5	4	0	0
17 Hydroxyprogesterone	1.8	5	5	0
Δ4-Androstenedione	1.6	28	10	12
Cortisone	1.0	4	2	5
Cortisol	0.7	7	7	0
Dehydroepiandrosterone	0.4	7	20	8
Glycochenodeoxycholic Acid	1.9	5	20	12

*All steroids were at 0.1 mM and glycochenodeoxycholic acid at 2.0 mM concentration.

FIGURE 19

The effect of varying concentrations of progesterone (□ - □) and Δ^4-androstenedione (■ - ■), on the Na^+/K^+-ATPase activity of isolated erythrocyte membranes.


% inhibition of erythrocyte membrane Na^+/K^+ - ATPase
50
40
30
20
10
0
0.00
0.05
0.10
0.15
0.20
0.25
Steroid concentration (mM/L)

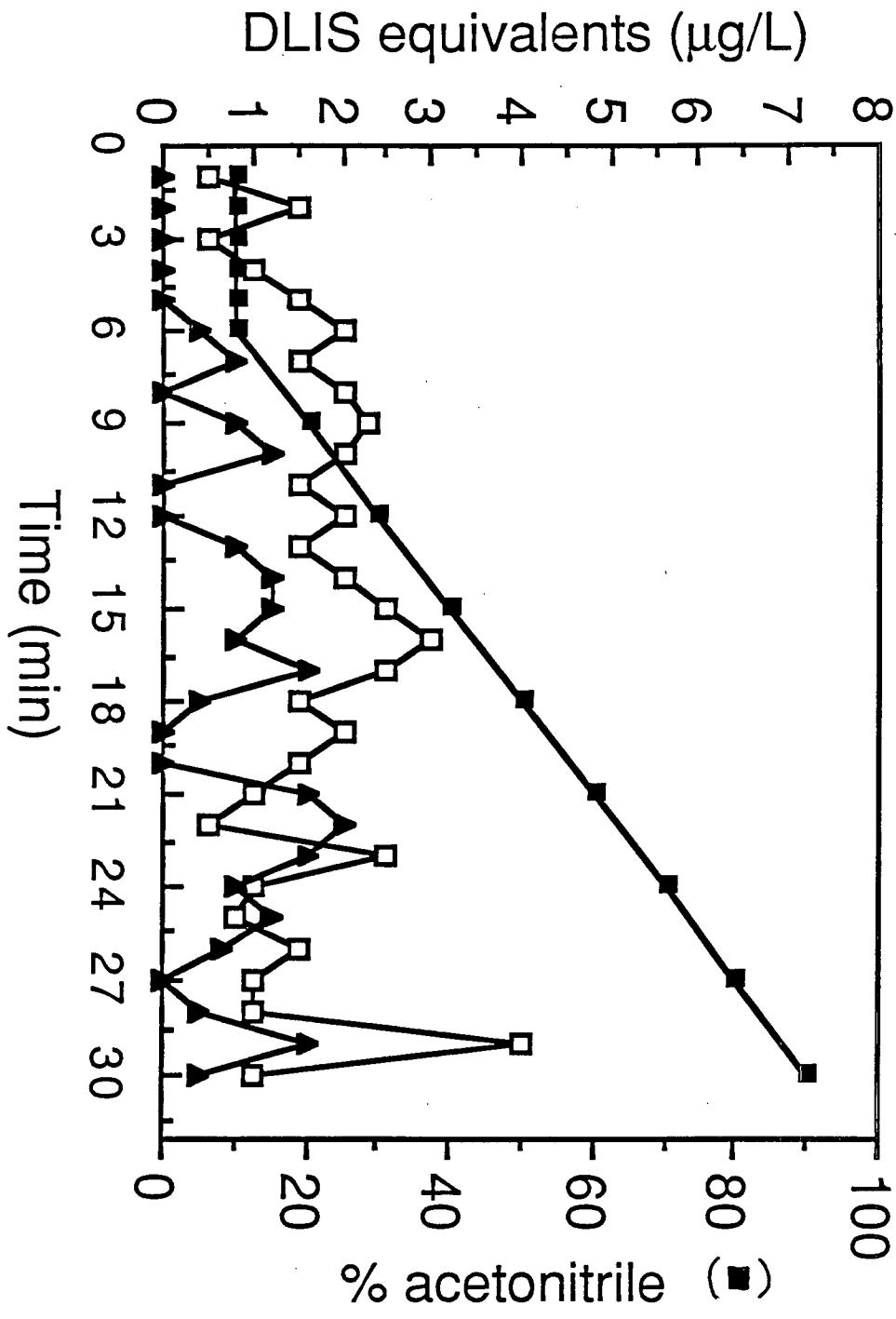
quadruples and inhibits the activity of erythrocyte membrane Na^+/K^+-ATPase by 40%. These results do not correlate with the inhibitory activity expressed by progesterone and Δ^4-androstenedione on erythrocyte uptake of rubidium (Figure 8). In that case, these steroids inhibited the activity by 5 and 30% respectively (n=2).

