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REVIEW ARTICLE

Synthesis and Biological Activity of Semisynthetic Digitalis Analogs

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Keyphrases □ Digitalis—synthesis and biological activity of semisynthetic analogs, review □ Cardiotonic steroids—synthesis and biological activity of semisynthetic digitalis analogs, review □ Structure–activity relationships—digitalis analogs, model for digitalis receptor, review □ Guanylhydrazones—synthesis and biological activity, review □ Receptors, digitalis—model proposed, structure–activity relationships of digitalis-like compounds, review □ Steroids, cardiotonic and cardioactive guanylhydrazones—synthesis and biological activity, structure–activity relationships, digitalis receptor proposed, review

CONTENTS

The cardiac glycosides are one of the most important and widely used groups of drugs in clinical medicine. These vegetable drugs act on the failing heart to produce a marked inotropic effect, and for this purpose they are unrivaled by any synthetic or semisynthetic substitute. The cardiac glycosides are also one of the most toxic groups of substances in general clinical use.

In a recent prospective study, Beller *et al.* (1) examined 931 consecutive patients admitted to the medical service at the Boston City Hospital. They found that 15% of the patients admitted were taking digitalis on admission; of these, 23% showed definite manifestations of digitalis toxicity and a further 6% showed possible digitalis toxicity. The mortality rates for the various groups of patients were: *(a)* patients receiving digitalis on admission and showing definite signs of digitalis toxicity, 41%; *(b)* patients receiving digitalis on admission and showing no signs of digitalis toxicity, 17%; and *(c)* patients not receiving digitalis, 9%.

Many factors other than the use of digitalis would have contributed to these mortality rates, but the results of this survey, one of the few prospective studies available, generally confirm previously reported rates of digitalis toxicity. Beller *et al.* (1) cited seven other studies in which the mortality rates of patients who

developed digitalis toxicity ranged from 7 to 50% (average of 22%). Recent accounts of the clinical pharmacology and toxicology of cardiac glycosides are given in Refs. 2–11.

The incidence of digitalis intoxication may be increasing (12). This apparent trend was discussed by Beller *et al.* (1) who cited several possible contributory factors, including an increase in the proportion of the population at risk. Older people generally show a higher rate of digitalis toxicity to "normal" doses of digitalis, possibly because of decreased kidney function (13). However, Beller and associates believe that the increase in the *reported* incidence of digitalis toxicity is due largely to improved methods of surveillance and to improved diagnosis of toxicity based on a more adequate knowledge of the electrocardiography of arrhythmias.

The need for an effective but safer substitute for digitalis is thus obvious. But there is another reason for the current interest in digitalis and digitalis-like compounds. The cardiac glycosides are unique specific inhibitors of the pumping mechanism which transports sodium and potassium across cell membranes against the electrochemical gradient. The pumping entity has been identified as a spatially oriented, membrane-bound adenosine triphosphatase which requires the presence of magnesium ions and is activated by changes in the concentrations of sodium and potassium ions. It is specifically inhibited by ouabain and other biologically active cardiac glycosides and related cardiotonic steroids. This enzyme, henceforth referred to as Na^+,K^+-ATPase, has been the subject of approximately 1500 published scientific papers in the 10-year period prior to 1972 [figure cited by Schwartz *et al.* (14) in their recent review].

The current interest in Na^+,K^+-ATPase arises because of its key role in controlling cell physiology and because it is the putative receptor mediating the effects of cardiac glycosides on myocardial contractility. A characteristic feature of almost all living cells is that the concentration of potassium is high in the intracellular fluid and low in the extracellular fluid and that the reverse applies in the case of sodium. The cell usually achieves this situation by actively exchanging three intracellular ions of sodium for two extracellular ions of potassium. The cell thereby undergoes a net loss of ions. It is believed that the development of this capacity was an essential step in the evolution of life. For millions of years, the primoidal cell must have struggled with the problem of accumulation of water as a result of the osmotic effects of nondiffusible organic molecules. The cell may have responded by developing a mechanism (Na^+,K^+-ATPase) whereby a net loss of ions was achieved (15). In doing so, it would have established an electropotential difference across the cell membrane and laid the basis for such excitatory events as the propagation of impulses in nerve and muscle. Other membrane phenomena that have been linked directly or indirectly with Na^+,K^+-ATPase activity include amino acid transport, sugar transport, antilipolytic effects, transport of *p*-aminohippuric acid, and transport of biogenic amines. (For references, see Ref. 14.)

