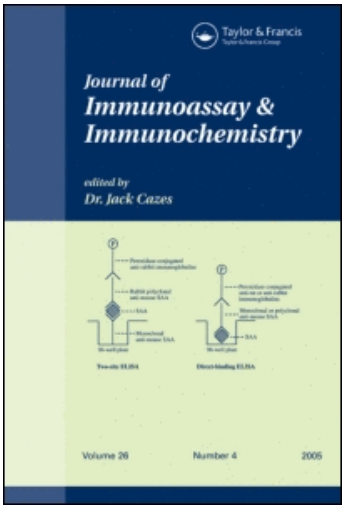

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Bufalin Radioimmunoassays: In Search of the Endogenous Digitalis-Like Substance

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BUFALIN RADIOIMMUNOASSAYS: IN SEARCH OF THE ENDOGENOUS DIGITALIS-LIKE SUBSTANCE

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ABSTRACT

The existence of a mammalian natriuretic substance similar to plant digitalis, which inhibits Na,K-ATPase, has been speculated about, but as yet no definite substance has been found. Digoxin-like immunoreactive substance (DLIS) has been reported in various clinical states including new born infants.

Using bufalin (a cardioactive substance of animal origin) as antigen, four polyclonal antisera have been produced from 2 separate rabbits and characterised for cross-reactivity with 32 compounds. One antiserum showed a marked change in its cross-reactivity after resting the animal for a year. Of the endogenous substances tested, progesterone was found to be the most cross-reactive. Radioimmunoassay of foetal cord sera with different antisera, gave different levels of bufalin-like immunoactivity. However, after a novel "affinity-immunoassay" procedure, this apparent bufalin-like immunoactivity disappeared.

It is concluded that bufalin-like immunoactivity in the cord blood is caused by the cross-reaction of endogenous steroids with bufalin antiserum, and the same may be true for DLIS.

KEYWORDS: Na, K-ATPase Inhibitor, Digoxin-like Immunoreactive Substance, Bufalin, Radioimmunoassay.

INTRODUCTION

Since de Wardener first demonstrated that natriuresis which follows volume expansion is induced in part by a circulating factor (1), several substances with natriuretic properties have been described (2). Based on the premise that natriuresis occurs as a result of inhibition of the Na, K-ATPase, this endogenous substance is thought to be related to digitalis, although digoxin given to normal subjects failed to cause natriuresis (3), but had such an effect when infused at high concentrations into the renal artery (4). A digoxin-like immunoreactive substance (DLIS) has been described in situations such as renal failure (5), liver failure (6), pregnancy (7) and newborn infants (8,9).

So far endogenous digitalis-like factor has not been identified although substances such as dehydroepiandrosterone sulphate, progesterone and fatty acids have been shown to cross-react with digoxin antibodies (10, 11, 12, 13). More recently Hamlyn's group (14, 15, 16, 17) claim to have identified ouabain, as the endogenous digitalis-like substance. Ouabain, a plant glycoside possesses a cis junction in the steroidal ring (CD cis), which is not found in the known endogenous steroid molecules in humans (18).

Unlike digitalis compounds which are of plant origin, bufadienolides are cardioactive substances of amphibian origin and have been shown to inhibit Na, K-ATPase, K^+ transport, and interfere in radioreceptor assay (19).

Bufalin is a cardiotonic steroid constituent of Ch'an Su, a galenical preparation of the dried venom of Chinese toad Bufo bufo gargarizans and is commercially available. Bufalin blocks potassium vasodilation, potentiates noradrenaline vasoconstriction and increases vascular resistence when administered locally, through inhibition of Na, K-ATPase (20). Bufalin has now been shown to be a more potent inhibitor of Na, K-ATPase than ouabain (21). Tal et al (22) reported a ouabain-like activity in bovine plasma which had similar retention time to a toad skin ouabain-like compound. Bufalin because of its animal origin, is a possible candidate for the role of an endogenous digitalis-like substance.

In this article, I describe the development of a radioimmunoassay (RIA) for bufalin, the characterisation of two polyclonal antisera after primary and secondary immunisation, and the use of them in RIAs for the assay of foetal cord blood before and after a novel "affinity immunoassay" procedure. The use of these two assays for the detection of bufalin in traditional Chinese medicine has been described elsewhere (23).

