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Chemistry and biochemical pharmacology of cardiac glycosides – A review

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Abstract

Cardiac glycosides constitute one of the most important categories of pharmacological agents of plant origin used in the clinical management of cardiac failure. The present review deals with the current ideas on the structure-activity relationships (SAR) and the biochemical mechanism of cardiac glycoside-induced positive inotropy. Divergent modern concepts on the role of calcium in the cardiac glycoside-induced cardiotonic effects are discussed.

Key words: Cardiac glycosides, cardenolides, Na, K-ATPase, positive inotropy, structure-activity relationships (SAR).

1. Introduction

Coronary atherosclerosis and hypertension are principal causes of heart disease in middle and old age. Coronary and hypertensive disorders constitute nearly 60% of the incidence of heart diseases. Industrialization has contributed significantly to the rise in pulmonary heart diseases in India[1,2]. Similarly, the incidence of ischemic heart diseases is increasing. Cardiac failure is the most characteristic clinical symptom of most of these heart diseases. Cardiac failure may be defined as a clinical condition when the output of the heart is inadequate to meet the peripheral blood circulation required for normal functioning of the tissue.

Cardiac failures are of two types, namely, (a) acute and (b) chronic or congestive heart failure. Congestive heart failure is a clinical syndrome resulting from chronic failure and is due to congestion in the peripheral circulation. The term congestive heart failure is, in fact, applicable whenever and wherever congestion occurs as a sequel to heart failure. In general, congestion due to increased blood results from the retention of sodium ion and water[3]. Clinically, heart may apparently fail as a

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whole, but in order to understand the physiology of the process, it is helpful to consider separately the failures of the left and right ventricles of the heart. In hypertension, coronary atherosclerosis and aortic valvular diseases, the left atrium cannot empty blood efficiently to the left ventricle, thus leading to the left-sided cardiac failure. Similarly, right-sided cardiac failure is secondary to left-sided failure, and is due to congenital heart diseases or to diseases of the lungs, like emphysema.

Cardiac failure is usually accompanied by a reduction in the cardiac output. The resultant increase in the blood volume, which occurs as a compensatory measure, further complicates this condition[3]. Increased metabolic rate as in thyrotoxicosis and decreased oxygen-carrying power as in anemia, precipitates 'high output' cardiac failure. On the other hand, hypertension and coronary atherosclerosis result in 'low output' cardiac failure.

Whatever might be the cause of the cardiac failure, cardiac glycosides are still the most useful drugs available for its treatment. Cardiac glycosides, otherwise known as cardenolides, are of plant origin and constitute an outstanding class of drugs. They exert a specific action on the heart and are of great clinical value in cases involving decompensation, where the heart is beating in a deficient condition[4]. Particularly, cardiac glycosides are most effective in the clinical management of (a) atrial fibrillation and tachycardia, (b) sinus tachycardia and failure, (c) paroxysmal atrial tachycardia, and (d) left ventricular failure due to hypertension and ischemic heart diseases.

The effect of cardiac glycosides primarily rests in its effects on myocardial efficiency. Marked increase in the myocardial efficiency occurs due to (i) an increased strength of contraction as a result of direct stimulation of myocardium, (ii) dilated ventricles empty more completely, thereby increasing the cardiac output, and (c) relatively less oxygen is consumed by myocardium for a given amount of work, thus leading to an enhanced efficiency of the heart.

Heart rate is usually above normal in heart failure. Cardiac glycosides might also serve to reduce the heart rate by direct depression of the arterio ventricular (AV) node and by vagal stimulation[3]. Further, improvement in funtion has been shown to be positively correlated with a slowing of the heart rate.

1.1. *Natural sources of cardiac glycosides*

Cardiac glycosides are of plant origin. Their occurrence has been reported in wide and unrelated plant families[5,6]. Cardiac glycosides appear to be confined to angiosperms. They are particularly abundant in Apocyanaceae, Asclepiadaceae families[7]. Table I lists the plant families and genus which are reported to contain cardiac glycosides[5,8]. Some of the well-known sources include the celebrated genus Digitalis (the source of digitalis glycosides), Strophanthus, Scilla, Convallaria, Thevetia, etc. These compounds are found in almost all parts of the plant, but the total amount or relative distribution in any given plant may vary with the stage of development, time of harvest, and mode of drying of the plant, apart from its ecological conditions[5].

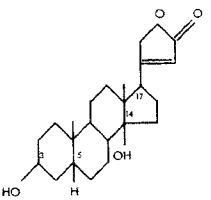
Table I
Plant families in which the occurrence of cardenolides has been reported

Apocyanaceae	*Asclepiadaceae*	*Liliaceae*
Adenium	Asclepias	Urginea
Acocanthera	Calotropis	Bowiea
Acocynum	Cryptolepis	Convallaria
Carissa	Cryptostegia	Ornithogalum
Cerebra	Gomphocarpus	Rhodea
Nerium	Meriabee	
Strophanthus	Panchycarpus	
Tanghinia	Periploca	
Thevetia	Xysmalobium	
Urechites		
Crucifere	*Celestraceae*	*Scrophylariaceae*
Erysimum	Euonymus	Digitalis
Cheiranthus		
Sterculiaceae	*Rununculaceae*	*Moraceae*
Monosonia	Adonis	Anthriaris
	Helleborus	Antiaropsis
		Castilla
		Ogeodeia
Leguminaceae	*Tiliaceae*	
Coronilla	Corchorus	

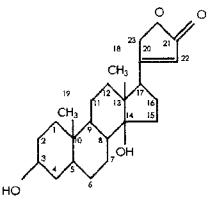
1.2. *Chemical nature of cardiac glycosides*

Cardiac glycosides consist of three moieties: a mono/oligo saccharide, a steroid (cyclopentanophenanthrene) and a five- or six-membered α-β unsaturated lactone ring. The glycosides are acetals in which the hydroxyl group of a non-sugar component and the secondary hydroxyl is condensed within the sugar molecule itself to form an oxide ring. Thus, glycosides may be considered as sugar ethers. The non-sugar component, termed as aglycone, is attached to the sugar component (glycone) through an oxygen atom. Both α and β glycosides are possible, the variations being dependent on the stearic configuration of the linkage between the sugar and the aglycone.

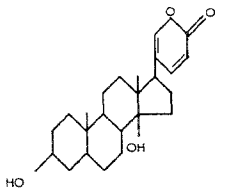
Naturally occurring cardio-active glycosides are classified into two broad groups, cardenolides (Fig. 1, I) and bufadienolides (Fig. 1, II) on the basis of the nature of the lactone ring at C-17 of the basic nucleus (Fig. 1). Cardenolides constitute the predominant group and are C-23 steroids having an α-β unsaturated γ-lactone (butanolide) ring, whereas bufadienolides are C-24 steroids consisting of a doubly unsaturated six-membered lactone (δ-pyrone or hexadienolide) ring. Structural and chemical variations of the steroid moiety are numerous, and usually arise from (a) different configuration centers at C-3', C-5 and C-17 (Fig. 1, I), and (b) the presence or absence of oxygen and double bonds in the molecule.



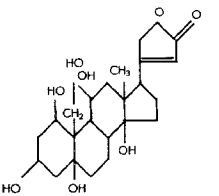
I. Cardenolide



III. Digitoxigenin



II. Bufodienolide



IV. Ouabain (genin)

FIG. 1.

Under proper conditions of hydrolysis, one or more saccharide groups can be removed from cardiac glycosides, resulting in compounds of simpler structure. The acetal linkage in the cardiac glycosides is susceptible to hydrolysis if they are boiled with mineral acids. However, the ease with which hydrolysis occurs depends on the chemical nature of the particular cardiac glycosides. In most cases, the glycoside is easily hydrolysed by an enzyme present in the same plant tissue but in cells different from those which contain the glycoside. Injury to the tissue, germination and perhaps other physiologic activities of the cells bring the enzyme in contact with the glycoside leading to its hydrolysis.

The most commonly encountered monosaccharide in a cardiac glycoside is a β-D-glucose. However, cardiac glycosides are reported to contain rare sugars such as those found in digitalis glycosides (Tables II and III). It is clear from Tables II and III that digitalis glycosides contain rare sugars such as digitoxose and digitalose. Further, cardenolides from other sources contain rare sugars (Fig. 2).