Considerable evidence supports the theory, first proposed by Repke and Portius (16), that the effects of cardiac glycosides on heart muscle contractility result from the inhibition of myocardial Na^+,K^+-ATPase. There is also much evidence to the contrary, including the fact that the cardiac glycosides seem to exert a wide range of primary effects, ranging from the release of intracellular bound calcium to direct effects on the polymerization of actin and myosin (17). The investigator is thus faced with the extremely difficult task of correlating one of several events at the biochemical level with events at the physiological level. One important body of evidence that links Na^+,K^+-ATPase with the active transport of Na^+ and K^+ and with the effects of cardiac glycosides on heart muscle contractility is the analysis of structure–activity relationships. A consistent parallelism has been claimed to exist between cardiotonic activity and the ability of cardiac glycosides to inhibit both Na^+,K^+-ATPase and the active transport of Na^+ and K^+. However, these structure–activity relationships are less impressive when viewed against the limited range of structural modifications that have been made of the basic cardiac glycoside molecule.

All that has been said so far emphasizes the need for a more extensive search for digitalis-like compounds. The objectives of the medicinal chemist in this area may be defined as follows:

1. To develop cardiotonic steroids with an improved therapeutic index. (This is based on the assumption that toxic and therapeutic effects can be separated.)
2. To contribute to the study of the mode of action of cardiac glycosides by developing systematic and graded series of compounds for use in structure–activity relationship studies. (The present authors believe that they have achieved this objective with respect to defining the essential structural features of the C-17β-lactone ring and its biologically active isosteres, as discussed under *Structure–Activity Relationships.*)
3. To search for new types of therapeutic agents based on the selective inhibition of Na^+,K^+-ATPase in tissues other than the heart.

The factors limiting such studies have been attributed to the difficulties in the chemistry of the cardiac glycosides. According to Thorp and Cobbin (18): ". . . the problem is an enormous one since the chemistry of the cardiac glycosides is so difficult. It is no exaggeration to say that the work of fifty chemists might be insufficient to occupy the full time of one pharmacologist." On the basis of personal experience, it is suggested that the ratio should be about 10 to one to synthesize and screen a series of analogs.

The purpose of the present review is to give an account of the major semisynthetic modifications that have been made on the cardiotonic steroids. Only brief mention is made of the many studies of structure–activity relationships among the naturally occurring cardiotonic steroids since this field has been covered by numerous reviews which will be cited. The mode of action of cardiotonic steroids will be discussed in some detail, even though there are many

up-to-date and far more comprehensive reviews of this subject. The reason for including this section is to highlight those aspects that are relevant to the interpretation of structure–activity relationships and the development of receptor concepts.

The review concludes with an account of the authors' own work in replacing the 17β-lactone with a series of open-chain substituents. On the basis of this work, a model is proposed for that part of the "digitalis receptor" that accommodates the lactone and its biologically active isosteres.

PROPERTIES OF NATURALLY OCCURRING CARDIOTONIC STEROIDS

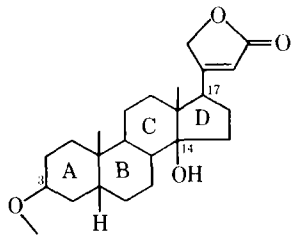
Any discussion of structure–activity relationships among cardiac glycosides and related compounds must take note of the problems associated with the determination of cardiotonic and cardiotoxic activity. Considerable variations in both actual and relative potency are observed when compounds are compared using different test preparations and different species (19).

The common practice of using toxicity measurements for the initial assessment of potency of cardioactive compounds can be misleading (19, 20). Wolff *et al.* (21) showed that newly synthesized compounds that displayed cardioactive properties when tested by the cat toxicity method showed no cardiotonic properties when tested on the isolated rabbit atrium preparation. This finding does not imply a separation of the two facets of digitalis-like activity but rather that the cardiotoxic properties measured were not of the digitalis-like type.

The significance of reported cardiotonic activity is thus difficult to assess, at least in quantitative terms. The determination of digitalis-like activity of new compounds needs to be based on a spectrum of test procedures, and a selection of well-tested reference compounds must be included in the test program. Details of various biological procedures used in estimating cardiotonic activity were extensively reviewed (18). Factors that modify the action of cardiotonic steroids on heart function also were reviewed recently (17).