D-3b The Effect of HPLC-Fractionated Cord Serum on Erythrocyte Membrane Na^+/K^+-ATPase Activity

Cord serum (500 mL) was processed as previously described and fractionated by reverse phase HPLC. Figure 20 compares the digoxin-like immunoreactivity expressed by each of the eluted fractions, with their inhibition of isolated erythrocyte membrane Na^+/K^+-ATPase activity. Fifty fractions were collected over thirty minutes (Materials and Methods, D-3e), dried, then reconstituted in assay buffer. There are a number of peaks which show mild inhibition of erythrocyte membrane Na^+/K^+-ATPase activity. Fractions that elute at 7, 10, 14, 17, 22, 25 and 29 minutes may correspond to the canine kidney-ATPase inhibiting fractions which elute at 7, 12, 16, 25 and 28 minutes (Figure 16). There is, however, no direct correlation with digoxin-like immunoreactivity, except in the peak which elute at 29

FIGURE 20

HPLC elution profile of the erythrocyte membrane Na^+/K^+-ATPase inhibitory activity (▲-▲) and the digoxin immunoreactivity (□-□) of cord blood.



% inhibition of erythrocyte membrane Na^+/K^+ - ATPase

minutes from which progesterone has been recovered. Although cord serum contains a number of digoxin-like immunoreactive substances, not all of them show inhibition of Na^+/K^+-ATPase, and there is no proof that the enzyme inhibition demonstrated in the laboratory is present in vivo.

XI. DISCUSSION

It has always been difficult to monitor patients on digoxin therapy, without the additional problem caused by endogenous materials that interfere with present methodologies for its analysis. Digoxin immunoreactivity has been found in the urine and plasma of individuals who had not taken the drug[5,19]. Patients with renal or hepatic failure[1,22,37], hypertension[24,25], and pre-eclampsia[23] had levels of DLIS twice those seen in the normal population[42,80]. Even these values are only half of those found in the serum of premature infants[13,16,18,21], and suggest that this would be an interesting population to study.

A. DIGOXIN RADIOIMMUNOASSAY

Assays for serum digoxin were developed to give a low degree of cross-reactivity with substances of a similar structure[69]. However, it is evident from the present study that digoxin radioimmunoassays can be affected by many steroids and bile acids if they are present in high enough quantities. Progesterone, deoxycorticosterone, 21-deoxycortisone (0.1 mmol/L) and glycochenodeoxycholic acid (2.0 mmol/L) cross-react with digoxin antisera DB-157 at levels which exceed the therapeutic range (0.8-2.0 µg/L) for the drug. The degree of this cross-reactivity is not only influenced by the antisera, but also by the assay conditions used. Increasing the temperature and incubation

time[82,83] can significantly reduce the immunoreactivity of digoxin-like substances in these assays. This observation suggests that the material in question has a low affinity for the digoxin antibody. It has been postulated that DLIS is an endogenous steroid with digitalis-like properties[69]. Although considerable evidence exists to support this notion[37], the exact chemical identity of DLIS remains to be elucidated.