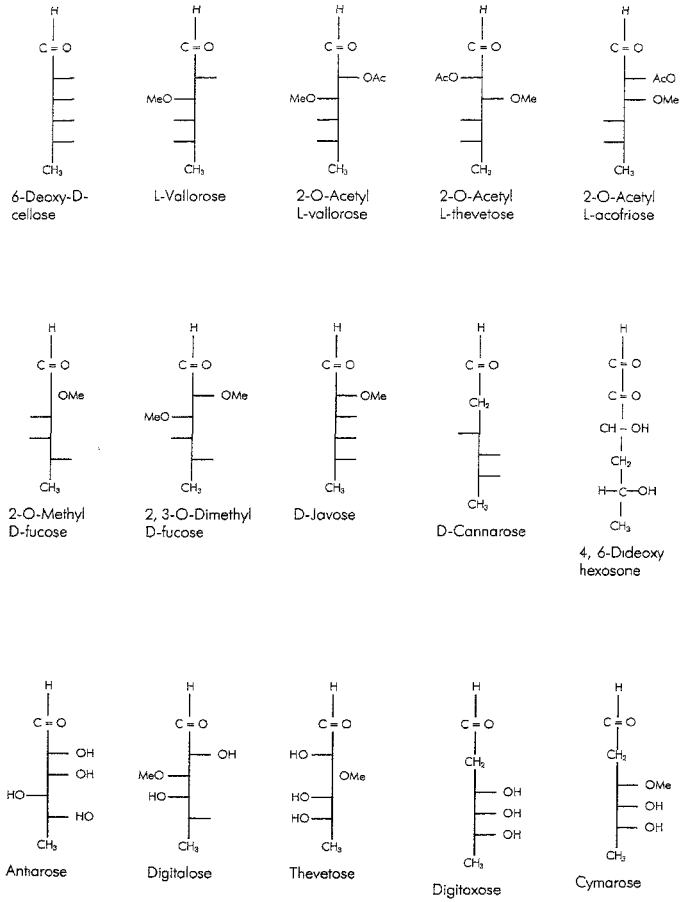


FIG. 2. Rare sugars found in cardiac glycosides.

Table II

Cardiac glycosides of *Digitalis lanata* and their glycones

Derivatives of digitoxigenin	
Lanatoside A	3DG, 1 acetyl group, 1G
Acetyl digitoxin (α & β)	3DG, 1 acetyl group
Gluco-evatromonoside	1DG, 1G
Gluco-digitoxigenin-glucomethyloside	1 glucomethylase, 1G
Gluco-digifucoside	1 fucose, 1G
Neo-gluco-digifucoside	1 fucose, 1G
Derivatives of gitoxigenin	
Lanatoside	3DG, 1 acetyl group, 1G
Gluco-gitroside	1DG, 1G
Digitalum verum	1DL, 1G
Derivatives of gitaloxigenin	
Lanatoside E	3DG, 1 acetyl group, 1G
Gluco-lanadoxin	1DG, 1G
Gluco-veradoxin	1DL, 1G
Derivatives of digoxigenin	
Lanatoside C	3DG, 1 acetyl group, 1G
Desacetyllanatoside C	3DG, 1G
Acetyl digoxin (α & β)	3DG, 1 acetyl group
Digoxin	3DG
Gluco-digoxigenin-*bis*-digitoxoside	2DG, 1G
Derivative of diginatigenin	
Lanatoside D	3DG, 1 acetyl group, 1G

Table III

Cardiac glycosides of *Digitalis purpurea* and their glycones

Derivatives of digitoxigenin	
Purpurea glycoside A	3DG, 1G
Digitoxin	3DG
Gluco-digitoxin-*bis*-digitoxoside	2DG, 1G
Gluco-evatromonoside	1DG, 1G
Derivatives of gitoxigenin	
Purpurea glycoside B	3 DG, 1G
Gitoxin	3DG
Gluco-gitoroside	1DG, 1G
Digitalum verum	1DL, 1G
Stropeside	1DL
Derivatives of gitaloxigenin	
Gluco-gitaloxin	3DG, 1G
Gitaloxin	3DG
Gluco-gitaloxigenin-*bis*-digitoxoside	2DG, 1G
Gluco-lanadoxin	1DG, 1G
Gluco-veradoxin	1DL, 1G
Veradoxin	1DL

DG – digitoxose; G – glucose; DL – digitalose.

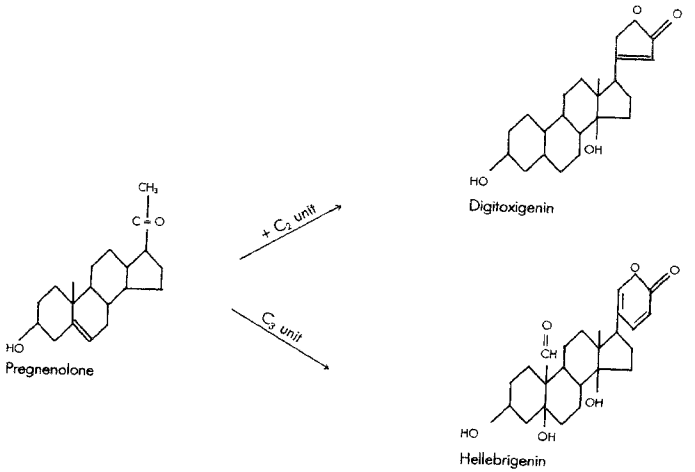


FIG. 3. Biogenesis of cardiac glycosides.

1.3. *Biogenesis of cardiac glycosides*

Figure 3 shows the biogenesis of cardiac glycosides. Pregnenolone was demonstrated to be the natural precursor of cardiac glycosides. The biogenesis of the cardiac glycoside aglycone moiety is derived from mevalonic acid pathway, the final molecule arising from a condensation of a C-21 steroid with a C-2 (as in cardenolide) or a C-3 unit (as in bufadienolides)[9] (*see* Fig. 3).

However, feeding experiments in *Digitalis lanata* indicated that progesterone itself can be a precursor for the biosynthesis of cardiac glycosides. Biotransformation studies conducted largely with *Strophanthus kombe*[10] and *D. purpurea* cultures[11-13] present evidence for the progesterone initial process of biosynthesis of cardenolide[9-11] (Fig. 4).

2. Structure-activity relationships (SAR)

The possible relationship between the three-dimensional structure of a cardiac glycoside and its therapeutic and toxic effects has been examined repeatedly. The major impetus for the sustained interest in this is the possibility of broadening the therapeutic range of this class of compounds.

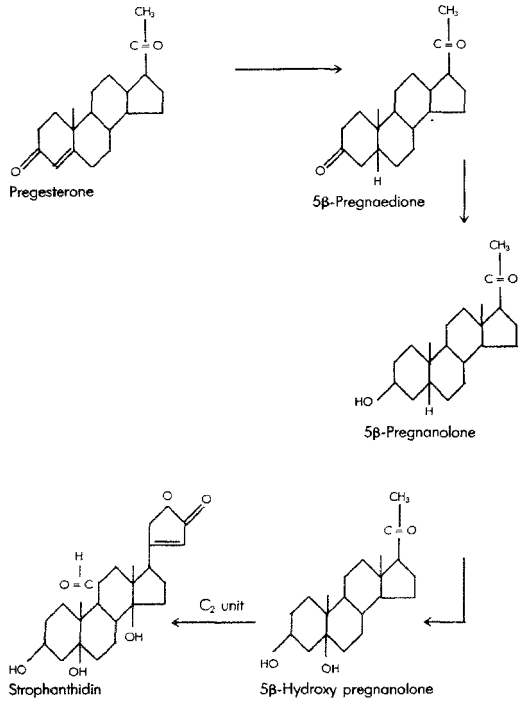


FIG. 4. Biosynthesis of cardenolides from progesterone.

It has been shown that the cardiotonic activity of the cardiac glycosides is qualitative, and resides in the steroidal aglycone portion[14]. Hence, the aglycones themselves have been examined for the cardiotonic activity. The most widely studied aglycones are digitoxigenin (Fig. 1, III) and ouabagenin (Fig. 1, IV).

In view of the above, digitoxigenin may be considered to possess the minimum structural requirements for cardiotonic actions of the corresponding cardiac

glycosides, digitoxin. Its structure is unique and differs totally from that of other class of (biologically important) saturated steroids, in that both A/B and C/D ring junctions are *cis*. Because of such stearic configuration, the β or 'upward' hydroxyl group at C-3 is axially oriented. The C-14 hydroxyl group has likewise β, or 'upward' configuration. The structural requirements for cardiotonic activity were found to be highly specific. For example, change in conformation at C-5 in digitoxigenin leads to aglycone uzarigenin in which A/B fusion is *trans*; this small change results in a sharp diminution in cardiotonic activity[6]. Similarly, epimerization at C-3 (to give 3-epidigitoxigenin)[15], or saturation of unsaturated lactone ring attached at C-17 of digitoxigenin[15,16] leads to a loss of activity.

In 1963, Tamm[17] proposed the minimum structural elements which are absolutely necessary for cardiotonic activity (*see* Fig. 5). As mentioned earlier, digitoxigenin appears to have the bare minimum structural requirements for a steroidal glycoside to possess cardio-active properties. As apparent from Fig. 5, the generalized requirements are (a) the presence of a steroidal 14-β-hydroxyl group in which A/B and C/D ring junctions are in *cis* conformation, and (b) B/C ring junction is in *trans* conformation. Furthermore, steroids should have an α-β unsaturated lactone (τ-lactone or δ-pyrone) as a C-17β-side chain and a 3β-oriented oxygen function (either a hydroxyl group or a glycosidic linkage). Tamm[17] further assumed that additional hydroxyl groups are only of minimal importance for their cardiotonic properties, but the distance between lactone carbonyl and oxygen function at C-3 was critical from the point of view of structure-activity relationship. After a preliminary treatment of the minimum structural requirements of a cardiac glycoside, we would like to review the literature on the SAR of cardiac glycoside in terms of the contribution of the steroidal (aglycone) and carbohydrate (glycone) moieties to physiological activity.

