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CARDIOTONIC STEROIDS: CORRELATION OF SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATE INHIBITION AND ION TRANSPORT *in vitro* WITH INOTROPIC ACTIVITY AND TOXICITY IN DOGS

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1 A new series of cardiotonics based on five steroid nuclei has been evaluated for inhibition of Na^+/K^+-ATPase and Rb uptake by red blood cells, and for inotropic activity and toxicity in dogs. Structure-activity relationships are discussed.

2 The *in vitro* tests can be used satisfactorily to predict inotropic activity, but not toxicity or therapeutic ratio.

3 Although compounds with greatly improved therapeutic ratios relative to ouabain and tolusin have been obtained, they proved to be strongly emetic in the conscious dog.

Introduction

The inhibition of Na^+-K^+ activated ATPase and the consequent alteration of Na^+ and K^+ fluxes by cardiac glycosides is well established, though whether this is the primary event in the inotropic effect of the glycosides is still not clear. The subject has been reviewed by Glynn (1964) and more recently by Lee & Klaus (1971). Some structure-activity relations for the inhibition of ATPase by sterols and glycosides have been demonstrated by Wilson, Sivitz & Hanna (1970) and by Shigei, Takeda, Tashima & Nakao (1971), while a large series of compounds has been investigated for ATPase inhibition, cardiotonic activity and cytotoxicity by Kupchan, Mokotoff, Sandha & Hokin (1967).

In the present work a new and more varied series of steroids has been evaluated in terms of both *in vitro* and *in vivo* activities. A primary object of the work was the improvement of the therapeutic ratio and for this a better measure of toxicity was required. The development of arrhythmias in dogs was adopted but since this test is time consuming, expensive and poor in reproducibility a correlation was sought with either ATPase inhibition or inhibition of ^{86}Rb uptake by red cells. Rubidium has been shown to be transported into red cells by the same mechanism as potassium (Bernstein & Israel, 1970) and the inhibition of this process by digoxin has been used as an assay for digoxin levels in plasma (Grahame-Smith & Everest, 1969).

Materials and Methods

Cardiotonic steroids based on the nuclei shown in Figure 1 were prepared by Mrs J. Bowler, Dr

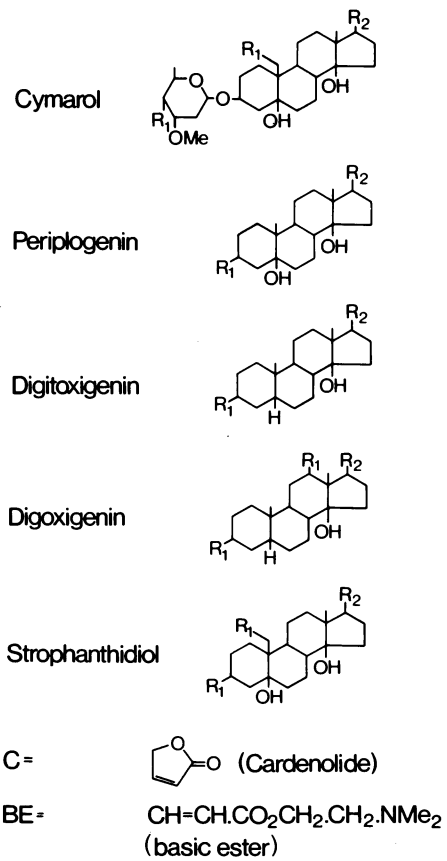


Figure 1 Structures of steroid nuclei.

R. Clarkson and Dr A. Eakin and details of their synthesis will be reported elsewhere.

Inotropic activity and arrhythmogenicity were measured in beagle dogs. The dogs were anaesthetized with pentobarbitone, 30 mg/kg, and the trachea was cannulated. The animals were artificially ventilated by intermittent positive pressure, and the thorax was opened via a right fourth intercostal space incision. A miniature pressure transducer, with frequency response uniform to 1500 Hz (Fry, 1960) was inserted into the left ventricle via the apical dimple. Left ventricular pressure (P_{LV}) so monitored was differentiated with respect to time using an active analogue differentiator circuit with output linear to 60 Hz, and to within 10% up to 90 Hz, to yield dP_{LV}/dt. Electrodes were sutured to the right atrium and intraventricular sulcus to enable atrio-ventricular conduction time (using a gated integrator) and ventricular rate (using a cardiotachometer) to be monitored. Lead II electrocardiogram was used to monitor arrhythmogenesis.