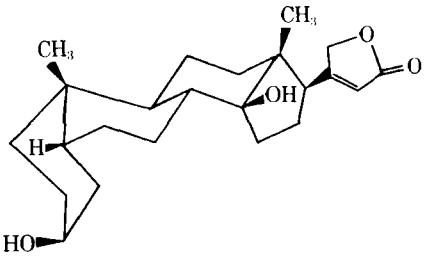
Within the constraints discussed, the following generalizations may be made. Qualitatively, the physiological activities of the naturally occurring digitalis-like compounds are similar, implying a fundamental similarity in their mechanism of action. Quantitatively, individual members of this class of compounds exhibit differences in potency and duration of action. However, the therapeutic ratios of most, if not all, of these compounds are similar, supporting the widely held beliefs that the toxic effects of digitalis-like compounds are an extension of the positive inotropic effects and, hence, that it would not be possible to improve therapeutic ratios by molecular modification. These assumptions have yet to be adequately tested, but it must be emphasized that the term therapeutic ratio has no absolute meaning and will vary according to what portions of the log dose–response curves are compared.

Digitoxin (a cardenolide) (I), digitoxigenin (II), and bufalin (a bufadienolide) (III) are representative examples of naturally occurring cardiotonic steroids. The biological activity of these molecules is thought to arise as a result of a specific interaction between a receptor and the aglycone (or genin) portion of the molecule. If the glycoside is cleaved, the genin retains activity whereas the isolated sugars are devoid of cardiotonic effects (22). However, the presence of the sugars is thought to retard detoxification effectively and to confer on the molecule certain physical properties important for uptake and distribution (23). These observations are important in the design of new cardiotonic steroids since a molecule with a free hydroxy group at C-3 may undergo rapid metabolism and show no useful activity (23). In other words, modification of the side chain at C-3 is an appropriate means of varying the pharmacokinetic properties of cardiotonic steroids.

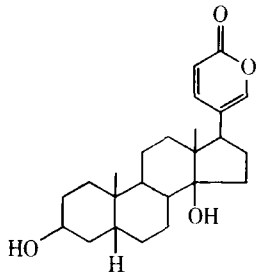


(digitoxose)$_3$

I: digitoxin (cardenolide)



II: digitoxigenin



III: bufalin (bufadienolide)

The stereochemistry of the steroid portion of cardiac glycosides is unusual in that the C/D and usually the A/B ring junctions have the *cis*-configuration (II). The general chemistry of the cardiac glycosides and the genins has been well documented (24, 25), and the chemistry of the sugar residues of natural and semisynthetic glycosides has been reviewed (26, 27).

For many years, studies on structure–activity relationships of cardiac glycosides have been limited to the determination of relative potency ratios of naturally occurring glycosides or to relatively minor semisynthetic modifications of functional groups com-

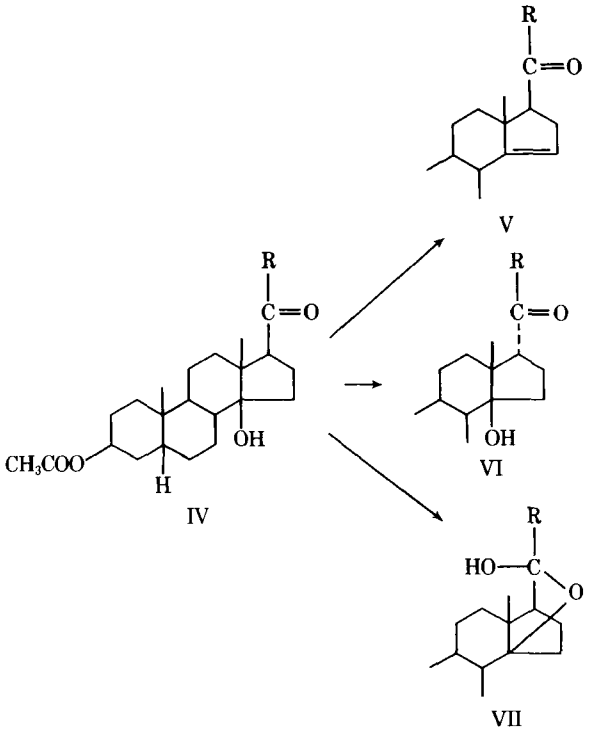
mon to this class. The early work in this field [reviewed by Tamm (22) and Chen (28)] established three structural features thought to be associated with cardiotonic activity (29):

1. *The unsaturated lactone at C-17.* This is present either as a butenolide ring in the cardenolides (*e.g.*, I and II) or as a 2-pyrone ring in the bufadienolides (*e.g.*, III). Saturation of the lactone ring results in considerable loss in activity (19, 30).