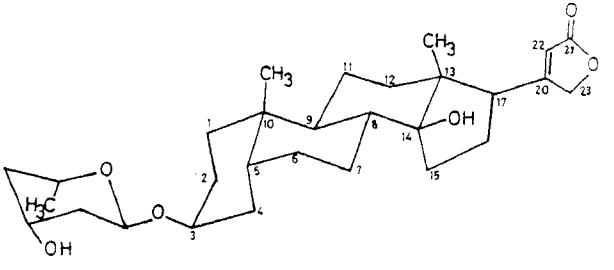


FIG. 5. Structural elements absolutely necessary for cardiotonic activity.

2.1. *Contribution of steroidal aglycone to physiologic activity*

The steroidal aglycones in general have the following structural features:

(i) C-17 cyclopentanophenantherene nucleus. Unless there are double bonds at C-4 and C-5, the rings A/B and C/D have *cis* configuration and B/C *trans* configuration.

(ii) presence of angular equatorial methyl groups at C-10 and C-13.

(iii) presence of β-oriented hydroxyl groups at C-14 and C-3.

(iv) presence of a β-oriented five-membered α-β unsaturated lactone ring.

2.1.1. *A/B and C/D ring junction*

The effect of modification of A/B and C/D ring junctions on the biological activity of cardiac glycosides is given in Table IV. A/B and C/D rings in cardiac glycosides have a *cis* conformation. As shown in Table IV, a change in the conformation at C-5 usually produces less active compounds and the loss of activity seems to be more pronounced in cardenolides than in bufadienolides[18]. However, other studies[19,20] have produced contrary results where A/B *trans* compounds were found to be more potent than digoxin which has a *cis* configuration with a wider margin of safety in therapeutic and toxic doses. In a recent study on 73 digitalis analogues, the lead structures of a cardiac glycoside were shown to have a 5β configuration[21] (Fig. 5). Further, the nature of substitution at C-5' and the orientation are not pharmacophoric substructures but merely amplifiers of the interaction energy of steroid lead[21]. Although a recent study indicates difference in potency of 5α and 5β cardiac glycosides (Table IV), the results in general indicate that there is no significant difference in the physiological effects of 5α and 5β epimers.

Although the hydroxyl group attached at C-14 (C/D ring junction) has been described by Tamm as the minimum structural requirement for cardiac glycosides[17], current opinion is that the C-14 group is not indispensable, but its substitution with hydrogen leads to a considerable loss of activity. Although in general, 14-deoxy compounds are less active (Table IV), it appears that the *cis* conformation of the C/D ring junction is more important than the 14β-OH group. A recent study on the relation between C-14 hydroxyl structure and biological activity, gluco-digitoxigen and its analogue that lacks C-14 hydroxyl group (named Dig-3) studied in anesthetized dog and Starling's heart-lung preparations support the observation that the C-14 hydroxyl group does not constitute a structural requirement for its inotropic and electrophysiological effects in heart, although its potency is greatly diminished[22] (*see* Table IV). In a recent study on 73 digitalis analogues, 14β-androstane-3β, 14 diols were found as the lead structure of cardiac glycosides at the level of drug-receptor interactions, as determined by their effect on Na, K-ATPase from human cardiac muscle[21].

Table IV
Structure-activity relationship (SAR) in cardiac glycosides I: Modification of A/B and C/D ring junctions

Modification locus	*Nature of modification*	*Remarks*	*Reference*
A/B and C/D ring junctions	Change in configuration at C_5 (α to β)	Loss of activity	19
	Double bond at C_4-C_5 or C_5-C_6	Effect on biological activity not clear	20
	5α or 5β Cardenolides	Biologically active	24
	Glucosidation of uzarigenin (5α compound) or digitoxigen (5β)	Potency decreases (5α) Potency increases (5β)	24
	Conjugation of uzarigenin (5α) or digitoxigenin (5β) with rhamnose	Potency increases (both 5α and 5β)	24
	Removal of β-OH at C_{14}	Loss of activity	18
	14-Deoxydigitoxigenin (5β) 14-Deoxydigitoxigenin (5α)	Loss of 60% activity Loss of 90% activity	26
	Prednisone (5α) Prednisone (5β)	Biologically active Biologically active	17
	Dig-3 (gluco-digitoxigenin without C_{14} hydroxyl)	Biologically active	30

2.1.2. *C-3β-OH group and other substitutions*

Naturally occurring cardiac glycosides have a β-oriented oxygen either as a hydroxyl group or as a glycosidic linkage at C-3. Table V describes the effect of modification of cardiac glycosides at C-3 on its activity. As shown in the table, 3-deoxy digitoxigenin was found to have comparable activity with that of digitoxigenin[23]. Further, this derivative appeared to lose only 50% activity in terms of the inhibition of Na, K-ATPase enzyme[24]. Hydrogenation of lactone in 3-deoxy digitoxigenin leads to a far greater loss of activity as determined by the same criterion. Changes in potency, resulting from alteration in the structure of side chain at C-3 suggested that out of 77 cardenolides tested, the geometries of the steroid nucleus contributed by the side chain at C-3 was integral to the inhibitory potency of the enzyme from two different sources[25]. This species difference in the responsiveness to different semi-synthetic cardiac glycosides was traced to inherent species differences in the complementarity of the steroidal binding sub-sites in Na,K-ATPase[25].

Gomphosides, a group of 5α-H cardiac glycosides isolated from *Asclepias fructicosa* have a unique, double glycosidic linkage between the glycone and 2α and 3β carbon atoms of the steroid *via* oxygen atoms. The 3'-axial hydroxyl of its conformationally rigid sugar residues appears to be responsible for its potent biological activity[26]. The effect of distribution of polar groups on the biological activity of cardiac glycosides indicates that the presence of acetate, free hydroxyl and glycosides at C-3

Table V
Structure-activity relationship in cardiac glycosides II: Modification of C_3-OH group

Modification locus	*Nature of modification*	*Remarks*	*Reference*
C_3-OH	3-Deoxy digitoxigenin	No loss of activity	31
	Hydrogenation of lactone in 3-deoxy-digitoxigenin	Almost complete loss of activity	28
	Change in configuration of oxygen at C_3 (β to α)	Loss of activity	28
	Branching at C_3	Biologically active on Na,K-ATPase enzyme	33
	Gomphoside (5α-H) double glycosidic linkage through oxygen at 2α and 3β of the steroid	Potent glycosides	35
	Acetate, hydroxyl and glycosidic substitution at C_3	Potency decreases with raise in polarity in A ring	36
	3α Amino compounds	Biologically active	37

inhibits Na,K-ATPase, and that the extent of inhibition decreases with rise in polarity in the A-ring region of the steroid nucleus. 3α- and 3β-amino analogues of digoxigenin were tested for biological activity in isolated guinea pig atrial preparations[27]. As shown in Table V, 3α-amino compounds demonstrated activities comparable to their 3β-hydroxy counterparts[27].

2.1.3. *Nature and stereochemistry of substituents at C-14, C-15 and C-16*

There is no uniformity of opinion in the reports on the substitution at C-14, C-15 and biological activities of the resulting compounds. As shown in Table VI, the introduction of an oxygen function in the form of hydroxyl substitution at C-15 of digitoxigenin always resulted in a decrease in biological activity[28,29]. Similarly, in a series of semi-synthetic analogues, the activity of 14-desoxy-14βH cardenolides, the activity usually decreased to a level of 4-15% of the original compounds. Although 15-oxo compounds had 10-30% activity of the original compound[29] (Table VI), both in the series of digitoxigenin and that of 14-desoxy-14βH digitoxigenin, the 15α-OH compound is ineffective. However, in a series of 14-desoxy-14β-chlorodigitoxigen derivatives, the 15β-hydroxy analogues and 15-oxo derivatives were inactive whereas the 15α-hydroxy analogues possessed up to 30% of the effects of digitoxigenin[28]. In spite of a fall in the potency, it was interesting to note that 14β-15β epoxides had a better therapeutic index[30]. A recent study observed that gluco-digitoxigen and its analogues devoid of C-14 hydroxyl group (named Dig-3) exert comparable effects on anesthetized dog[22]. This further supports the notion that the presence of a hydroxyl group attached at C-14 of digitalis does not constitute a structural requirement for the preservation of its pharmacological and electrophysiological effects on heart[22].

Table VI
Structure-activity relationship in cardiac glycosides III: Modification of substituents at C_{14}, C_{15} and C_{16}

Modification locus	*Nature of modificanon*	*Remarks*	*Reference*
C_{14}, C_{15} and C_{16}	-OH at C_{15} of digitoxigenin	Decrease in biological activity	26, 38
	C_{15} -oxo-compounds	Has 10-30% activity	38
	14β-15β Epoxide	Less effective	27
	16α-Hydroxyl group	Unfavourable effect	40
	16-Acetyl-16α gitoxin and 16α gitoxin	Enhanced efficacy	42
	Gitoxin		
	(1) 16β Formate	Increased activity by 30 fold	45
	(2) 16β Acetate	9-12 Fold increase	45
	(3) 16β Methoxy carbonate	60% Decrease in activity	45
	(4) 16α Acetate, nitrate and methyl ether	Increase in activity	46

16-Hydroxy-substituted analogues of cardiac glycosides have received much attention in recent years. Table VI gives a list of various C-16 substituted gitoxin analogues and their effect on biological activity. Although 16α acetate, nitrate and methyl ester substitution in gitoxin enhanced the activity of gitoxin[7,31,32] (Table VI), it was usually associated with enhanced toxic effects. Variations in the digitoxose moiety carried an increase or decrease in potency depending upon the position and number of substituted hydroxyl groups[32]. In general, 16α substitution in gitoxin produced unfavorable effects on myocardium[33]. However, 16-acetyl-16α-gitoxin was found to have a far greater degree of safety from toxic effects[34].