Aortic blood pressure was monitored via a stiff polyethylene catheter (1.5 mm bore) inserted down the right carotid artery to the level of the aortic arch. The catheter was attached to a Bell and Howell type 4-327-L221 pressure transducer driven by an SEL carrier amplifier system. All records were taken on a Mingograph 81 ink ejection oscillograph.

Increases in peak dP_{LV}/dt were taken to indicate contractility increases. Dose-response curves for peak dP_{LV}/dt and the cumulative total amount of compound given were determined in three dogs, for each individual compound and the mean amount of compound (μg/kg) necessary to cause a fixed level of peak dP_{LV}/dt increase was taken to indicate the activity of the compound. The level of increase of peak dP_{LV}/dt was set at 50% of control level since full sigmoid dose-response curve determinations are not possible with arrhythmogenic cardiotonic compounds such as those under investigation.

Peak dP_{LV}/dt changes were recorded only as percentages in all experiments with the exception of seven, in which the animals were given ouabain. In these experiments, mean control peak dP_{LV}/dt was 1777 ± 173 mmHg/second. From these experiments, peak dP_{LV}/dt, aortic blood pressure and heart rate were plotted against time. When peak dP_{LV}/dt had reached 50% over control value, changes in aortic blood pressure and heart rate were noted, and corrections were applied to peak dP_{LV}/dt using the regression relationships cited by Furnival, Linden & Snow (1970). Left ventricular end diastolic pressure changes were ignored as having little and uncertain influence on peak dP_{LV}/dt. The results of these corrections are listed in Table 1. Generally, heart rate decreased and aortic blood pressure increased as ouabain was administered at progressively higher doses. There was no significant difference between uncorrected and corrected values of peak dP_{LV}/dt. Therefore, in subsequent experiments only uncorrected percentage peak dP_{LV}/dt changes were considered in order to derive compound dosages to cause 50% peak dP_{LV}/dt increases.

Toxicity of compounds was determined in animals prepared in the same way, but compounds were infused at three different rates, each in three dogs. The time of infusion for the compound given to cause regular ventricular tachycardia, with A–V dissociation, was determined, and together with the infusion rate, enabled the quantity of compound necessary to cause the specified arrhythmia to be calculated. Mean values were taken including results at all infusion rates. This assisted in overcoming some of the inherent difficulties caused by the differences in compartmental distribution rates, excretion rates, and metabolic rates possible with the different compounds. The differences between compounds in these latter respects could not be determined because of limited quantities of compound available.

With figures for both active doses and toxic doses, it was possible to determine therapeutic ratios, defined as (dose to cause arrhythmias)/(dose to cause 50% increase in peak dP_{LV}/dt).

Rate of hydroxylamine attack

The rates of hydroxylamine (0.2M) attack on cymarol analogues (5.10^{-5}M) at pH 7.3 ± 0.05 (controlled using 0.014M phosphate buffer) and $25 \pm 0.1^{\circ}$C were obtained spectrophotometrically. The decrease in absorbance at 235 nm was measured as a function of time using a Perkin Elmer 402 u.v. recording spectrophotometer equipped with a thermostatted cell block.

The products resulting from attack were not characterized but are believed to result from addition to the double bond at C 20.

Preparation of Na^+/K^+-activated, ouabain-inhibited ATPase

Preliminary experiments showed that rat and guinea-pig heart contained only a small proportion of the Na^+/K^+-dependent enzyme as compared with dog heart and that inhibition by ouabain was poor. The majority of the rat and guinea-pig heart enzyme was Mg^{++}-dependent, Na^+/K^+-independent. It was also found that LiBr extraction by the method of Akera, Larsen & Brody (1969) gave better yields of enzyme than

Table 1 Results from seven male dogs given ouabain, with corrections to measured peak dP_{LV}/dt values according to regressions derived by Furnival *et al.* (1970).