MATERIALS AND METHODS

Bufalin-3-adipate for conjugation to bovine serum albumin (BSA) was a gift from Prof K Shimada, Kanazawa University, Japan. All chemicals and reagents were purchased either from Sigma Chemical Company, St. Louis, USA or E. Merck, Darmstadt, Germany. Radioactive [^{3}H]-bufalin (specific

activity 69.5 Ci/mmol) was custom labelled by Amersham International PLC, Amersham, England.

Blood Specimens

Cord blood samples sent for neonatal hypothyroidism screening were collected into plain tubes and the sera separated and stored at -20°C. Some of the cord sera were pooled for the "affinity immunoassay".

Bovine blood was obtained from a local abattoir.

Development of Bufalin Radioimmunoassay

i) Conjugation of bufalin-3-adipate to bovine serum albumin (BSA)

Bufalin-3-adipate was conjugated to bovine serum albumin according to the method of Erlanger et al (24) using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide. Due to the low yield it was not possible to determine the conjugation ratio of bufalin molecules to BSA.

ii) Immunisation

For the initial (primary) immunisation 100 µg of the conjugate was dissolved in 0.5 ml physiological saline, emulsified with 0.5 ml Freund's Complete Adjuvant and injected subcutaneously at five

different sites on the back of two New Zealand white rabbits (4-5 months old). For the second booster injection at 4 weeks, the antigen (100 μg) was emulsified with Freund's Incomplete Adjuvant and for the final injection, the antigen (100 μg) was injected dissolved in physiological saline.

Blood sample was taken on the third day after the final injection and compared with pre-immunisation serum for the presence of antibodies to bufalin. More blood was withdrawn from the ear vein on the 8th day after the final injection for the development of the immunoassay. Two animals were immunised and although both showed a similar antibody titre on day 3 (approximately 1/4000 at 50% binding of the tracer) animal 2 had an increased titre by day 8 (1/6400). The primary antisera from the two animals are referred to as B1 & B2, respectively.

Both animals were rested for almost a year before being re-immunised (secondary immunisation) with a single injection of bufalin conjugate. Blood was collected ten days later. The antibody titre on this occasion had risen to 1/12800 at 50% binding of the tracer for both antisera, and the antisera are referred to as B1.Y1 and B2.Y1, respectively.

iii) Radioimmunoassay

The RIA standard curve ranged from 0 - 200 pg (0 to 520 fmoles) of bufalin per tube. The antiserum dilutions chosen were 1/3200 for B1 and 1/5000 for B2, which gave binding of [^{3}H] bufalin ranging from 60% - 20% between the zero and the highest standard. The secondary antisera (B1.Y1 & B2.Y1) were used at dilutions of 1/12800 with the binding ranging from 75% to 25%. In order to standardise the matrix effect of solvent residues, all tubes were appropriately compensated with either ethanol (used for standards) or diethyl ether (used as an extractant).

The RIA procedure involved addition of bufalin standards in duplicate to borosilicate glass tubes (10 x 75 mm) dissolved in 0.1 ml of ethanol followed by the addition of 1 ml diethyl ether to each tube. Samples (0.5 ml of serum) were extracted with 5 ml diethyl ether, vortexing for 3 minutes using SMI multivortex (Emeryville, CA). Four 1 ml aliquots were removed for assay in duplicate by the two antisera. 0.1 ml of ethanol was added to all extracts. The organic solvents were evaporated under reduced pressure in a vacuum oven at 40°C. 0.1 ml of [^{3}H] bufalin (20000 dpm ≡ 50 pg bufalin) in 0.05 M phosphate buffer pH 7.4 with 0.1% gelatin and 0.01% sodium azide (buffer A) was added to each tube and the tubes vortexed. 0.1 ml of antiserum solution in buffer A was added to each tube, the tubes were vortexed

and incubated at 4°C for 3 hours. 0.5 ml of cold dextran coated charcoal solution (activated charcoal (0.5% w/v) and dextran (0.05% w/v)) in buffer A was added to each tube, the tubes were vortexed for 10 seconds and centrifuged (4000 xg) at 4°C for 15 minutes. The supernatant representing the bound fraction was decanted into 6 ml of scintillation fluid (xylene (75% v/v), Triton X-100 (25% v/v), p-bis-2-(-5phenyloxazole)(0.02% w/v) and 2,5-diphenyloxazole (0.3% w/v)) and counted in a liquid scintillation counter (Beckman LS9800) with an automatic quench correction.