The increased levels of digoxin-like substances in some clinical states (such as hypertension, pre-eclampsia, liver and kidney disease) suggests that there is either an increased production and/or a decreased clearance of this material in these states. A portion of DLIS is protein bound[42]. It is possible that the foregoing clinical conditions are accompanied by a decrease in the relative amount of DLIS-binding proteins. This may increase that fraction of DLIS that is circulating in a free form, thereby increasing the degree of DLIS which cross-reacts in the radio-immunoassay[84]. Serum that has been boiled prior to analysis[19,23,42,80,81] has shown increased DLIS levels. It was suggested that this procedure releases DLIS from its binding protein. It must be remembered, however, that this procedure also removes albumin and causes the release of non-esterified fatty acids[80] which are known to cross-react with digoxin-specific antisera[80]. In addition, boiling removes significant amounts of protein from the sample. If this protein matrix is not restored

prior to the assay for digoxin, levels of DLIS will be artifactually increased[85]. When I took these conditions of pre-boiling into account, there were no appreciable improvement in recoveries of DLIS from mixed cord blood. It is possible that the DLIS of cord serum may be different from that found in adult disease states, and as such have altered protein binding affinities which demonstrate a different response to this treatment.

Different commercial RIA systems used for serum digoxin estimation have shown variations in their immunoreactivity to DLIS depending on the source and specificity of antisera used[17]. Antisera DB-157 obtained from Nuclear Medical Laboratories demonstrated the highest levels of cross-reactivity with cord serum[17]. Experiments carried out in other laboratories did not use this system, which may account for some of the discrepancies in my findings. Many researchers have speculated on the nature of DLIS. Diamandis *et al*[43] suggested that cortisone, present in umbilical vein plasma, may account for the digoxin immunoreactivity seen in cord blood. This was not, however, a major cross-reacting steroid in the radioimmunoassay used here. Progesterone and deoxycorticosterone, also present in cord serum, showed the highest levels of immunoreactivity to antisera DB-157. There are other substances also known to interfere in digoxin radioimmunoassays[86]; lipids and fatty acids (such as oleic and linoleic acid) can form micelles which sequester labelled digoxin and cause false positive assay results.

B. ISOLATION OF DLIS

A number of different procedures for the extraction of DLIS from serum were examined. Dichloromethane was used in early methods of digoxin analysis[12] to eliminate the interference by endogenous materials. Organic solvents such as ethanol and methanol have been used to recover DLIS from a number of biological sources[87,88,89]. More than 85% of DLIS was recovered from serum with either of these solvents, whereas only 45% of digoxin was recovered from the serum of patients on drug therapy. When normal serum was spiked with digoxin, the recovery (80%) was much higher. This discrepancy in digoxin recovery may have something to do with the drug metabolism. Digoxin is absorbed by the small intestine where 25% is bound to albumin[30] and tissue proteins. Drug elimination from the body (37% daily) is mainly through renal excretion. Some of the drug is recycled in the enterohepatic circulation. The major route of digoxin metabolism involves a reduction of the lactone ring to form dihydrodigoxin, sequential stepwise removal of sugar molecules and epimerization to form polar metabolites[5] with various degrees of biological activity and protein binding capacity. There is considerable inter-individual differences in the biotransformation of digoxin, digoxigenin, digoxigenin-bisdigitoxide, monodigitoxide, and dihydrodigoxigenin may account for over 20% of the total concentration[5,34] in some patients. The more polar metabolites have an altered response to organic solvent treatment.