2. *The stereochemistry of the steroid molecule.* A C/D *cis*-configuration in conjunction with β-orientation of the lactone ring at C-17 is the basic stereochemical requirement for cardiotonic activity. Alteration of the stereochemistry at either of these centers results in abolition of activity. The 17α-cardenolides, for example, are inactive (22), as is the recently synthesized 14α-digitoxigenin (31). The 14α,17α-cardenolides also have been shown to be devoid of activity (32). The *cis*-configuration of the A/B ring junction, when present, confers maximum activity. Epimerization to the 5α-configuration, as found in uzarigenin (5α-digitoxigenin), results in a reduction in potency. Uzarigenin is approximately one-half as potent as digitoxigenin (22).

3. *The hydroxy groups at C-3 and C-14.* Potency is greatest when the β-hydroxy group at C-3 is combined as a glycoside and is generally reduced when this group is oxidized or esterified (19). Epimerization to the 3α-configuration leads to almost complete loss in activity (23). Recent studies on the importance of the oxygen function at C-3 showed that 3-desoxydigitoxigenin, synthesized independently by Zürcher *et al.* (31) and by Saito *et al.* (33), is almost equipotent with digitoxigenin when tested for its effects on isolated cardiac muscle (34) or for its inhibitory action on Na^+,K^+-ATPase (31). This indicates that the 3β-hydroxy is not an indispensable requirement for cardiotonic activity.

Likewise, the 14β-hydroxy group is not an absolute requirement for activity, but its presence enhances potency. This group may be replaced by a 14β,15β-oxido group as occurs in resibufogenin or in 14β,15β-epoxy-β-anhydrodigitoxigenin with the retention of activity (35–37). The fact that a 14β-oxygen function is not an essential requirement for cardiotonic activity was confirmed by the synthesis of 14-desoxy-14β-uzarigenin (38), which was shown to possess a definite cardiotonic activity, somewhat less than that of uzarigenin (39).



Scheme I

DEVELOPMENT OF SYNTHETIC METHODS

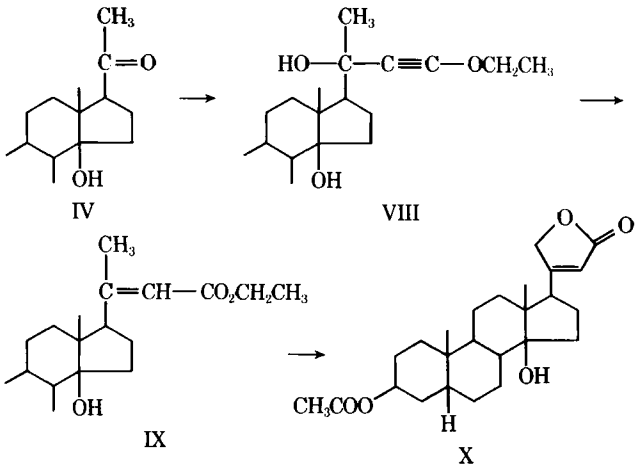
The difficulties presented by the chemistry of the cardioactive genins are demonstrated by the considerable amount of work carried out over many years aimed at the total synthesis of naturally occurring cardenolides. In the 1940's, Ruzika, Plattner, and colleagues and Elderfield and coworkers achieved efficient methods for the synthesis of the 17β-butenolide ring and the introduction of the 14β-hydroxy group into model substances. But the goal of introducing both of these functional groups into the one steroid molecule while still maintaining the C/D ring junction in the *cis*-configuration was not achieved.

These studies were resumed in 1956, and in 1962 the successful synthesis of digitoxigenin (II) was reported (40). In a review, Sondheimer (41) drew attention to three potential difficulties involved in the construction of the 17β-lactone from 14β-hydroxy-20-oxosteroids (Scheme I). First, 14β-hydroxy steroids (IV) are readily dehydrated with acids to give the corresponding Δ^{14}-compounds (V), and no practical method for protection of this group is known*. Reactions in which strong Lewis acids are present, *e.g.*, the Reformatsky reaction, are, therefore, excluded. Second, the 17β-side chain of 14β-hydroxy steroids exists in a thermodynamically unstable configuration, and conversion to the more stable 17α-isomer (VI) occurs if possible. Third, interaction between the 14β-hydroxy group and the 17β-side chain can take place and, in the case of 20-oxosteroids, the relatively unreactive hemiketal (VII) is produced.