Dog No.	Weight (kg)	Age (Months)	Control values: Peak dP_{LV}/dt (mmHg/s)	Control values: HR (beats/min)	Control values: P_{AO} (mmHg)	Values when peak dP_{LV}/dt increase was 50%: HR (beats/min)	Values when peak dP_{LV}/dt increase was 50%: P_{AO} (mmHg)	Corrections to peak dP_{LV}/dt (mmHg/s): For HR change	For P_{AO} change	Combined change	% Peak dP_{LV}/dt (correction at 150% control)
1	9.5	15.5	1215	122	90	113	100	−189	+120	−69	−3.8
2	10	16.5	1975	118	118	107	144	−231	+312	+81	+2.7
3	9	16.0	2400	115	120	108	117	−147	−36	−183	−4.8
4	9	16.6	1130	125	110	125	112	0	+24	+24	+1.4
5	10	15.9	1920	130	105	119	108	−231	+36	−195	−6.7
6	9	16.2	1760	131	80	130	104	0	+288	+288	+11
7	9.5	16.7	2040	117	98	111	104	−126	+72	−54	−1.8
Means	9.4	16.2	1777	123	103	116	113				−0.28
s.e. mean	±0.2	±0.2	±173	±2.4	±5.5	±3.3	±5.6				±2.6

HR = heart rate; P_{AO} = aortic blood pressure.

NaI extraction according to Nakao, Tashima, Nagano & Nakao (1965). Dog heart extracted by the method of Akera *et al.* (1969) was, therefore, used routinely throughout this work: 20 g left ventricular muscle from a dog killed by pentobarbitone injection was minced finely with scissors and homogenized with a motor-driven Potter-Elvehjem type homogenizer (Teflon–glass) in 4 vol. of the following buffer: 0.25 M sucrose, 5 mM histidine, 5 mM disodium edetate (EDTA), 0.15% sodium deoxycholate, adjusted to pH 6.8 with tris base. The homogenate was centrifuged at 12,000 *g* for 30 min and the supernatant re-centrifuged at 100,000 *g* for 1 hour. The high speed pellet was washed by re-suspending in the same buffer and re-centrifuging at 100,000 *g* for 1 hour. This pellet was then suspended in 1 vol. 0.25 M sucrose containing 5 mM histamine and 1 mM tris EDTA and mixed with an equal vol. of LiBr (2.0 M). After stirring for 1 h at 2°C the suspension was centrifuged at 100,000 *g* for 1 h and the pellet washed by re-suspension in the same buffer and re-centrifuging under the same conditions. The final pellet was suspended in 20 ml of the sucrose/histidine/tris EDTA buffer, divided into 1 ml aliquots and stored at −20°C. Three batches prepared in this way had protein contents of 1.66, 1.07 and 1.26 mg/ml (estimated by the method of Lowry, Rosebrough, Farr & Randall, 1951). They were diluted to a standard activity before use in the assay of inhibitors.

Estimation of ATPase activity

Tris ATP (γ-^{32}P) was obtained from the Radiochemical Centre, Amersham, (to special order) as a 10 mM solution pH 7.4, 2 μCi/ml and sufficient cold 10 mM tris ATP was added before use so that 10 μl contained between 50,000 and 300,000 ct/minute. The standard incubation mixture contained: 200 μl buffer (250 mM Tris, 250 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, pH 7.4), 100 μl enzyme suspension (25 to 36 μg protein), 100 μl H_2O (containing inhibitor if water-soluble), and 20 μl dimethyl formamide (containing inhibitor when not water-soluble). After 30 min pre-incubation at 37°C the reaction was started by addition of 100 μl ATP and stopped 20 min later by the addition of 100 μl 11% Na_3PO_4 in 7% $HClO_4$ followed by 500 μl of a suspension of charcoal in water (Norit SX 11, 60 mg/ml). After mixing rapidly in a Vortex mixer, the suspension was centrifuged in a high speed microcentrifuge and 100 μl of the supernatant added to 20 ml water for counting by Cerenkov radiation at an average efficiency of 37%. Blanks were run by adding perchlorate to the enzyme before ATP, and the Na^+/K^+ independent enzyme was estimated by

omitting Na^+ and K^+ from the incubation buffer. The total counts in the incubation were also monitored by adding water instead of charcoal suspension. Per cent inhibition was calculated as:

$$100\left(1-\frac{\text{ct/min (inhibited)} - \text{ct/min (no } Na^+/K^+)}{\text{ct/min (control)} - \text{ct/min (no } Na^+/K^+)}\right)$$

Uptake of Rb by red cells

$^{86}RbCl$ was received from the Radiochemical Centre, Amersham, as an aqueous solution containing 1.08 mg/ml and 5.09 mCi/ml; 10 µl of this was diluted to 1.5 ml in Krebs-Ringer-phosphate (Umbreit, Burris & Stauffer, 1957) containing 1 mg glucose/ml and 0.3 mg cold RbCl/ml, making a stock solution of 2.2 mM and 33 µCi/ml (initially) which was stored frozen at −20° C.