For cross-reaction studies, all compounds (Table 1) were dissolved in ethanol at an initial concentration of 1 mg/mL, from which appropriate dilutions were made. Cross-reactivities were determined by the 50% displacement method (25).

iv) "Affinity Immunoassay" Procedure

In an immunoassay, substances in biological fluids can prevent a tracer from binding an antibody because of structural similarities to an antigen, being present in large quantities, allosteric inhibition etc. All of this translates into an apparent antigen concentration in a biological fluid. In order to determine if the apparent immunoreactive substance in the foetal cord serum was structurally similar to bufalin the following assay procedure was developed. This is based on the

TABLE 1

Percentage cross-reactivities of listed compounds with two bufalin antisera, after primary immunisation (B1, B2) and after secondary immunisation (B1.Y1, B2.Y1)

	Antiserum 1		Antiserum 2	
Cross Reactant	**B1**	**B1.Y1**	**B2**	**B2.Y1**
17α-hydroxyprogesterone	0.04	0.00002	0.0031	0.0081
18-Hydroxycorticosterone	0.002	0.00006	0.0006	0.00034
Aldosterone	0.002	0.00027	0.002	0.0003
Androstane-17ß-ol-3-one	0.02	<	0.0067	0.0085
Androstenedione	0.026	0.0022	0.0073	0.0052
Androsterone	0.0005	<	0.00038	<
Cholesterol	<	<	<	<
Cholic acid	<	<	<	<
Cinobufagin	N.D.	2.1	N.D.	1.75
Cinobufotalin	N.D.	0.38	N.D.	0.23
Corticosterone	0.004	0.00028	0.001	0.00076
Cortisol	0.002	0.000059	0.0005	0.00023
Cortisone	0.007	0.00022	0.003	0.00021
Dehydrocholic acid	<	<	0.000044	<
Dehydroepiandrosterone Sulphate	0.0008	0.000096	0.00069	0.0003
Dehydroepiandrosterone	0.002	<	0.001	<
Deoxycholic acid	<	<	0.000022	<
Digitonin	0.026	0.006	0.08	0.047
Digitoxigenin	14	8.6	71	48
Digitoxin	36	17	100	100
Digoxin	0.38	0.14	2.5	1
Glycocholic acid	<	<	<	<
Lignoceric acid	<	<	<	<
Oestradiol	<	<	<	<
Oestriol	0.001	0.00052	0.0004	0.0024
Oestrone	<	<	<	<
Ouabain	0.05	0.002	0.057	0.006
Pregnenolone	<	<	<	<
Progesterone	0.15	0.001	0.059	0.063
Stearic acid	<	<	0.000083	<
Testosterone	0.035	0.002	0.014	0.0065
Tridecanoic acid	<	<	0.000008	<

< upto 100μg per assay of a compound had no effect on the displacement of bufalin
ND Not tested

principle that in an immunoassay, an antibody binds a limited amount of an antigen and as the amount of antigen is increased, the amount bound to the antibody increases until the antibody binding sites are fully saturated. When a given amount of antigen (eg 200 pg) is incubated with a constant amount of antibody, a finite amount of antigen (eg 50 pg-deduced from percentage tracer bound) will be bound to the antibody at equilibrium. This bound fraction can be separated from the non-antibody bound fraction by means of dextran-coated charcoal. If this antibody bound fraction is re-extracted with diethyl ether and assayed in an RIA, then the result obtained (i.e. amount of tracer bound) will be equivalent to competition by 50 pg of the antigen (standard) in the present example. However if the analyte is structurally different from the antigen, then the value would be different depending on the relative affinity of the antigen and analyte for the antibody. Essentially, this procedure removes all interferring substances, retaining only substances with high affinity for an antibody.

"Affinity extraction" of either bufalin (50 & 200 pg) or pooled cord serum was performed using various dilutions of pre- or post-immune B2 antiserum because of its higher specificity for cardioactive substances followed by bufalin RIA with B1.Y1 and B2.Y1 antisera.

v) Quality Control

The detection limit was determined by using the conventional two S.D. of mean for ten replicates of zero standard.

The interassay imprecision of the assay was determined by the effective dose values at 50 and 80 percent binding of 10 separate RIA runs.

The extraction recovery was monitored using charcoal stripped serum spiked with 1948 and 3896 pmol of bufalin per litre.

RESULTS

The sensitivity of the assay was 50 pmol/L. The interassay cv at 50% and 80% binding were less than 6%. The recoveries of bufalin spiked in charcoal stripped serum were 86 ± 10% and 82 ± 6% at concentrations of 1948 and 3896 pmol/L, respectively.