Sondheimer's successful synthesis was eventually achieved by reacting the ketone (IV, R = CH_3) with lithium ethoxyacetylide in tetrahydrofuran followed by mild treatment of the ethoxyacetylenic carbinol (VIII) with dilute acid. Oxidation of the resulting α,β-unsaturated ester (IX) with selenium dioxide resulted in about 30% yield of digitoxigenin acetate (X) (Scheme II). Since the work of Sondheimer, other successful syntheses of naturally occurring cardenolides have been reported, and some useful synthetic methods have emerged (42–45).

Similarly, much work has also been directed at the total syntheses of the second group of cardioactive

**Note added in press:* It was recently shown that the 14β-hydroxy group of digitoxigenin can be acetylated using isopropenyl acetate and *p*-toluenesulfonic acid (method developed by R. Rawson, Syntex Research, Palo Alto, Calif., personal communication).



Scheme II

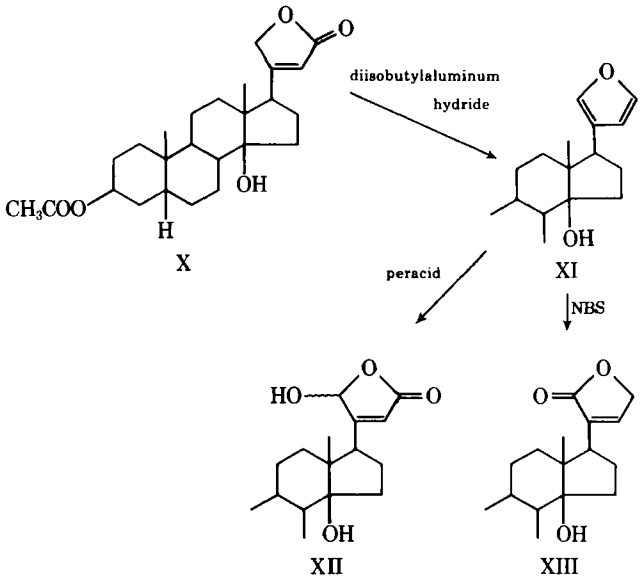
steroids, the bufadienolides. Only recently has this goal been achieved (46) by the synthesis of bufalin (III) and resibufogenin from common steroids. Following this report, other workers have also reported the successful syntheses of naturally occurring bufadienolides and other steroidal 5′-substituted 2-pyrones (47–50).

STRUCTURAL MODIFICATIONS OF CARDIOTONIC GENINS

The value of steroids as hormones and as hormonal-type drugs is probably due to the fact that the deployment of a number of simple functional groups around the rigid steroid moiety enables a wide variety of unique coding systems to be achieved. It is not surprising then that the bulk of the efforts devoted to the chemical modification of cardioactive genins has been designed to delineate the effects of the various substituents and functional groups on physiological potency. Recent publications containing somewhat limited reviews on new cardioactive steroids have been published (51–58).

The major emphasis in this section centers on the chemistry of new compounds synthesized as potential digitalis-like substances. Brief reference to biological effects is given where the information is available and seems of significance. In many cases, biological data were not given or, in the case of compounds found in the patents section of *Chemical Abstracts*, were not readily accessible. The transformation of cardioactive genins into structural isomers of steroid hormones has also received some attention (59, 60); but since these compounds are not cardiac stimulant substances, they will not be discussed further.

Modification of 17β-Lactone Ring—This functional group is common to all cardiac glycosides, and several studies have established its importance in the biological activity of this compound class. Until recently, only a few modifications of the lactone ring had been made, including saturation of the double bond and epimerization to the 17α-configuration. Methods of replacing the lactone ring with other isosteric hetero rings have been investigated (61). A route has been devised to convert the butenolide ring of the cardenolide genins to a furan ring bonded at