Whole human blood was collected in heparinized tubes and the red cells separated by centrifugation and washed twice with Krebs-Ringer-phosphate containing 1 mg glucose/ml. In the standard incubation procedure 0.2 ml red cells were diluted with 0.2 ml Krebs-Ringer-phosphate in a plastic centrifuge tube and pre-incubated with the inhibitor in 10 µl dimethyl formamide (DMF) for 45 min at 37° C; 50 µl stock RbCl solution was then added and the incubation continued for 2 h at 37° C. Ice cold 0.9% w/v NaCl solution (saline) was then added (1 ml) and the cells collected by centrifugation. After discarding the supernatant, the cells were washed three times by gentle re-suspension in 1 ml cold saline and centrifugation. The tube containing the final pellet of cells was drained and placed in a scintillation vial for counting in a scintillation counter using ^{32}P settings. About 10^5 ct/min were obtained when the ^{86}Rb was fresh and no inhibitor was present. Complete inhibition of uptake was obtained with 10^{-4}M ouabain and this was routinely used to obtain blank values (about 12,000 ct/min). Standards of 5×10^{-9}M ouabain (giving 50% inhibition) were included with each batch of compounds assayed. Per cent inhibition was calculated as:

$$100\left(1-\frac{\text{ct/min (inhibited)} - \text{blank}}{\text{ct/min (control)} - \text{blank}}\right)$$

Fifty percent inhibition values for both ATPase and Rb uptake were obtained from plots of % inhibition vs. log concentration.

Results

The ATPase prepared from dog heart by the above method has a very low content of Mg^{++} dependent

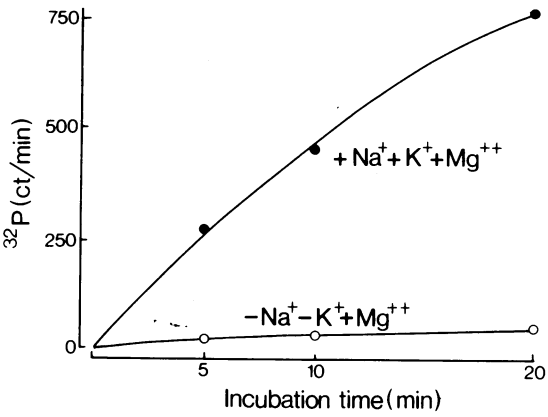


Figure 2 Dog heart ATPase. Time course of Na^+/K^+ activation.

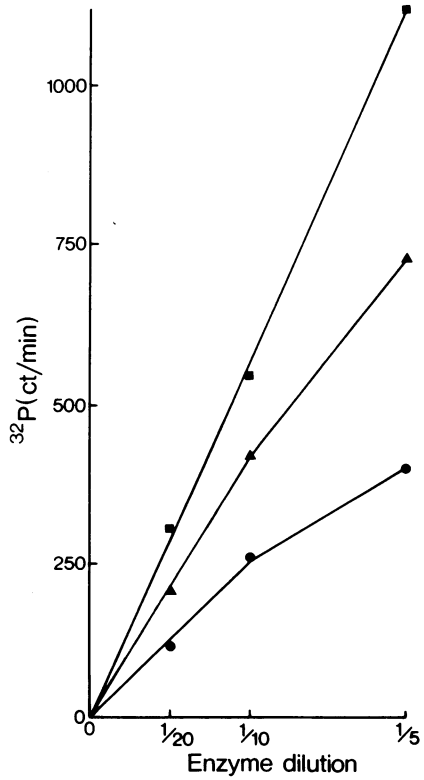


Figure 3 Dog heart Na^+/K^+ ATPase. Effects of substrate concentration and enzyme dilution: ATP 0.1 mM (●), 0.2 mM (▲), 0.4 mM (■).