Immunoadsorbent columns have been used to isolate many substances

from serum. Affinity column chromatography could efficiently isolate digoxin from serum with recoveries of approximately 80%. Yet, when this same affinity column is used to isolate DLIS, less than 20% of the immunoreactivity is recovered. This observation is consistent with results obtained in the radioimmunoassay for DLIS which indicated that although this material cross-reacts with digoxin-specific antibodies, it does so with a very low affinity. It is evident from these data that very large amounts of coupled antisera would be required to isolate significant amounts of DLIS. Affinity column chromatographic isolation of DLIS was therefore abandoned.

The use of C_{18} Sep-Pak cartridges was the most effective method for DLIS isolation from large volumes (500 mL) of serum. More than 75% of DLIS was recovered in the methanol extract and could then be concentrated one hundred-fold by evaporation. Resolubilization of the methanol extract with either 50 mM Tris buffer or phosphate-buffered-saline (pH 7.4) could be carried out with equal efficiency. Prior to the analysis of DLIS recovery, however, digoxin-free serum must be added to the sample to eliminate non-specific interference which occurs in the absence of a protein matrix[85]. When human serum albumin (5%) is used as the protein matrix for the assay, it cross-reacts with digoxin antisera and gives rise to digoxin-like interference. In keeping with the findings of others, I therefore elected to use a Tris buffer in our Na^+/K^+-ATPase assays. This

buffer was a satisfactory solvent for DLIS extracts and suitable for the assay of digoxin-like immunoreactivity.

C. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Cord blood extracts were fractionated by HPLC and the eluate monitored at 210 nm. These extracts contained many different substances with significant absorbance at this wavelength. The examination of individual fractions for digoxin- like immunoreactivity demonstrated at least six areas of notable digoxin immunoreactivity. These fractions eluted at 3, 6, 14, 17, 22 and 29 minutes. Many of the steroids and bile acids that we studied also elute in this area. Progesterone was recovered from the fraction which eluted at 29 minutes. The concentration of progesterone in this peak was determined by a specific radioimmunoassay. When this concentration was added to serum, 72% of the digoxin-like immunoreactivity which eluted at 29 minutes could be accounted for on the basis of the progesterone content of this fraction. The nature of the remaining DLIS material is unknown. Complete solubilization of progesterone is often difficult and requires absolute alcohol. The dilution of a concentrated standard of the steroid prior to analysis by radioimmunoassay is necessary to eliminate the effects alcohol has on the assay, but dilutions of DLIS when measured by RIA do not follow a linear curve. The evaluation of digoxin-like cross-reactivity exhibited by high

concentrations of pure progesterone may therefore not be accurate. La Bella et al[37,90] have presented some data which suggest that the most likely candidate for endogenous DLIS is a derivative of progesterone. They have postulated that 7-chloro-6-dehydro-17-alpha-acetoxyprogesterone is one such compound. This material has been shown to possess weak digoxin immunoreactivity.

D. NATRIURESIS

The first methods used to study the function of DLIS involved the evaluation of its natriuretic properties[3,4,27,28,40,50]. DLIS isolated from one source was injected into a test animal, the resulting degree of natriuresis was then measured. This technique is laborious and does not differentiate a response due to DLIS from one caused by steroids, dopamine, prostaglandins or the atrial natriuretic peptide produced by myocytes[84].

E. SODIUM/POTASSIUM-ADENOSINE TRIPHOSPHATASE INHIBITION ASSAYS

An accepted approach for studying DLIS is to measure its effect on Na^+/K^+-ATPase activity. This enzyme regulates cation transport across the cell membrane. It can be studied by measuring either sodium (^{22}Na) efflux or potassium (^{86}Rb) influx[26,84,91] across the cell membrane. This function is dependent on the intracellular sodium content and can thus be affected by the presence of insulin, thyroxin and mineralcorticoids[84] which alter cation concentrations.