16β Substitutions generally decrease the potency of cardiac glycosides (Table VI). However, 3β digitoxoside increases the activity of gitoxin by 15 times, but the effect is less if the 16β group is esterified. These data suggest an important role for C-16 esters and possibly the presence of a separate binding site on Na,K-ATPase corresponding to the C-16 position[7].

2.1.4. *C-17 Side chain*

Cardiac glycosides are characterized by the presence of 5-membered α-β unsaturated τ-lactone or a doubly unsaturated δ-pyrone ring attached to the steroid nucleus at C-17. The lactone side chain is commonly regarded as the most essential functional group required for the biological activity of cardiac glycosides[17]. Further, the C-17 side chain must be β-oriented. Any change from the β-conformation results in compounds with diminished activity[17,35]. However, Denghenghi[36] was able to show that isomeric cardenolides differing only in the position of attachment of the lactone ring to the steroidal 17β position were still active and even had a safer therapeutic index than the naturally occurring glycosides. However, recent studies contradict the above

Table VII
Structure-activity relationships in cardiac glycoside IV: Modification of C_{17} side chain

Modification locus	*Nature of modification*	*Remarks*	*Reference*
C_{17}	Change in configuration (α to β)	Inactive compounds	18, 41
	trans acrylic acid *trans* acrylonitrile Acrylic acid ester	Active compounds	48, 51
	Guanyl hydrazone derivatives of lactone	Activity slightly lost	49
	Nitrile group substitution for carbonyl group	Effective compounds	48, 49
	Substitution at C_{22} by halogen, alkyl and alkoxyl group	Enhanced activity	53
	Substitution at C_{21} by bromo, hydroxy, fluoride amino ester	Usually less active compounds	55
	Furano derivatives	Significant cardiotonic activity	56
	17β Substitution with COOH, $CONH_2$, $COCH_3$	All active except amide substitution	57
	Actodigin (lactone ring saturated)	Active	58
	β-Methyl actodigin	More potent than actodigin	58
	$C_{17}\beta$ Lactone substituted with 3'-furyl ring or 4'-pyridazinyl ring	Reduction in cardiotonic activity	37

view that the lactone group is an absolute requirement for cardio-active property. Table VII lists a variety of synthetic analogues where the lactone at C-17 side chain is substituted by different functional groups. It is evident from the table that *trans* acrylic acid, acrylonitrile and acrylic acid ester derivatives were active[37]. Similarly, guanyl hydrazone derivatives of the lactone were still active[38]. All these results indicate that lactone group is not an absolute requirement. Instead, the structural element -C=CH-C-A appears to be the basic structure required at C-17 for cardiotonic activity, where A is a heteroatom (=O, =N), R and R-1 being an H or an alkoxyl group[37,38]. Paradoxically, saturated lactone, which does not have this structural element, yet produces positive inotropic effect, only at higher concentrations[39]. In general, substitution of halogens or alkyl groups at C-22 (of the lactone ring) enhanced cardiotonic activity[40-43]. A recent study on the digitoxigenin derivatives compared the cardiotonic effects of C-17 side chain, changed by substitutions such as CH=CHX (where X = COOH, $CONH_2$, $COCH_3$, CN, COOR)[44]. All the compounds were active except for acid and amide substituents. In spite of a favorable shape and high capacity for their intramolecular bond formation, the effectiveness of the amide appears to be an exception to the observations made earlier. The potency decreased

when R group in the CH=CH-COOR had a longer chain length. This supports the previous suggestion that the portion of the digitalis receptor that interacts with the C-17 side chain lies within a cleft[44].

A survey of the recent literature shows that the relative orientation of the lactone ring in a cardiac glycoside influences its interaction energy with its receptor. An attempt to calculate the difference in interaction energy of cardio-active steroids with their receptor using different steroid conformations (with respect to 17β side chain orientation) revealed a linear relationship with Na,K-ATPase activity and interaction energy[45]. Further, studies on the activity of eight cardiotonic steroids with C=O at C-17 position support this observation[45]. A recent study on 73 digitalis analogues revealed that C-17 substitution simply serves as an amplifier of the interaction energy of the steroid with its receptor[21]. Another study on the potency of 35 steroidal glycosides on human cardiac muscle revealed that the geometries of the steroidal nucleus determine the differential contribution of the side chain at C-3 and C-17 to the integral inhibitory potency on enzymes from different species[25].

2.2. *Contribution of the aglycone linkage and nature of glycone to physiological activity*

Glycone and aglycone in cardiac glycosides follow a general pattern, *viz.*, aglycone–(rare sugar)$_n$–(glucose)$_n$. Among the known isomeric aldohexoses, only D-glucose has been found as a terminal carbohydrate component of cardenolides (Tables II and III). Usually, it is present in combination with other sugars. Rarely, cardenolides, in which the D-glucose residue is joined directly to the aglycone moiety, are found in nature. The synthetic analogues containing β-D-glucosides of strophanthidin and digitoxigen[46] showed high potency when compared with the corresponding hexosides of natural origin. Based on this, a relationship was proved between the degree of hydroxylation on the pyranose component of cardenolide and the cardiotonic activity.

The other sugars normally present in the cardenolides are shown in Fig. 2. It is evident from the figure that the cardiac glycosides contain 2-oxy- and 2-deoxy sugars and carbocyclic sugar derivatives[8]. D-Fucose occurs only in cardiac glycosides whereas L-fucose is present in other types of naturally occurring glycosides[47]. D-Xylose was the first aldopentose identified in cardiac glycosides. Cannarose is the only 2,6-dideoxy, 3-O-methyl ether reported to be present in cardiac glycosides[48]. Both D- and L-diginose occur in Strophanthus species, the D-form exclusively in the African and L-form in the Asian varieties[49]. The novel carbocyclic sugar components from *Asclepiadaceae* glycosides can neither be obtained in free form nor be isolated without decomposition. For example, in *A. fructicosa,* the glycosides have two ether bridges formed by the interaction of C-2 and C-3 hydroxyls of the genins with C-1' and C-2' of the sugar moiety, whereas calactin, calotropin and uscharidin from *Calotropis procera* arise by normal glycosidation at C-1' of the 4,6-dideoxy sugar.

Table VIII
Structure-activity relationships in cardiac glycoside V: Modification of the glycone

Pyranosyl moiety	*Lethal dose (μ mole)*	*Reference*
Digitoxigenin	0.8	
Glucose, β-D[b]-	4.3	14
6-Deoxy glucose-D[b]-	2.3	65
6-Deoxy-3-O-methyl glucose-α-L[b]-	2.7	14
6-Deoxy-3-O-methyl glucose-β-D[b]-	2.5	14
Mannose, 6-deoxy, α-L[b]-	1.9	14
6-Deoxy -β, D[b]-	1.5	66
6-Deoxy mannose, α-D-	0.8	66
Arabino hexose, 2-deoxy-β-D[b]-	2.7	66
Ribo-hexose-2-deoxy-β-D[b]-	2.7	66
2,6-Dideoxy-β-D[b]-	2.3	14
2,6-Dideoxy-α-D-	0.9	66
Glycero-pentose, 2,3,4-trideoxy, D-(or L-)	0.2	66
Strophanthidin	0.2	
Glucose, β-D[b]-	6.3	14
Mannose, α-L[b]-	8.2	66
α-D Mannose	2.2	66
6-Deoxy-α-L[b]-	7.0	14
6-Deoxy-β-D[b]-	5.6	66
6-Deoxy-α-L[b]-	4.0	66
Allose, 6-deoxy, -D[b]-	5.5	14
Arabinose-α-L[b]-	6.2	14
Xylose, β-D[b]-	5.4	14
Lyxose, α-D-	3.8	14
Glycero-pentose, 2,3,4-trideoxy, D-(or L-)	0.9	66

The discovery that the synthetic digitoxigenin-α-digitoxoside shows significantly less activity than that of its glycone led to the preparation of a series of hexosides with 'unnatural' glycosidic linkage in order to explain such loss of potency as a function of structural features. Table VIII is a list of hexosides of digitoxigen and strophanthidin glycosides. The table is arranged in the increasing order of 'deoxygenation' in the carbohydrate component. For the purpose of comparison, the potency values given are molar potencies. The most active glycosides appear to have the conformation in which the hydroxyl group in C-2', C-3 and C-4 of the pyranoid ring are equatorial, as seen with β-D-glucoside. Also, the 6-deoxy-β-D-glucoside had a higher potency than 6-deoxy-β-D-mannoside[50].

That the enhancement of cardiotonic activity by the carbohydrate component as a function of hydroxylation is suggested from a comparison of the potencies of the completely deoxygenated, 3-tetrahydropyronyl derivatives[51] of digitoxigenin with those of strophanthidin. As shown in Table VIII the value obtained is lower than

that of the corresponding glycone, which suggests that pyranoid ring makes no contribution of its own but merely acts as a vehicle for carrying hydroxyl functions, and that the unsubstituted tetrahydropyronyl ring serves only to 'dilute' the cardiotonic activity of the aglycone.