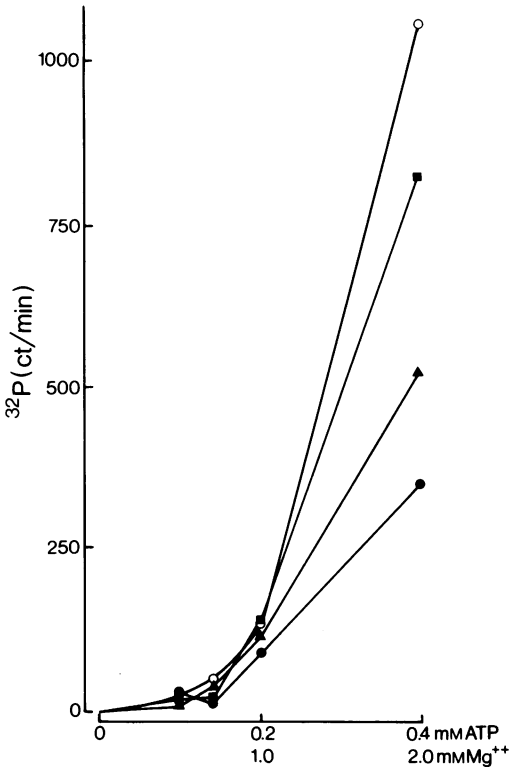


Figure 4 Dog heart Na^+/K^+ ATPase. Inhibition by ouabain at various ATP and Mg^{++} concentrations. Ouabain 10^{-6} M (●), 5×10^{-7} M (▲), 2×10^{-7} M (■), no ouabain (○).

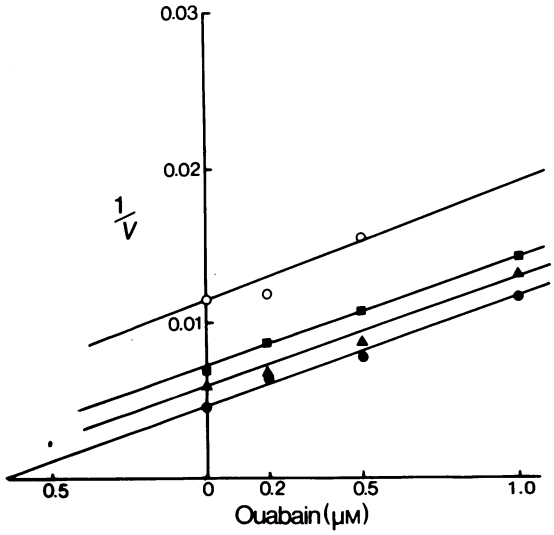


Figure 5 Dog heart Na^+/K^+ ATPase: Dixon plot for ouabain inhibition. ATP 0.4 mM (●), 0.2 mM (▲), 0.14 mM (■) and 0.1 mM (○).

enzyme and is considerably stimulated by Na^+ and K^+ (Figure 2). Its activity varies regularly with dilution and with ATP concentration up to at least 1 mM (at constant Mg^{++} concentration, Figure 3). Above 2 mM ATP there is evidence of substrate inhibition. The Na^+-K^+-dependent enzyme still requires Mg^{++} for full activity, since the substrate is believed to be an $Mg^{++}-$ATP complex (Hexum, Samson & Himes (1970)) and Figure 4 shows that activity below 1 mM Mg^{++}/0.2 mM ATP is so low that it is not possible to measure inhibition by ouabain accurately; 2.9 mM Mg and 1.9 mM ATP were adopted as standard for the assay of inhibitors.

The activity of the dog heart enzyme is markedly affected by an increase in temperature from 30° to 37°C although a corresponding preparation from guinea-pig heart was not. All incubations were therefore run at 37°C. A pre-incubation time of 30 min with the inhibitor before addition of ATP was employed as early experiments with guinea-pig and rat enzyme suggested that inhibition was lower without it. In fact later work with dog enzyme showed that pre-incubation made little difference. A Dixon plot (Figure 5, $1/v$ against inhibitor concentration) for ouabain inhibition gives a series of parallel lines for various ATP concentrations indicating uncompetitive (coupling) inhibition i.e. inhibition when the enzyme-inhibitor (EI) complex is not formed but the inhibitor combines only with the enzyme-substrate (ES) complex (Webb, 1963).

Inhibition of Rb uptake by ouabain is illustrated in Figure 6. Inhibition is complete at 10^{-7} M and the 50% value is 2.8×10^{-9} M. Some stimulation is apparent at concentrations of 10^{-10} M ouabain and below. A similar stimulation of chicken kidney ATPase by low concentrations of ouabain has been observed and correlated with increased ion transport in the chicken kidney tubule (Palmer & Nechay, 1964; Palmer, Lassiter & Melvin, 1966). These authors have explained the biphasic effect of ouabain in terms of two receptors on the ATPase. We have not found stimulation of our ATPase at concentrations down to 10^{-9} M ouabain.