E-1 Rubidium Uptake by Erythrocytes

Erythrocyte uptake of ^{86}Rb is inhibited by digoxin, ouabain, Δ^4-androstenedione, deoxycorticosterone, 21-deoxycortisone, cortisol and is sensitive to the detergent effects of bile acids[85]. All the bile acids studied in the present work caused hemolysis. This significantly interfered with the estimation of ^{86}Rb uptake. Hemolysis and inhibition occurred simultaneously at concentrations of glycochenodeoxycholic acid above 0.2 mmol/L. The levels of bile acids normally found in serum are 0.79 ± 0.82 μM/L[92]. When samples were diluted to eliminate hemolysis, there was no inhibition of ^{86}Rb uptake.

None of the DLIS-positive HPLC fractions isolated from cord serum (500 mL) inhibited ^{86}Rb uptake by erythrocytes. My data conflict with those reported by a number of different centers[4,39,43,44,93]. This discrepancy may be due to the source of sample studied. We looked at DLIS derived from cord serum which may not be identical to that derived from hypertensive serum, urine, CSF or the hypothalamus. The sample preparation may also be quite important. My experimental data showed that either the presence of ethanol at 0.5%, or potassium (0.9 mmol/L) inhibited ^{86}Rb uptake by 10%. We resolubilized our extracts in a buffer (0.05 M Tris pH 7.4) containing neither of these substances. Normal serum

contains 4.0 ± 0.25 Meq/L potassium. This ion should be removed by extraction procedures prior to Na^+/K^+-ATPase analysis in order to eliminate the influence of K^+ on this assay. Ethanol was frequently used in many of these earlier studies to ensure complete solubilization of DLIS. As stated earlier alcohol interferes in the ^{86}Rb uptake assay. In order to eliminate this non-specific effect, samples which contain ethanol must be diluted so that they contain less than 0.1% alcohol. Dilution curves for DLIS as measured by digoxin immunoassay are not linear[43]. This procedure may also eliminate any inhibitory effects the extract may have expressed. Diamandis and Soldin et al[43] also characterized DLIS from cord serum with a ^{86}Rb uptake assay. They concentrated 25 mL cord serum with a C_{18} Sep-Pak cartridge to 0.25 mL methanol extract prior to injection on an HPLC column. A step-wise gradient of methanol/water[43] was employed in contrast to the linear water/acetonitrile gradient I used. Eluted fractions were pooled into five groups, evaporated, then redissolved in 1.0 mL potassium-free Ringer's buffer. DLIS was recovered from two of the groups, but only their last pool consisting of fractions 33 to 37 expressed significant inhibition of ^{86}Rb uptake by erythrocytes. Their results most likely differ from ours because of the concentration of extract studied. Although we concentrated 500 mL cord serum to 4 mL methanol extract, only 2 mL of this was applied to the

HPLC column. Each eluted fraction was then separately redissolved in 2.0 mL of the assay buffer and 1.0 mL of this, examined for its effect on ^{86}Rb uptake. Diamandis' group[43] combined the material present in five fractions into one-half this volume (1.0 mL), to show 55% inhibition of ^{86}Rb uptake. Their use of a step gradient is also significant because it combines the effects of a number of substances such as bile acids, into one fraction.

There is a receptor for glycosides at the membrane Na /K -ATPase site. Other compounds may either bind to this receptor, or allosterically alter its affinity for the drug[84]. All unsaturated fatty acids and monoglycerides (mono-11-eicosenoin) cross-react with digoxin antibodies, displace membrane-bound ^{3}H-ouabain and inhibit rubidium uptake by erythrocytes[86]. There are various compounds that are immunoreactive to digoxin antibodies and able to influence potassium influx (^{86}Rb uptake) across the cell membrane: steroids, bile acids, fatty acids and lipids. Because of the non-specificity of this assay, it is not possible to prove that DLIS isolated from concentrated cord serum functions as an endogenous cardiac glycoside.