Influence of the diastereoisomeric nature of the glycone on the activity of glycosides is interesting. Table VIII gives the results and a comparison of three pairs of α-L and α-D isomers of strophanthidin and digitoxigenin. It is pertinent to note that the α-L isomer is always slightly more potent than the α-D isomer. On the contrary, the glycosides which contain the unsaturated α-D linkage show very low potency, compared with the corresponding β-D and β-L isomers. However, it is reasonable to suggest that, for all cases, the physical property responsible for the cellular uptake of the glycoside would be significantly different in different cases, which might account for their physiological divergence in cardiotonic activity.

In a recent study, digitoxigenin α-L, β-L, α-D and β-D glycosides; α-L, β-L, α-D and β-D mannosides and α-L and β-L rhamnosides were stereoselectively synthesized and the inhibitory effect of these glycosides on Na,K-ATPase was tested and compared with that of digitoxigenin, digitoxigenin-β-D-galactoside and D-digitoxoside[52]. The observations indicate that the sugar substituents may play a role in the binding of some glycoside stereo isomers only. With α-L rhamnosides, the C-5' -CH_3 and C-4' -OH appear to have a predominant role in the binding to receptor. Addition of a C-6' -OH to form corresponding monosides dramatically disrupts the effect of both C-5' -CH_3 and C-4' -OH in promoting receptor binding of α-L isomer. However, in the case of β-L isomers, some influence of C-4' -OH, C-3' -OH and C-2' -OH on binding still remains. As for β-D-glycosides, binding *via* a C-5' -CH_3 site appears to be of little importance and the addition of C-6' -OH diminishes the activity only slightly. An equatorial C-4'-OH, axial, C-3'-OH and equatorial C-2'-OH appear to contribute to binding in β-D glycosides[52].

In a recent study on the digitoxigen-3-O-β-D furanosides and several analogues, the furanoside analogues of cardiac glycosides were shown to have a weak to moderate cardiotonic activity when compared with their genins[53].

A number of studies on the binding and inhibition of Na,K-ATPase by cardiac glycosides indicated that the nature of sugar moiety also influences the dissociation rate constant[54]. Wilson *et al*[55] suggested that the monoglycosides fit the receptor site best and hence have the highest affinity[56]. For a given cardiac glycoside, the association rate constant increases with monosaccharides and decreases as the number of sugar molecules attached increase. It was suggested that steric hindrance prevents fast binding of *bis* and *tris* glycosides. The first sugar binds to the enzyme and contributes to a tight complex in the presence of Na^+, Mg^{++}, and ATP[57]. For example, digoxin monoglycoside is several times more potent than digitoxigenin in binding to ATPase[56]. The addition of a second, third and fourth sugar progressively decreases the affinity for the ATPase[58]. This was further demonstrated in an elaborate study on the kinetics of cardiac glycoside-receptor binding to several species and

tissues[55]. The dissociation rate constant also depends on the nature of the glycoside. Apparently, the sensitivity of various species to ouabain is determined primarily by the dissociation rate constant for the receptor-ouabain interaction[59]. Aglycone had a higher dissociation constant although the association rate constant did not change significantly, thus indicating the importance of the number of sugar moieties upon the velocity of the dissociation step[54,57]. It, therefore, appears from these studies that the sub-site of the cardiac glycoside receptor interacting with the sugar part of the steroid has a limited space. Acetyl groups on the sugar reduce the affinity considerably. For optimal binding, the aglycones need to be bound to one or two sugars only; if more than three, the affinity decreases as a result of increase in the dissociation rate constant.

Conformational possibilities for sugar components of cardiac monoglycosides contributing to the biological activity of cardiac glycosides have been studied[60]. It was shown that conformational lability and the presence of several oxygen-containing groups in the first monosaccharide residue underlie the possibility for coexistence of several productive conformations. Based on conformational parameters alone a number of monosaccharide residues were distinguished that should have either favorable or unfavorable effect on the biological activity of cardiac glycosides[60].

Literature on the interaction of sugar components with cardiac glycosides reveals certain interesting features discussed below[26,61,62]. The suggestion that the pyranose moiety simply serves as a hydroxyl-carrying structure was further substantiated in a recent study on the inotropic effect of 19 cardio-active glycosides on guinea pig left atrium. The compounds tested included five α-L rhamnosides and four α-L-thevetosides[63]. These were compared with their respective genins. The study showed that rhamnosides and thevetosides were amongst the most active of all cardiac glycosides. Further, the high activity of these compounds is probably related to the α-L-glycosidic linkage and the configuration of C-4'-hydroxyl and C-5' methyl groups. There was a stepwise loss of activity when the hydroxyl groups of the sugars were acetylated. The extent to which rhamnose enhanced the potency of different genins varied with the nature of genins and ranged from 6 to 35 fold[63,64]. Palytoxin, a highly toxic sugar-containing active principle isolated from *Palythoa tuberculosa*, caused K^+ release from rabbit red blood cells. Cardiac glycosides such as ouabain, digoxin, digitoxin, convallotoxin and cymarin inhibited the palytoxin-induced K^+ release[65]. The result of this study led to a suggestion that the sugar moiety of cardiac glycosides is important for the inhibitory effect of the K^+ release induced by palytoxin and that the inhibition is not related to their inhibition of Na,K-ATPase[65,66].

Gomphoside is a 5α-H cardiac glycoside isolated from *Asclepias fructicosa* and has a unique double glycosidic linkage to the aglycone through oxygen atoms, at C-2α and C-3α of the steroid. The C-3'-axial hydroxyl of its conformationally rigid sugar residue appears to be the functional group responsible for its potent inotropic activity. Using gomphoside as the model compound, the conformation of flexible glycosidic linkages of 5β-H was compared with that of 5α-H cardenolides with the

aid of computer graphics and conformationary potential energy calculations[26]. The relative biological potencies of these compounds can be accounted for in terms of their active binding conformations with their potential energy distributions. The conformational distributions of the glycosidic moiety were postulated to be the major determinant of the biological activity of cardiac glycosides[26]. Another report on the effect of gomphosides on guinea pig left atrial preparations indicated that the compound had very high potency comparable to the most active 5β-H cardiac glycosides[67]. Removal of the sugar moiety of gomphoside reduces the biological activity almost 500 fold indicating that it was the presence of sugar moiety that was mainly responsible for the high potency of gomphoside. Modification of the steroid, or sugar residue of gomphoside altered the potency. It therefore appears that the non-rotatable glycosidic linkage of gomphoside may be responsible for its high potency[67]. A recent study on 43 cardiac glycosides with doubly linked sugar, demonstrated that doubly linked glycosides show higher activities than their respective genins[61]. The inhibitory activity depends on the C-ring substitution also. Rhamnosides exhibited an effect at least three times higher than their respective genins[61].

Schölz and Schmitz for the first time compared the effects of 16'-glucuronide derivatives of digitoxin and digoxin, the metabolite form of cardiac glycoside, on human isolated ventricular preparations and guinea pig isolated papillary muscles[68]. Interestingly, glucuronide of digitoxin and digoxin, which are believed to be detoxicated in their conjugated forms, were biologically active on the human heart. The metabolites were slightly less potent but more effective than the parent compound[68]. This study demonstrates its clinical relevance with respect to inotropic action and plasma level determination of cardiac glycosides. However, it is worth mentioning that positive inotropic properties need not be confined to cardio-active steroids alone. Table IX gives a list of structurally divergent, yet potent positive inotropic agents, reported in the recent literature[69-74].

3. Cardiac glycosides and positive inotropy

3.1. *Inotropic agents*

Positive inotropy involves an increase in peak force of contraction as a consequence of the acceleration of force developed in the myocardium. Therefore, both the increase in the force of contraction (F) and the rate of force development (dP/dt) are quantitative parameters for the measurement of positive inotropy[75].

Investigations on isolated atrial preparations indicate qualitative differentiation and characterization of cardiac glycoside in comparison to other positive inotropic agents. It is also possible to quantitate the effectiveness of various glycosides with respect to activity, potency, speed of onset and duration of action, using isolated atrial preparations.

Positive inotropic agents may be classified on the basis of the biochemical mechanisms they invoke to induce inotropy. They are:

(*i*) *The adrenergic agents:* The actions of this class are mediated through the cardiac α- and β- adrenoceptors. An adrenergic agent essentially binds to the β-adrenoceptor

Table IX
Compounds with divergent structures possessing cardio-active properties reported in recent literature

Compound	*Reference*
Imidazo [4,5-b] pyridines such as AR-L57, AR-L100, AR-L115	86
Dibenzazepine derivatives, Bonnecor	85
Pirlimycin adenylate (Clindamycin analogue)	88
Trimecaine (quarternary derivative)	89
Hepatacaine (N-[2-(2-heptyloxy-phenyl carbamoyloxy-ethyl] piperidinium chloride)	90
Mono- and diaryl-2-quinuclidinyl carbinols	91

and enhances the levels of cAMP through the activation of adenylate cyclase enzyme. Examples include catecholamines, β-adrenergic agents, etc.