The compounds tested were derived from the

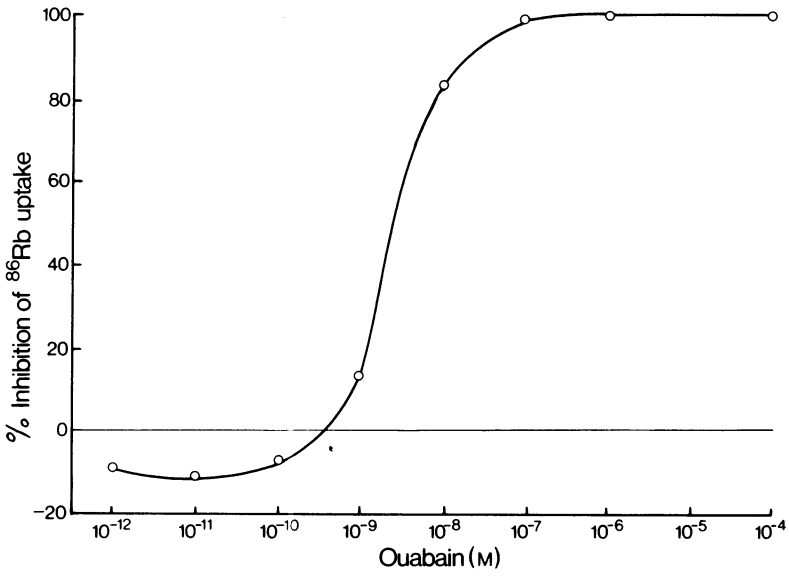


Figure 6 Inhibition of ^{86}Rb uptake into red blood cells by ouabain (for conditions see text).

five steroid nuclei shown in Figure 1. The results for ATPase inhibition, inotropic activity and inhibition of Rb uptake are given in Tables 2A-E, which are arranged to illustrate certain structure activity relations.

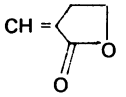
From Table 2A it can be seen that the basic esters are about as active as the corresponding cardenolides. A comparison of the activities of the compounds in Tables 2B and C shows that the activity of the cymarol analogues is markedly reduced following 4', 19 diacylation. This is probably the effect of a bulk group on C19 since variation of the group attached to C3 has only a slight effect (compare the five periplogenin analogues in Table 2D). The high activity of the cymarol analogues (Table 2B) appears to confirm the assumption that the butenolide ring of the natural compounds may simply be replaced by an electronegatively substituted ethylene residue without loss of activity. It will be noted that the

Table 2A Comparison of basic esters and cardenolides.

Compound	*Steroid*	R_1	R_2	ATP_{50} *(μM)*	Rb_{50} *(nM)*	Δ_{50} *(μmol/kg)*	*DA (μmol/kg)*	DA/Δ_{50}
63,632	Cymarol	HO	C	.42	2.55	.055	.114	2.07
63,605	Cymarol	HO	BE	1.2	14.2	.063	.292	4.63
62,655	Cymarol	CH_3CO_2	C	47	1183			
62,838	Cymarol	CH_3CO_2	BE	21.7	391	1.3	2.83	2.18
69,654	Periplogenin	HO	C	1.2	93			
58,622	Periplogenin	HO	BE	.73	–	.095	.388	4.08
61,374	Digitoxigenin	HO	C	.32	1.07			
57,267	Digitoxigenin	HO	BE	.27	8.6	.03		
61,424	Digoxigenin	HO	C	.71	128	.21		
61,411	Digoxigenin	HO	BE	1.8	65	.056		

C = Cardenolide; BE = Basic ester.
ATP_{50} : concentration required for 50% inhibition of Na^+/K^+ ATPase.
Rb_{50} : concentration required for 50% inhibition of ^{86}Rb uptake into red blood cells.
Δ_{50} : concentration required to produce a 50% increase in dP_{LV}/dt.
DA : dose to cause arrhythmias.

Table 2B Cymarol derivatives with modified side chains (R_1 = HO).

Compound	R_2	ATP_{50} (μM)	Rb_{50} (nM)	Δ_{50} (μmol/kg)	*DA* (μmol/kg)	DA/Δ_{50}
*63,632	C	.42	2.55	.055	.114	2.07
*63,605	BE	1.2	14.2	.063	.292	4.63
65,199	CH=CH . CO_2H		929			
70,898	CH=CH . CN	.14	1.93	.058	.106	1.83
70,899	CH=CH . CO_2Et	.55	12.2	.16	1.055	6.59
70,900	CH=CH . CO . CH_3	.32	–	.18	1.39	7.72
70,901		5.5	69	.49		

* denotes compound mentioned in previous table.