E-2 Canine-Kidney Na^+/K^+-ATPase Assay

The most widely used assay for Na^+/K^+-ATPase activity

measures the hydrolysis of ATP[84]. It is believed that an intact cell membrane can protect the Na^+/K^+-ATPase enzyme[84] from non-receptor-mediated interference by compounds such as steroids, bile acids, ascorbic acid or DLIS. Therefore, the canine kidney enzyme should be more susceptible to steroid inhibition than the intact erythrocyte. My data supports this: Δ^4-androstenedione and deoxycorticosterone (0.20 mmol/L) caused approximately 40% more inhibition of the canine kidney Na^+/K^+-ATPase than the ^{86}Rb uptake assay. Progesterone, interestingly enough, caused 45% inhibition of the canine kidney enzyme whereas it had negligible effects on ^{86}Rb uptake (5% inhibition). The red cell membrane-bound enzyme may not have an affinity for this steroid or it may protect the enzyme from any direct interference from it. When the isolated erythrocyte membrane Na^+/K^+-ATPase was examined, progesterone inhibited enzyme activity by over 40% (0.2 mmol/L). The erythrocyte ^{86}Rb uptake assay was more responsive to the presence of receptor-specific ouabain and digoxin than was the canine kidney enzyme. The canine kidney Na^+/K^+-ATPase assay is sensitive to 10^{-7}M glycoside while ^{86}Rb uptake by erythrocyte is inhibited by 10^{-9}M concentrations. Perhaps the human red cell is more sensitive to low concentrations of cardiac glycosides than the canine kidney cell enzyme because of the nature of its surrounding

lipid[63].

Cord Serum (500 mL) extracted with C_{18} Sep-Paks and then fractionated by HPLC demonstrated a number of peaks which inhibited the activity of canine kidney Na^+/K^+-ATPase by more than 20% (fractions eluting at 3, 7, 12, 16, 25 and 29 minutes). Two of these fractions (3 and 29 minutes) also demonstrated immunoreactivity to digoxin antisera DB-157. Progesterone has been recovered from the peak eluting at 29 minutes (see earlier data). Many steroids which have the ability to inhibit canine kidney Na^+/K^+-ATPase activity elute from the HPLC column with retention times similar to some of these other peaks. Dehydroepiandrosterone-sulfate (15.5 minutes) contains little digoxin immunoreactivity (0.4 µg/L), but inhibits canine kidney Na^+/K^+-ATPase activity[45]. 17-Alpha-hydroxyprogesterone has a retention time of 25.5 minutes, demonstrates 1.8 µg/L digoxin equivalent activity at 0.1 mM/L concentration, but only inhibits canine kidney Na^+/K^+-ATPase activity by 5%. It has been suggested, however, that a derivative of this steroid 14-beta-hydroxyprogesterone is many times more potent[90] as an inhibitor of enzyme activity.

E-3 Erythrocyte Membrane Na^+/K^+-ATPase

The erythrocyte membrane Na^+/K^+-ATPase assay was more

sensitive to cardiac glycoside concentrations (10^{-9}M) than the canine kidney enzyme (10^{-7}M) assay. In order to make the appropriate measurements, however, it was necessary to use five times the concentration of membrane-bound enzyme protein (3 mg/mL versus 0.6 mg/mL purified kidney Na^+/K^+-ATPase) and measure the product of ATP hydrolysis after 90 minutes. The canine kidney enzyme expressed a linear reaction curve in 15 minutes. The glycoside-insensitive ATP hydrolysis as measured in the absence of sodium and potassium and presence of either digoxin or ouabain, for both the canine kidney and erythrocyte-derived ATPase enzymes was less than 10% of the total, a finding that is not inconsistent with the findings of others[(94)].