(*ii*) *Phosphodiesterase inhibitors:* Cardio-active bipyridines such as Amrinone and Milrinone are phosphodiesterase inhibitors. Phosphodiesterase inhibitor inhibits the breakdown of cAMP as a consequence of which the levels of cAMP are enhanced. The effects of phosphodiesterase cannot be blocked by α- and β-blocking agents, histamine-receptor(H-1 and H-2)-blocking agents and inhibitors of prostaglandin synthesis. Other examples of phosphodiesterase inhibitors are MDL 17043, Buquineran, Vardax, USU 2776.

(*iii*) *Inhibitors of Na-,K-dependent adenosine triphosphatase:* These cover a wide variety of chemical entities including cardiac glycosides. Some non-cardiac glycosides, which are Na,K-ATPase inhibitors include erythrophloem alkaloids (like erythropheleine, cassaine), sulhydryl inhibitors (like *p*-chloromercuribenzote, methylmaleiamide, ethacrinic acid, chloropromazine), rubidium and vanadium (as vanadate).

(*iv*) *Agents affecting Na^+ currents:* Toxins like Grayanotoxin and veratrum alkaloids prolong the cardiac action potential by their action upon the sodium current. Studies on purkenji fibers and cardiac muscle cells suggest that these toxins increase the sodium influx by increasing the resting sodium permeability.

(*v*) *Agents affecting Na^+/Ca^{++} exchange:* Anothopleurin-A (AP-A), a polypeptide of 48 amino acids, isolated from sea anemone, *Anthopleura xanthogrammica*[76], seems to exert potent inotropic action on cardiac tissue and is as effective as the most potent catecholamine, isoproterenol (IC_{50}, 2.5×10^{-9}M). Electro-physiological data suggest that AP-A has a major effect on prolonging sodium current. This would cause an increase in Na^+ entry and indirectly activate Na^+/Ca^{++}-exchange mechanism.

Cardiac glycosides differ in their inotropic induction and development from other positive inotropic substances such as catecholamines, xanthine derivatives and glucagon. A quantitative evaluation involves such parameters as dynamics of contraction, concentration of extracellular calcium, frequency of stimulation and temperature of the system[75].

3.2. *Mechanism of cardiac glycoside-induced inotropy*

The demonstration of specific inhibition of Na-,K-dependent adenosine triphosphatase enzyme by cardiac glycosides[77,78] ensued a great surge in the study of cardiac glycoside–Na,K-ATPase interaction to understand the pharmacological and toxicological action of these compounds. Extensive literature is now available on the interdependence of the inhibition of Na,K-ATPase and inotropic development[77-79]. In general, factors that influence the interactions of cardiac glycoside with Na,K-ATPase include: (a) chemical structure of the glycoside, (b) source of the enzyme, (c) pathological condition, (d) temperature of the system, and (e) nature of membrane lipids.

In experiments, cardiac glycosides inhibit the active transport of Na^+ and K^+ ions across erythrocyte membrane, only when these are present on the outside of the cell membrane[80], thereby indicating that the drug molecule must interact with the outside of the cell membrane only, to elicit its pharmacological effect.

Most of the studies point out that the positive inotropic action of cardiac glycosides, and their toxic effects, are intimately related to their binding to the sodium pump. Such a relation was indicated from the factors which influence digitalis binding to Na,K-ATPase and development of positive inotropy[81]. It is widely held that this direct link with sodium-pump inhibition results in an enhancement in contractile force of myocardium due to an increase in the intracellular transient $[Ca^{++}]$ following membrane excitation, the sequence being, increased $[Na^+]$ leading to increased Na^+/Ca^{++} exchange. Alternatively, a displacement of Ca^{++} from membrane-associated pools of Ca^{++} was also implicated[78,79].

The term Na,K-ATPase is usually applied to more or less purified preparations where the enzyme is still bound to a fragment of the plasma membrane. Highly purified Na,K-ATPase preparations consist mainly of two major polypeptides[78]. Photo-affinity labeling of cardiac glycoside-binding site on Na, K-ATPase by ethyl diazomalonyl derivative of cymarin revealed that the binding site is on the larger polypeptide unit[82] which also has a phosphorylation site[83]. However, a recent study has reported that the larger polypeptide of Na,K-ATPase accounts for only 50% of the covalently bound photo-affinity label, while the other 50% was found in a proteolipidic fraction (M_r 12,000 daltons) which appears to represent a hitherto unknown component of Na,K-ATPase[84]. An important feature of the inhibitory action of a cardiac glycoside upon the enzyme is that the inhibition could be reversed by the addition of K^+ at a low concentration[85] which suggests a competitive mode of inhibition of Na,K-ATPase by K^+ and cardiac glycosides[86].

Although K^+ is believed to bind to the conformationally distinct phosphoenzyme facilitating the dephosphorylation and interfere in ouabain binding promoted by Mg^{++}, Na^+ and ATP, the role of K^+ with respect to P_i phosphorylation is not clear[78]. A steady-state level of phosphoenzyme which seems to exist in the presence of P_i, Mg^{++} and K^+ could facilitate the binding of cardiac glycoside if the conformation of the ATPase2 form is the only criterion. The K^+-cardiac glycoside antagonism is not simply due to K^+-induced dephosphorylation, but can be explained by a

reduced affinity of the K^+-complexed phosphoenzyme for cardiac glycoside. In a recent study, it has been demonstrated that K^+-complexed enzyme binds to cardiac glycoside but with reduced affinity. However, at high concentrations of cardiac glycoside, hardly any inhibition by K^+ to glycoside binding is observed[87].

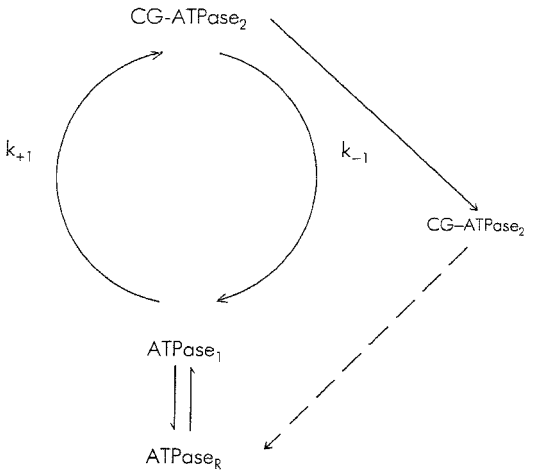
With the availability of radiolabeled cardiac glycoside, it was possible to show that the saturable component of bound-labeled cardiac glycoside parallels the inhibition of Na^+ efflux, a function associated with ATPase activity[81]. The inhibition develops slowly when the extracellular [Na^+] is low. Elevated extracellular [Na^+] enhances and rubidium (Rb^+) ion antagonizes the glycoside-induced inhibition of the sodium pump[79].

Scatchard analysis of the interaction of ouabin with Na,K-ATPase in the presence of Mg^{++} and ATP demonstrated a single type of ouabain-binding site in the enzyme preparations from a number of sources[88]. Similar results were observed for the interaction of different cardiac glycosides with Na,K-ATPase. The presence of a single high affinity non-interacting type of binding site appears to be the general feature of the cardiac glycoside-binding site[89].

Charnock and Post[90], for the first time, demonstrated that ouabain preferentially binds to the phosphorylated Na,K-ATPase. Further, ATP binds to the enzyme in the presence of Na^+, independent of the presence of ouabain, whereas dephosphorylation of K^+ was blocked by cardiac glycosides[91]. This suggests that cardiac glycosides bind to the phosphorylated conformation of the enzyme. The binding required ATP (or other nucleotide triphosphates) and Mg^{++}, and was stimulated by Na^+ and depressed by K^+.

The binding of cardiac glycosides to Na,K-ATPase, supported either by the combination of (Na^+, Mg^{++}, P_i), (Mg^{++}, K^+, P_i), (Mg^{++}, Na^+, ATP) or (Mg^{++}, P_i) stabilizes the phosphoenzyme and renders the complex resistant to K^+-induced dephosphorylation[92]. This leads to the cardiac glycoside binding, and consequently to a stoichiometric inhibition of the enzyme activity[93] and active Na^+ and K^+ transport. The capacity of various enzyme preparations to bind to cardiac glycosides strictly parallels the enzyme activity for various enzyme preparations of different purity[94] suggesting that Na,K-ATPase has only one type of binding site for cardiac glycoside.

Tobin and Sen[95] found that while ouabain binding to Na,K-ATPase was readily reversible at 37°C, glycosides were essentially irreversibly bound at 0°C. The dissociation rate constant increased exponentially with increasing temperature, the half-life of ouabain-enzyme complex obtained with the guinea pig kidney Na,K-ATPase being 9 hours at 0°C and 3 minutes at 37°C. The activation energy calculated from Arrhenius plot and the free energy of interaction of enzyme and ouabain indicated a high entropy change and a large conformational change associated with the binding of ouabain[95]. The dissociation of cardiac glycosides from its complex with Na,K-ATPase and the regeneration of the enzyme activity was demonstrated in red blood cell demonstrating the reversibility in an *in vivo* system[96]. The regeneration of enzyme activity previously inhibited by ouabain, digoxin and digitoxin took place at 37°C.