Table 2C Cymarol-4′,19-diacetate derivatives with modified side chains (R_1 = CH_3CO_2).

Compound	R_2	ATP_{50} (μM)	Rb_{50} (nM)	Δ_{50} (μmol/kg)	*DA* (μmol/kg)	DA/Δ_{50}
*62,655	C	47	1183			
*62,838	BE	21.7	391	1.3	2.83	2.18
†62,966	BE	13	183	.42		
65,210	CH=CH . CO_2H	NA	NA			
†63,116	CH=CH . CN	6.8	349	1.09		
62,936	CH=CH . CO_2Et	NA	NA			
65,551	CH=CH . CO . CH_3	105	3548	9.7		
63,978	CH	NA	1698			

* denotes compound mentioned in previous table; † 4′,19-dipropionates (R_1 = $C_2H_5CO_2$); NA = not active

Table 2D Basic esters (R_2 = BE).

Compound	*Steroid*	R_1	ATP_{50} (μM)	Rb_{50} (nM)	Δ_{50} (μmol/kg)	*DA* (μmol/kg)	DA/Δ_{50}
*57,267	Digitoxigenin	HO	.27	8.6	.03		
62,276	Strophanthidiol	CH_3CO_2	18	–	.94	6.78	7.21
*63,605	Cymarol	HO	1.2	14.2	.063	.292	4.63
*62,966	Cymarol	$C_2H_5CO_2$	13	183	.42		
*58,622	Periplogenin	HO	.73	–	.095	.388	4.08
63,056	Periplogenin	CH_3CO_2	.41	22	.08	.409	5.11
67,135	Periplogenin	Pr^nCO_2	.62	22.6	.09	.975	10.83
67,167	Periplogenin	$C_5H_{11}^nCO_2$	.91	2.9	.13	1.228	9.45
67,134	Periplogenin	O	.76	–	.16	1.818	11.36

Table 2E Miscellaneous compounds.

Compounds	*Steroid*	R_1	R_2	ATP_{50} (μM)	Rb_{50} (nM)	Δ_{50} (μmol/kg)	*DA* (μmol/kg)	DA/Δ_{50}
67,875	Digitoxigenin	OH	CH_2NH_2	27	241	1.9	>18.5	>9.74
67,880	Digitoxigenin	OH	$CH_2NH . CO_2 -(CH_2)_2NMe_2$	3.2	–	1.4	>13.9	>9.93
61,558	Digitoxigenin	$PhCO_2$	BE	2.55	–		1.497	
Ouabain	–	–	–	.38	2.82	.028	.099	3.54
Tolusin	–	–	–	.066	.83	.075	.208	2.77

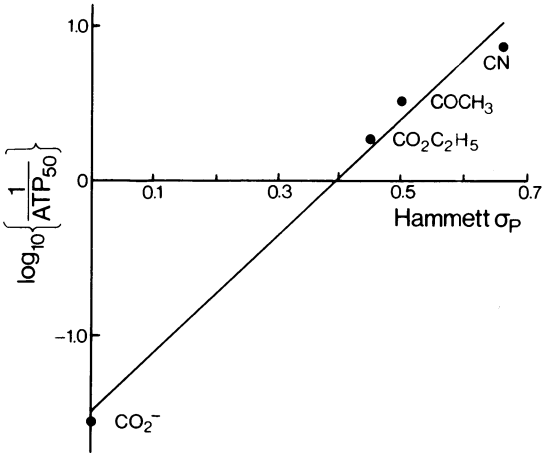


Figure 7 The relationship between ATPase inhibition data and electron withdrawal at C_{21} for cymarol analogues. Values for the Hammett σ constants are taken from Jaffe (1953).