After review of the effects of eight steroids on the four assay systems studied (digoxin-like immunoreactivity, Digi-tab RIA, ^{86}Rb uptake by erythrocytes, canine kidney and erythrocyte membrane Na^+/K^+-ATPase assays (Table V)), one could see little correlation between cross-reactivity with antisera DB-157 and the degree of enzyme inhibition. Progesterone, the most immunoreactive steroid studied, strongly inhibited canine kidney Na^+/K^+-ATPase activity but did not significantly affect the red cell in either the ^{86}Rb uptake or erythrocyte-derived membrane assay. At 0.1 mmol/L concentration its inhibition of erythrocyte membrane Na^+/K^+-ATPase (10%) is

similar to that of Δ^4-androstenedione (12%), but this activity quadruples at double the concentration (0.2 mmol/L). The same concentration of progesterone inhibits ^{86}Rb uptake by 5%. The erythrocyte may protect Na^+/K^+-ATPase activity up to a point at which the concentration of this steroid overcomes any protective effect expressed by the isolated membrane. Δ^4-Androstenedione with the fifth highest level of cross-reactivity, shows the fourth highest inhibition of canine kidney Na^+/K^+-ATPase activity of the eight steroids studied. It has, however, the greatest effect on the intact red cell giving 28% inhibition of ^{86}Rb-uptake. The lipophilic characteristics of Δ^4-androstenedione may attract it to the lipid-rich component of erythrocytes[95] bringing the steroid into contact with glycoside-specific sites on the membrane.

Cord serum (500 mL) extracted on C_{18} Sep-Paks and fractionated by HPLC showed a number of peaks (Figure 20) which cross-reacted with digoxin antisera DB-157 (retention times of 4, 9, 16, 19, 23 and 29 minutes). These may correspond to the peaks eluted at 3, 6, 14, 17, 22 and 29 minutes shown in Figures 3, 12 and 16 of a previous experiment. Erythrocyte membrane-derived Na^+/K^+-ATPase activity was inhibited (more than 10%) by substances in the fractions eluted at 10, 14, 17, 22, 25 and 29 minutes. Only the last peak which eluted at 29 minutes

exhibited both enzyme inhibition and immunoreactivity. This peak likely contains progesterone as shown in an earlier experiment. Canine kidney Na^+/K^+-ATPase was inhibited more than 20% by material recovered in the fractions eluted at 3, 7, 12, 16, 25 and 29 minutes (Figure 16). Some of these peaks may correlate with those seen in Figure 20, eluted at 14, 17, 25 and 29 minutes, but their effect on the erythrocyte membrane-derived enzyme is less. DLIS demonstrates a low affinity for digoxin antibodies and may also have a lower affinity for human erythrocyte membrane-bound Na^+/K^+-ATPase than for the purified enzyme used in the canine kidney assay.

Dehydroepiandrosterone-sulfate (retention time 15.5 minutes) a component of cord serum, can both displace ^{3}H-ouabain and inhibit canine kidney Na^+/K^+-ATPase[45,96], but only mildly cross-reacts with digoxin antisera and does not affect the erythrocyte membrane assay. Derivatives of progesterone also displace ^{3}H-ouabain and inhibit Na^+/K^+-ATPase activity[37,90]. Alpha acetylation and modification of the beta ring of chlormadinone acetate[37] creates a synthetic derivative of hydroxyprogesterone (7-chloro-6-dehydro-17-alpha-acetoxyprogesterone) which inhibits ^{86}Rb uptake, displaces ^{3}H-ouabain, inhibits Na^+/K^+-ATPase activity and is weakly immunoreactive to digoxin antibodies[37]. A natural

counterpart of this compound has not been identified.

In view of the nature of cardiac glycosides, it is reasonable to expect that the endogenous form (DLIS) of these drugs may also be a steroid. Many researchers have reported on the presence of an endogenous sodium pump inhibitor in biological fluids[94,97] with immunoreactive glycoside-like properties. Others have demonstrated inhibition without immunoreactivity. There is evidence that DLIS increases in response to fluid or salt loading[98] and could play a major role in disease states characterized by fluid retention[4,37,56,84,99]. The premature infant exists in a state of negative sodium balance due to a high rate of sodium excretion[63,100]. Does this explain the presence of DLIS in this population, or has it another role? Nifedipine, a calcium antagonist that is used as an antihypertensive agent[101], has been shown not only to lower the blood pressure in certain hypertensive patients but also to decrease the amount of detectable DLIS in these patients.