ATPase$_R$=Resting state (conformation); ATPase$_2$=CG-susceptible state (conformation); CG–ATPase^2=ATPase^2–cardiac glycoside complex.

FIG. 6. Schematic representation of different forms of Na,K-ATPase.

The activity could be accelerated considerably by the presence of Na^+, ATP and in the absence of Mg^{++}[81].

Strophanthidin-3-bromoacetate, an alkylating derivative of strophanthidin, inhibited isolated Na,K-ATPase irreversibly but 70% of positive inotropic effect was reversible in isolated papillary muscle preparation of guinea pig heart suggesting a dissociation of these two events[81]. We could, therefore, generalize, as shown in Fig. 6, that cardiac glycosides bind preferentially to the phosphorylated, conformationally distinct ATPase2 form of the Na,K-ATPase by a reversible process. K^+ binding to the enzyme antagonizes cardiac glycoside, but does not exclude the glycoside interaction with Na,K-ATPase. In other words, K^+ and cardiac glycoside do not compete for the same site, although K^+-induced reduction in the affinity of the cardiac glycoside binding cannot explain the increased dephosphorylation rate caused by K^+.

4. Cardiac glycosides and their receptors

During the last decade a judicious use of different binding techniques employing radiolabeled ligands has led to considerable progress in the identification of pharmacological receptors. Drugs that interact specifically and selectively with receptors

have become indispensable tools to define receptors and to investigate their properties. A pharmacologic receptor for drugs and hormones may be defined as a macromolecule (usually a protein), which with high affinity and selectivity, binds certain drugs or hormones eliciting (for instance, by a conformational change of the macromolecule) their pharmacologic effects. Since receptor is a part of the cell membrane, environmental factors such as membrane fluidity, ions, etc., may influence the drug-receptor interactions. Usually, a membrane-bound receptor is in some way coupled to an enzyme, the stimulation or inhibition of which passes the information on to the interior of the cell[97]. Classification of receptors according to the effector system to which they are coupled indicates that different receptors may be coupled to similar effector system. For example, receptors for divergent ligands such as glycogen, vasointestinal peptides (VIP) and adrenaline are coupled to adenylate cyclase.

The sequence of events in the action of a receptor may be divided into four steps: (a) occupancy of the receptor by the ligand, (b) coupling of the receptor and the effector systems, (c) triggering of a cascade of intracellular biochemical events that lead to the physiological response in the target cell, and (d) desensitization or down-regulation leading to a diminished response to prolonged occupancy of the ligand and receptor.

Numerous studies on the binding of cardiac glycosides with Na,K-ATPase indicate that it is the 'receptor' enzyme for cardiac glycoside[98]. Binding sites for cardiac glycosides on isolated cell membranes or isolated heart preparations have been measured using radiolabeled digoxin[99]. These membrane-bound sites closely associated with Na,K-ATPase activity have been postulated as pharmacologic receptors for cardiac glycosides[100]. In fact, it has been demonstrated that the binding sites for cardiac glycosides fulfilled the requirements of sensitivity at low serum concentrations, saturability, reversibility of binding, high affinity, and selectivity[101]. Techniques such as monoclonal antibodies[102], photolabel[103], immunoassay[104] have been employed to understand the quantitative structure-activity relationship as a function of receptor affinity and selectivity[105-107].

4.1. *Measurement of cardiac glycoside-receptor binding*

The techniques commonly employed in the assay of cardiac glycoside-receptor binding involved filtration binding assay[108]. Since receptors are embedded within membranes, they can be separated by filtration or centrifugation from the soluble ligand. Filtration assay involves separation of the ligand-receptor complex at equilibrium on a glass fibre filter under reduced pressure. In order to give a clear experimental definition for specific binding of cardiac glycoside to its receptor, ouabain was used as the binding ligand[109]. Ouabain is found almost exclusively bound by specific binding to its binding sites in isolated cell membranes (97%) and cardiac homogenates (80%)[110]. Lipophilic glycosides were found to bind to a greater extent usually in non-specific sites. Since they may penetrate intact cells, it is difficult to estimate specific binding of these lipophilic glycosides. Cardiac glycoside-receptor binding was shown to be a reversible process[78]. Using scatchard analysis several authors have demonstrated the presence of uniform, non-interacting binding sites for cardiac

glycosides in the presence of Mg^{++} and ATP. Interestingly enough, in the presence of low inorganic phosphate, the ouabain-binding sites were demonstrated to be nonuniform[78]. A similar observation was made with low Mg^{++} concentrations. A reversal of a curvilinear scatchard to a linear scatchard plot was observed in Mg^{++}-supported ouabain binding in the presence of low K^+ concentrations[78]. Therefore, the overall binding reaction appears to take place only in the presence of P_i and/or K^+.

Several experiments have repeatedly confirmed the inhibition of Na,K-ATPase activity following the binding of cardiac glycoside[111]. In fact, a constant relationship between ouabain-binding capacity of the membrane and Na,K-ATPase activity was confirmed by the observation of the location of the ouabain-binding site and the enzymatically active site on the same polypeptide chain of the enzyme[82]. Thus, the quantitation of specific receptor for cardiac glycoside binding in cell membranes seems to be the quantitation of the Na,K-ATPase molecule[112]. These findings support the view that Na,K-ATPase is the receptor enzyme for cardiac glycosides.

Several pieces of evidence have been produced to prove a correlation between cardiac glycoside binding and the development of positive inotropy[100,113]. The evidence, however, is always obtained by indirect methods. The indirect method involved perfusion of the heart or isolated tissue preparation with labeled cardiac glycosides. The tissue-bound ^3H-glycoside was correlated with increased contractility. However, there is a quantitative correlation between cardiac glycoside-receptor binding and an increase in the force of contraction of isolated heart preparations[109]. These authors have further confirmed that there was no receptor occupancy by cardiac glycoside when the inotropic effect was washed out[110]. It, therefore, appears that cardiac glycosides elicit their physiological response by binding to their specific receptor and activating an effector system (Na,K-ATPase), in cardiac membranes, and that there is adequate quantitative evidence for the coupling of the receptor-effector system and physiological response in cardiac glycoside-receptor interactions. Qualitatively, the specificity of cardiac glycosides to its receptors appears to be a function of the structure of the glycoside molecule. Modification of the structural features of cardiac glycosides drastically affects their binding to the receptors[114]. Such modifications include: (a) lactone ring: saturation of the α-β unsaturated lactone ring leads to a ten-fold decrease in the affinity of the glycoside to its receptor[114], (b) steroid ring: the modification of normal *cis* A/B ring junction to a *trans* configuration reduces the binding thirty fold[114], (c) the number of sugar moiety of the cardiac glycosides is inversely related to the affinity of the ligand to its binding site (*see* Section 2.2. for a detailed discussion). Thus, monoglycosides have the highest affinity followed by diglycosides. This observation suggests that the sub-site of the cardiac glycoside interacting with sugar has a limited space. Even acetylation of the sugar reduces the affinity considerably[54,57]. Using photo-affinity labeling of digitoxin further indicated the spatial limitation of the sugar-binding sub-site[103].

A recent study on the potency of 17 digitalis derivatives on cardiac Na,K-ATPase from cat, bovine and human heart indicated that the binding of the whole molecule was necessary for optimal potency. Further, the kinetics of binding were similar in

feline and human systems, corroborating the physiological similarities of feline and human systems to cardiac glycosides[115]. Studies on the structure-activity relationship, from indirect binding experiments with bovine and guinea pig cardiac Na,K-ATPase, show a significant correlation between the K_d values of guinea pig Na,K-ATPase and inotropy, demonstrating that the pharmacologic receptor for inotropy is a part of the enzyme[116]. Secondly, the inhibition of binding of ^{3}[H]-ouabain and ^{3}[H]-digitoxin can be used to probe the relative potencies of unlabeled cardiac glycosides. Finally, studies of structure-activity relationship indicate that the functional groups on the steroid, as reflected by affinity for the site, determine the potency and inotropic effects[114].

5. Contrary views on the mode of action of cardiac glycosides

Positive inotropic effect of cardiac glycosides is temporarily linked to and even dependent upon their ability to bind to and inhibit the activity of Na,K-ATPase. The evidence for a correlation between the binding of cardiac glycosides, inhibition of Na,K-ATPase and development of positive inotropy was always obtained by indirect methods[100,113]. It has not been possible to establish a direct quantitative relationship between specific cardiac glycoside binding and its effect. Hence, several authors have questioned the relationship between the effect of cardiac glycoside on Na,K-ATPase and the force of contraction[117-119]. It appears that the cardiac glycoside-induced inhibition of Na,K-ATPase as the sole basis of positive inotropy is an oversimplified mechanism in the context of recent developments. A growing amount of data indicates a dissociation of the two effects[117,120]. In fact, therapeutically effective doses of cardiac glycosides are even reported to activate Na,K-ATPase[121], and toxic effects of cardiac glycosides may be due to altered ionic composition caused by inhibition of Na^+, K^+ pump[122]. The attempts to establish a direct relationship between the interactions of cardiac glycoside with ATPase and inotropy get further complicated if we reexamine the processes underlying the inotropic development, particularly the role of Ca^{++} in the process of inotropy.