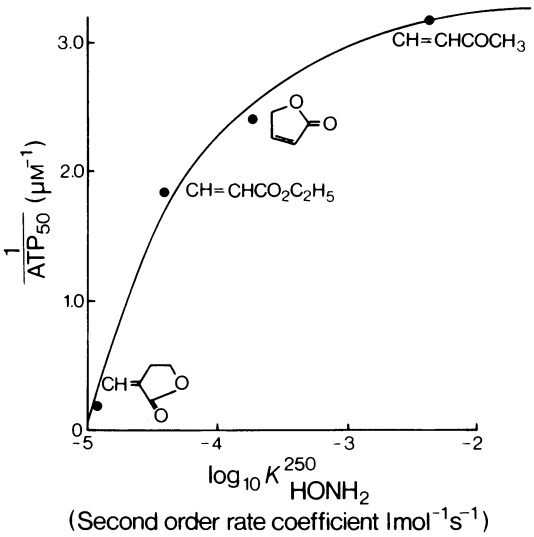


Figure 8 The relationship between ATPase inhibition data and the rate of hydroxylamine attack on the double bond of cymarol analogues. Reaction rates were measured spectrophotometrically as described in the text.

nitrile (70,898) and the methyl ketone (70,900) are both more active than cymarol itself. In fact the ATP_{50} values fall in line with the Hammett σ values of the electronegative group (Figure 7). For these cymarol analogues a correlation was also obtained between ATPase inhibition and the rate of nucleophilic attack on the double bond (Figure 8). If one assumes that increased reactivity towards nucleophilic attack only leads to increased ATPase inhibition up to a certain maximal level then a plot of

$$\log_{10}\left\{\frac{1}{ATP_{50}}_{max} - \frac{1}{ATP_{50}}\right\}$$

versus $\log k_{HONH_2}$ is linear.

$$\left(\frac{1}{ATP_{50}}_{max} = 3.5\ \mu M^{-1}\right)$$

Most of the compounds reported by Kupchan *et al.* (1967) were reversible inhibitors and we tested the effect of washing the enzyme on the inhibition obtained with a number of our compounds. Concentrations of drug giving about 50% inhibition were used and half the enzyme samples were washed twice after incubation with the drug and before adding substrate. The other half were treated normally. The results in Table 3 show that only 63,605 (basic ester) and 70,900 (methyl ketone) are reversible. At present we have no explanation for this.

Table 3 Effect of washing on inhibition of ATPase by cardiotonic steroids.

Drug	*Conc. (μg/ml)*	*% inhibition of ATPase*	
		Control	*Washed*
Ouabain	.73	69.3	65.9
70,898	.1	47	48
63,116	5	44.5	49
65,199	50	69.5	78
65,210	200	83.1	71.9
70,900	.5	77	0
63,605	1.0	68.1	0
62,655	50	57.6	72.5

Discussion

The desired improvement in therapeutic ratio (DA/Δ_{50}) has been achieved with a number of the compounds reported here, but on further testing in the conscious dog they proved to be highly emetic and no further development was possible. A secondary object of the work was to establish correlations between the *in vitro* and *in vivo* tests and Table 4 shows a correlation matrix from which it appears that ATPase inhibition (ATP_{50}) and rubidium uptake inhibition (Rb_{50}) correlate well with each other and with inotropic activity (Δ_{50}). The correlation of either test with toxicity

Table 4 Correlation coefficients (No. of points).

	ATP_{50}	Rb_{50}	Δ_{50}	DA	DA/Δ_{50}
ATP_{50}		.97 (21)	.98 (23)	.69 (17)	.09 (16)
Rb_{50}			.99 (18)	.56 (11)	−.01 (11)
Δ_{50}				.91 (16)	.28 (16)
DA					.48 (16)
DA/Δ_{50}					

Headings as Table 1.

(DA) is less good but still significant with Rb uptake showing greater discrimination between activity and toxicity than ATPase though this may be due to the smaller number of samples for which full data were available. Neither test correlates with therapeutic ratio (DA/Δ_{50}). It is therefore not possible to use either *in vitro* test as a more precise indicator of toxicity. Haustein, Markwardt & Repke (1970) have claimed that 16-epi-gitoxin has an improved therapeutic ratio over ouabain (as measured on an *in vitro* system) which is associated with a biphasic dose-response curve for ATPase inhibition. We have obtained a sample of this compound (by courtesy of Dr Repke) and can confirm the biphasic inhibition of ATPase but in our dogs very little positive inotropic activity appeared at doses up to 100 μg/kg while arrhythmias were induced at doses as low as 5 μg/kg. All the compounds described above had linear, parallel dose-response curves (with the exception of 63,978 which had only very low activity) although many of them have greatly improved therapeutic ratios compared with ouabain. This pattern of ATPase inhibition cannot therefore be taken as indicative in general of improved therapeutic ratio.

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