The dose of digoxin prescribed to children with cardiac insufficiencies[67,102,103] is 3 to 5 times that of the adult patient to maintain the same serum levels. The infant appears to tolerate these higher levels, but symptoms of toxicity in this age group differ from those of the adult[104] so this

may not be true. A higher dose does not appear to clinically benefit the child and tends to accumulate in the tissues. The heart to serum ratio for digoxin seen in adult patients (28:1), has been measured at 149:1 in children on the drug[102]. This tissue-bound digoxin is released into the blood post-mortem[104]. Although the normal heart can tolerate levels of digoxin ten times the therapeutic range[104,105], in the compromised individual, values one-quarter of this can be dangerous. It has, therefore, become very important to be able to accurately measure serum digoxin levels. DLIS, when present in serum, has an additive effect on the levels of drug measured by radioimmunoassay thereby giving an erroneously high estimate of plasma drug levels. Whether or not there is also an additional biological effect in infants has not been proven.

XII. CONCLUSIONS

High levels of digoxin-like immunoreactive substances have been detected in serum from premature infants. DLIS is also present in cord serum. The isolation of this material from large volumes of cord serum revealed a number of fractions that cross-reacted with antisera to digoxin.

Digoxin is a cardiac glycoside that inhibits Na^+/K^+-ATPase activity. HPLC-fractionated cord serum was examined for its ability to inhibit Na^+/K^+-ATPase activity as measured by three different assay systems: ^{86}Rb uptake by erythrocytes, canine kidney Na^+/K^+-ATPase, and erythrocyte membrane-derived Na^+/K^+-ATPase assays. Of the six major fractions which cross-reacted with digoxin antisera, two were found to inhibit canine kidney Na^+/K^+-ATPase activity and one of these inhibited both the canine kidney and erythrocyte membrane enzyme. This latter fraction was shown to contain progesterone. There were four non immunoreactive fractions that inhibited Na^+/K^+-ATPase activity, but none of the HPLC-eluted cord blood fractions inhibited ^{86}Rb uptake.

There are a number of immunoreactive steroids present in cord serum which, if present in significantly high concentrations, could also inhibit Na^+/K^+-ATPase activity: progesterone, deoxycorticosterone, 21-deoxycortisone, 17 alpha-hydroxyprogesterone, Δ^4androstenedione and dehydroepiandrosterone-sulfate. Some of the inhibitory activity recovered from cord serum may well be attributable to the presence of

such steroids or other substances (such as bile acids and fatty acids). A number of these substances could be present in any one fraction and act synergistically to inhibit Na^+/K^+-ATPase activity. It is important to realize that in order to measure the Na^+/K^+-ATPase inhibitory activity of these fractions, large volumes of cord serum had to be processed. Given the low levels of inhibition measured in this study, it is likely that DLIS has limited physiological significance as a modulator of the Na^+ pump activity.

Although the greatest limitation to this study was the non-specificity of laboratory assay procedures available, there are five conclusions that can be drawn. The expression of DLIS is influenced by the source of antisera used to measure it. Cord blood contains many digoxin-like immunoreactive substances but large volumes of serum must be concentrated five hundred fold in order for them to effectively inhibit Na^+/K^+-ATPase activity. There is no obvious correlation between cross-reactivity with digoxin antisera DB-157 and the enzyme inhibitory characteristics. The purification procedure was based on polarity; therefore, each of the eluted HPLC fractions isolated may have contained a number of different substances that either acted together or in opposition to each other. It appears that cord blood DLIS does not possess Na^+/K^+-ATPase inhibitory activity of clinical significance because of the low concentrations found <u>in vivo</u>.

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