There is a general agreement that at molecular level positive inotropic effect does not involve their direct interaction with contractile proteins[123] or myocardial energy metabolism[124]. However, many investigators have shown a correlation between positive inotropic effect and an increase in Ca^{++} exchangeability[125,126], which means positive inotropic response enhances intracellular Ca^{++} availability so that more Ca^{++} is available to interact with contractile and regulatory proteins.

Since Na,K-ATPase enzyme is responsible for maintaining differential distribution of Na^+ and K^+ across cardiac cell membrane, its inhibition would result in a raised intracellular concentration of Na^+, particularly in the immediate vicinity of the cytosolic side of the cell membrane. Under this condition, and particularly because cardiac muscle cell has the ability to exchange Ca^{++} for Na^+[127], the intracellular availability of Ca^{++} may increase (Fig. 7). Although the increase in intracellular Ca^{++} may be insufficient to activate or influence tension development[128], it may supplement the supply of trigger Ca^{++}, thus promoting additional release of Ca^{++}

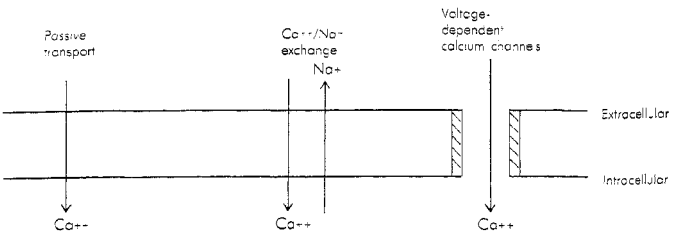


FIG. 7. Diagrammatic representation of the process by which Ca^{++} is mobilized into the cell.

and producing positive inotropic response (Fig. 8). The cardiotonic effect of cardiac glycosides closely resembles that of calcium. Studies aimed at establishing the influence of extracellular concentration of Ca^{++} on cardiac glycoside-induced positive inotropy has been demonstrated in cat, frog and guinea pig heart muscle[129]. Raised extracellular Ca^{++} also potentiates the cardio-toxicity of cardiac glycosides. However, at low concentration of Ca^{++}, the time necessary for a known concentration of cardiac glycoside to produce maximum inotropic response is reduced (Fig. 9).

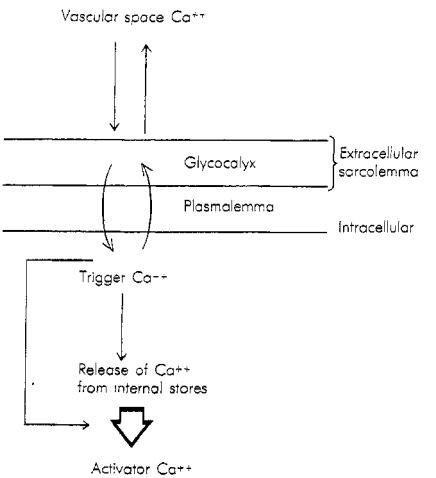


FIG. 8. Diagrammatic representation of mobilization of Ca^{++} by a small amount of trigger Ca^{++}.

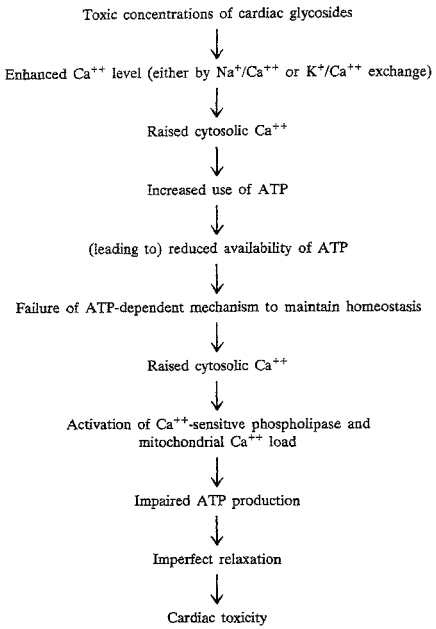


FIG. 9. Hypothesis for cardiac glycoside-induced cardio-toxicity involving cellular calcium.

By applying recently developed sensitive techniques such as the measurement of uptake of labeled Ca^{++} [130] and voltage clamp studies[131], it was found that cardiac glycosides enhance slow inward current for which Ca^{++} is the predominant charge carrier (Fig. 7). The small but significant increase in Ca^{++} influx may involve transport of Ca^{++} through slow channels[131], but it seems unlikely that it is of sufficient magnitude to account for positive inotropic response. In addition to an increase in slow inward current, positive inotropic effect of cardiac glycoside may affect a redistribution of Ca^{++} associated with superficial pools[132]. The interaction of cardiac glycoside with cell membranes may increase the exchangeability of the superficial pool of Ca^{++} associated with cell membrane. Since cardiac glycoside-induced positive inotropy alters the slow inward current, the interaction of Ca^{++} antagonists such as verapamil and nifedipine offers further evidence implicating the role of Ca^{++} channels in cardiac glycoside-induced positive inotropy. Calcium channel antagonists reduce the amount of active Ca^{++} in heart cells by inhibiting the ion flux across the

voltage-dependent Ca^{++} channels[133-134]. The positive inotropic effect of cardiac glycosides may, at least, in part, involve calcium channels in the development of positive inotropy. Verapamil was found to antagonize arrhythmic and atrial fibrillation caused by cardiac glycosides[135,136], further validating the observation that the toxic effects of cardiac glycosides are due to altered ionic composition.

5.1. *Na,K-ATPase as calcium labilizing structure*

The binding kinetics of cardiac glycosides to specific binding sites indicate that only one particular conformation of Na,K-ATPase displays a high affinity towards the drug. This conformation occurs only transiently during the pump cycle[101], the concentration of which depends on the functional state of the cardiac cells[122]. Therefore, both the concentration and the subsceptible conformation of the ATPase determine the extent of receptor-cardiac glycoside interaction. Na,K-ATPase is a proteolipidic enzyme integrated into the plasma membrane. Apart from its function as an ionic pump, this enzyme acts as a Ca^{++}-binding structure. The lipid moiety of the ATPase essentially consists of phospholipids such as phosphotidyl serine[137-140]. As an integral part of the enzyme, phosphotidyl serine constitutes the inner annulus of the lipids which display a high affinity for Ca^{++} provided that the plasmalemma is polarized. The protein part of the Na,K-ATPase was shown to undergo conformational changes in the structure during the process of recycling[141-143]. Further, the lipids participate in the conformational changes associated with the transport cycle of the protein part of Na,K-ATPase[137], which involve a change in the spatial arrangements of the lipids during each transport cycle. Experimental evidences from purified Na,K-ATPase and cardiac plasmalemma indicate the involvement of proteins associated with the protein part of the enzyme in the conformational changes[141,144]. Along with depolarization, the high affinity of the phospholipids to Ca^{++} is lost, releasing Ca^{++} into cytosol[145,146]. The extent of release of Ca^{++} depends on the spatial arrangement of the complexing ligands. The amount of Ca^{++} bound to the depolarized membrane which constitutes the 'residual affinity' determines the amount of Ca^{++} released per excitation. The residual affinity which is due to the spatial arrangement of the lipids is changed during the activity of the Na,K-ATPase. With increased ATPase activity, the residual affinity will decline, resulting in an increased release of Ca^{++} during each excitation, where the driving force for the Na,K-ATPase activity is Na^{+} load.

A schematic representation of the functional states of Na,K-ATPase in intact heart muscle cells is shown in Fig. 6. The binding of cardiac glycosides to ATPase2 conformation results in the preservation of this conformation which leads to (a) the exclusion of this complex from the normal transport activity of the enzyme, and (b) the transient sustaining of the labilization of Ca^{++} binding inherent to this conformation allows a transient labilization of Ca^{++} which is due to reduced residual affinity. The coupling of the ion-pumping activity of Na,K-ATPase and the Ca^{++}-binding nature of the transient conformation, probably explains the enhanced Ca^{++} binding by cardiac muscle microsomes treated with cardiac glycoside[129,147]. The results obtained with Na,K-ATPase and plasmalemma membrane could be correlated to findings in

intact cardiac tissue[129]. In fact, in ventricular muscle, the size of the superficial Ca^{++} fraction was shown to increase with concentration[147].

The alternative hypothesis could be summarized as follows: Cardiac glycosides bind only to one conformation of the Na,K-ATPase, which exists during the cycling of the ATPase (probably the ATPase2 form). The amount of bound cardiac glycosides depends not only on its concentration, but also on the amount of susceptible conformation, which depends on the activity of the enzyme. Therefore, cardiac glycosides bind also to resting muscle (resting Na^+, flux), but at a low rate due to low pump activity. However, binding enhances the Ca^{++} exchange rate and produces positive inotropic effect. However, if Na,K-ATPase is maximally burdened due to Na^+ influx, cardiac glycoside will become rapidly bound but only induce toxic effects, since ion gradients completely escape the control. This explains the beat dependency and the requirement of extracellular Ca^{++} for cardiac glycoside binding and production of inotropic and toxic effects. This also explains the positive inotropic effect as a Ca^{++} binding function of Na,K-ATPase whose normal ion-pumping effect is coupled to Ca^{++} mobilization. Currently attempts are being made towards an understanding of cardiac glycoside-induced positive inotropy as a function of Ca^{++} interaction with specific conformations of Na,K-ATPase.

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