Table 1 lists cross-reactivities of the two antisera with various compounds after primary and secondary immunisation. Antiserum B2 was more specific for cardioactive agents as shown by higher cross-reactivities compared with antiserum B1. On the other hand, antiserum B1 reacted more extensively with some of the endogenous steroids. Progesterone was found to be the most cross-reactive of the endogenous steroids tested. Antiserum B1.Y1 obtained in the secondary response showed a marked reduction in cross reactivity to all compounds tested. In contrast antiserum B2.Y1 obtained in the secondary response was as reactive as the primary antiserum B2.

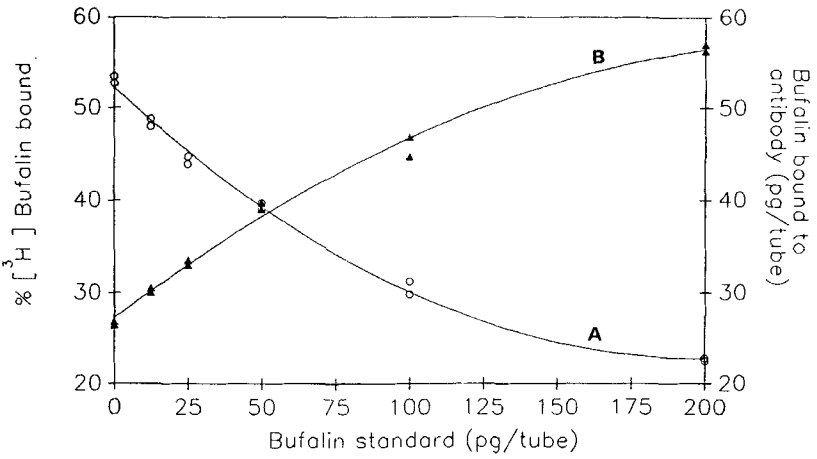

FIGURE 1 A typical bufalin RIA standard curve for antiserum B2 (1:5000 dilution) representing %[³H] bufalin bound (Curve A, y axis, left) and a curve derived from curve A representing mass of bufalin bound to the antibody per tube (curve B, y axis, right). Total amount of bufalin incubated is 50 pg tracer plus a standard.

Bufalin-like immunoreactive substance in foetal cord blood

An apparent bufalin-like immunoreactive substance was found in foetal cord sera with both primary and secondary antisera. The apparent immunoreactivity was higher with antiserum B1 (2104 ± 345 pmol/L) than antiserum B2 (1411 ± 208 pmol/L). Significantly ($p < 0.00001$, Student's t-test) lower immunoreactivity (367 ± 72 pmol/L) was obtained with secondary antiserum B1.Y1, whereas secondary antiserum B2.Y1 gave similar results to primary antiserum B2. Similar results were obtained when pooled cord sera were assayed simultaneously.

Figure 1 shows a typical RIA standard curve (curve A) and a curve

TABLE 2

Immunoassayable bufalin (pg) following "affinity-immunoassay" of known amounts of bufalin or foetal cord extract using various dilutions of pre- or post-immune antiserum B2 for affinity extraction. Lower table shows "affinity-immunoassay" results of pooled sera at 0h and 5h after oral intake of Chinese traditional pills containing bufalin. Mean of two assays except lower table. (See curve B, figure 1, for assessing the expected amount of bufalin as a function of total bufalin incubated. Note 50 pg = 0 standard and 200 pg = 150 standard because tracer constitutes 50 pg of bufalin).

	50 pg bufalin		200 pg bufalin		Pooled cord serum (= 48 pg apparent bufalin)	
RIA antiserum →	B1.Y1	B2.Y1	B1.Y1	B2.Y1	B1.Y1	B2.Y1
Pre 1:500	4.6	4.8	16	15	0.4	6.8
Post 1:500	23	22	99	99	0	4.0
Pre 1:5000	2.8	4.8	16	10	1.3	11
Post 1:5000	25	26	52	50	0.3	8.0
Pre 1:10000	2	3.8	13	9.8	0	8.0
Post 1:10000	17	16	33	31	0.6	7.8

	Basal pooled LSW serum sample		5h pooled LSW serum sample*		Pooled cord serum	
Pre 1:500	0	0	4	9	0.5	3.5
Post 1:500	0	4	18	24	0	10

* LSW - Chinese traditional pills containing bufalin (concentration in blood: 58 pg/0.1 mL).

representing mass of bufalin bound to the antibodies as a function of total mass incubated (curve B). The apparent bufalin-like immunoreactivity detected in the pooled cord serum which was equivalent to 48 pg/0.1 mL serum, after "affinity immunoassay" procedure, was indistinguishable from assay with pre-immune serum. Table 2 shows results of a typical "affinity immunoassay" using antiserum B2 for extracting endogenous bufalin-like

substances in foetal serum. Incubation of known amount of bufalin or pooled serum containing bufalin derived from Chinese medicine yielded results around the expected value (see curve B, figure 1). Preincubation with pre-immune serum caused some binding of bufalin or steroids in cord blood, most probably due to binding proteins in serum.

Bufalin-like immunoreactivity in bovine serum

103 and 259 pmol/L of apparent bufalin were obtained using B1.Y1 and B2.Y1, respectively. These results were similar to basal values for seven human volunteers (134±43 & 238±75 pmol/L for B1.Y1 and B2.Y1, respectively).

DISCUSSION

Immunoassays for steroidal compounds have become indispensible in biological and clinical research. However the specificity of an antiserum can be a problem because although the presence of different functional groups may impart different biological properties, these differences maybe too subtle to be recognised by an antibody. Undoubtedly, even with a properly "tailored" antiserum against a steroid molecule, there are some cross-reactions with biologically unrelated compounds. This cross-reaction may have a significant impact on an immunoassay, if the relative concentration of a cross-reactant is high and is especially relevant for direct (non-extraction) immunoassays. However the specificity of an antiserum maybe improved by separating the

cross-reactant from the steroid of interest by means of extraction or chromatography.

Because of structure-function similarities between digitalis compounds and bufadienolides, the latter are suitable candidates for the role of the endogenous Na, K-ATPase inhibitor, because of their animal origin. Kieval et al (26) using a bufalin immunoassay, reported an immunoreactive substance in human bile which had electro-physiological properties ascribable to digitalis. However, two other bile samples, which were positive for bufalin-like immunoreactivity failed to elicit electro-physiological response. They did not speculate as to the nature of the bufalin-like substance in bile, but reported cross-reactivities of equal or less than 0.1% for progesterone and bile acids, respectively. In the present study, all antisera had very little cross-reaction with dehydrocholic, deoxycholic and glycocholic acids (less than 0.000044%).

The four digitalis substances (digoxin, digitoxin, digitoxigenin and digitonin) showed marked cross-reaction with the bufalin antisera. Antiserum B2, could not distinguish between digitoxin and bufalin. Digitoxigenin had a cross-reaction of 71%, digoxin 2.5% and digitonin 0.08%. There was not much change in the cross-reactivity for digitalis substances after a year. The digitalis compounds have a sugar residue at position C-3 and have a 6 membered lactone instead of bufalin's 5 membered lactone at position C-17

(figure 2). Surprisingly, digitoxigen which is almost identical to bufalin barring the 6 membered lactone ring showed slightly lower cross-reactivity than digitoxin which also has a glycoside residue at position C-3. Antiserum B2 therefore bound compounds with both 5 and 6 membered lactone rings. However, the presence of the hydroxyl group at position C-12 in digoxin hindered binding. Antiserum B1 showed greater specificity for bufalin when using digitalis compounds as cross-reactants, but on the other hand had higher cross-reaction with other compounds initially, but this changed markedly after re-immunisation a year later. In search for the "putative" endogenous digitalis-like substance, antiserum B1 because of its lower cross reactivity with cardioactive substances might not be suitable. Also since the lactone ring is essential for the inotropic activity of the cardioactive agents (26) as well as for binding to the Na, K-ATPase (27), the antiserum should preferably bind both 5 and 6 membered lactone rings. Antiserum B2 therefore has the properties of binding both 5 and 6 membered lactone cardioactive agents and is also less cross-reactive with other steroids.

As with digoxin-like immunoreactive substances in foetal cord blood (8, 9), different amounts of bufalin-like immunoreactive substance was detected in foetal cord serum with different bufalin antisera. The values obtained with antiserum B1 were higher than antiserum B2 initially, but decreased significantly after resting the animal for a year, which was also reflected by the change in cross-reactions with individual compounds.

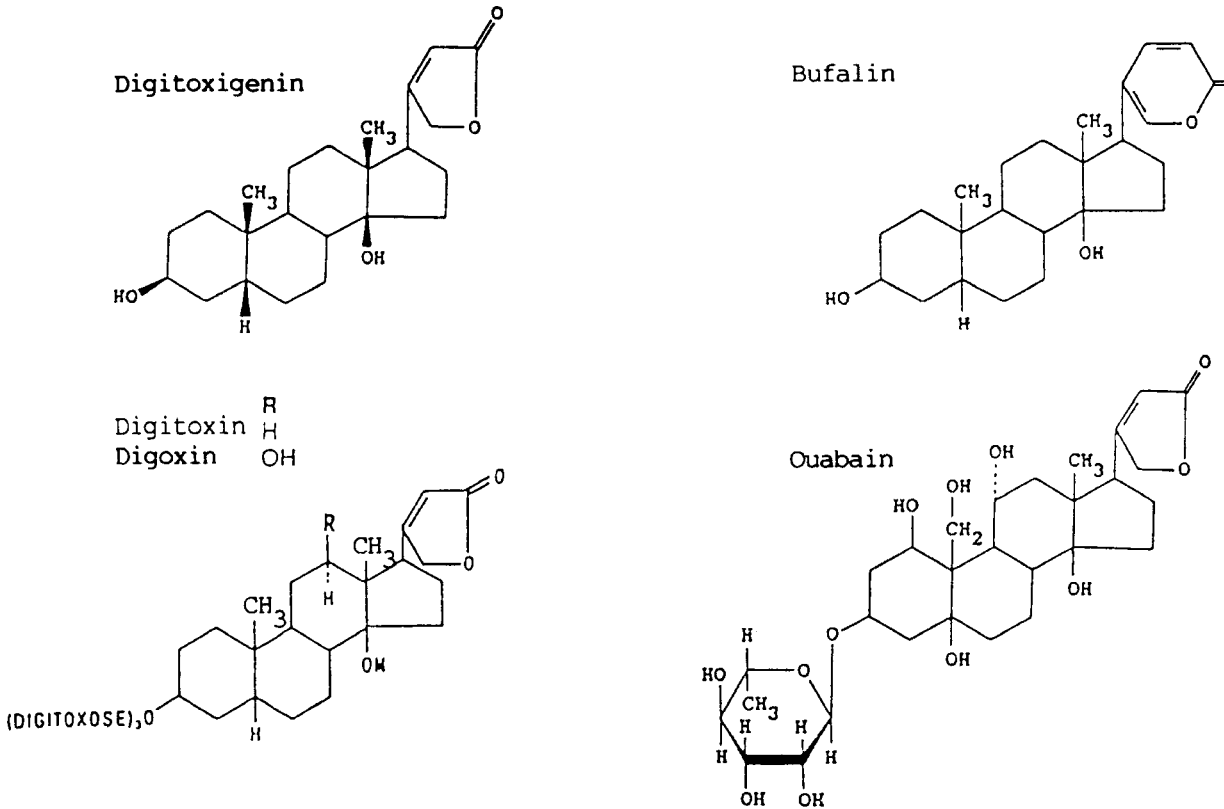

FIGURE 2 Chemical structure of bufalin and some plant cardenolides.

Although individual steroid hormones were not measured in cord blood in the present study, in the previous study (9) we measured eight known steroid hormones in foetal cord blood, and using the mean values obtained in that study, it can be surmised that progesterone contributed substantially to the apparent bufalin-like immunoactivity in the present study. However, using "affinity immunoassay" procedure, the apparent bufalin-like immunoreactivity disappeared suggesting that the results obtained are due to cross-reactants in the foetal cord sera, of which there are many, but none have have structural similarties to bufalin. Recently Naomi et al (28) also ruled out bufalin as the endogenous digitalis-like factor, but it was interesting to note that the cross-reaction profile of bufalin with seven commercial digoxin antisera resembled the profile of DLIS in various clinical states including cord blood. This further confirms that different antisera athough yielding similar results for the antigen, have different propensities for cross-reaction. Although Hamlyn et al (14, 15, 16) claim to have isolated ouabain from human blood, the levels reported (138±43 pmol/L), which coincidentally are similar to adult basal values of the apparent bufalin for B1.Y1, would be too low to influence the current bufalin assays, considering the cross-reactivity of ouabain with the current bufalin antisera.

The ouabain-like activity in bovine blood with a similar HPLC retention time to a toad ouabain-like substance reported by Tal et al (22), would appear not to be due to bufalin or related substances, since neither

bufalin RIAs yielded values for bovine serum greater than basal human results.

In conclusion, polyclonal antisera against bufalin with different cross-reactivities with steroids and other cardioactive substances have been produced and radioimmunoassays developed. The apparent bufalin-like immunoreactive substance in foetal cord blood is due to cross-reaction from mainly steroidal compounds present in the cord serum, and the same may be true for DLIS.

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