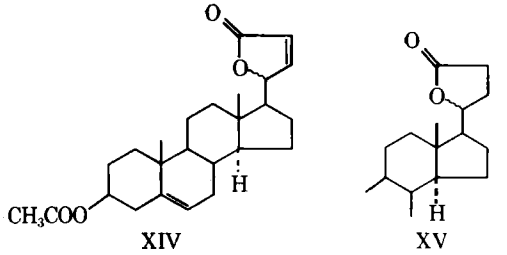


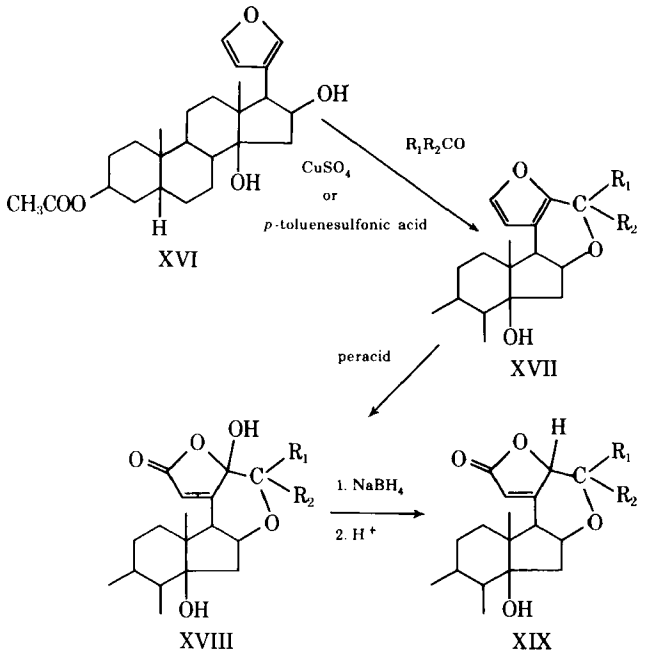
Scheme III

the 3-position to C-17 of the steroid (62) (XI). For example, diisobutylaluminum hydride reduction of digitoxigenin 3-acetate (X) gave the corresponding 17β-(3-furyl) derivative (XI). Such a compound can be oxidized in different ways to give either the 21-hydroxy derivative (XII) of the starting cardenolide or an isomeric cardenolide (XIII) (Scheme III) (63). The latter compound is of particular interest because it differs from the natural cardenolide only in the position of attachment of the butenolide ring to the steroid nucleus.

Several 17β-(3-furyl) steroids derived from naturally occurring cardenolides have been prepared for biological evaluation (64). Minesita *et al.* (65) reported that 17β-(3-furyl) steroids such as XI exhibit cardiotonic activity comparable to the cardenolides. This suggests that the unsaturated lactone is not essential for physiological activity and can be substituted by an isosteric hetero ring. Similarly, many isomeric cardenolides have been prepared by oxidation of 17-furyl steroids (66). Deghenghi (53) reported that the isomeric cardenolides such as XIII increased the force of myocardial contraction in dogs with pentobarbital-induced heart failure, with a potency comparable to digitoxigenin (II). It was also claimed that this compound elicited an inotropic activity at a dose considerably smaller than that producing toxic effects. This property is interesting because the main problem involving the clinical use of cardiac glycosides is related to their narrow therapeutic ratio.

Pettit *et al.* (67) described another isomeric cardenolide (XIV) in which the lactone ring was substi-





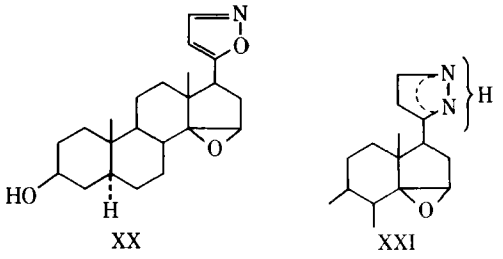
Scheme IV

tuted as the γ-carbon instead of the β- or α-carbon found, respectively, in the natural cardenolides or in the previously described isomeric cardenolide (XIII). Compound XIV and its butenolide analog (XV) (68) also differ from the natural series in that they lack an hydroxy group at C-14 and the C/D ring junction has the *trans*-configuration. Both these compounds were biologically inactive.

Further investigation of the 17β-furyl steroids (69) showed that 17β-(3-furyl) derivatives of 16β-hydroxycardenolides (XVI) underwent a novel condensation with simple carbonyl compounds in the presence of copper sulfate or *p*-toluenesulfonic acid to form the bicyclic derivative (XVII). Oxidation of XVII with peracid, as previously described, gave the lactol (XVIII) which, on reduction with sodium borohydride, gave the bicyclic derivative (XIX) which incorporates the original unsaturated lactone ring (Scheme IV). The biological activity of these compounds was not reported.

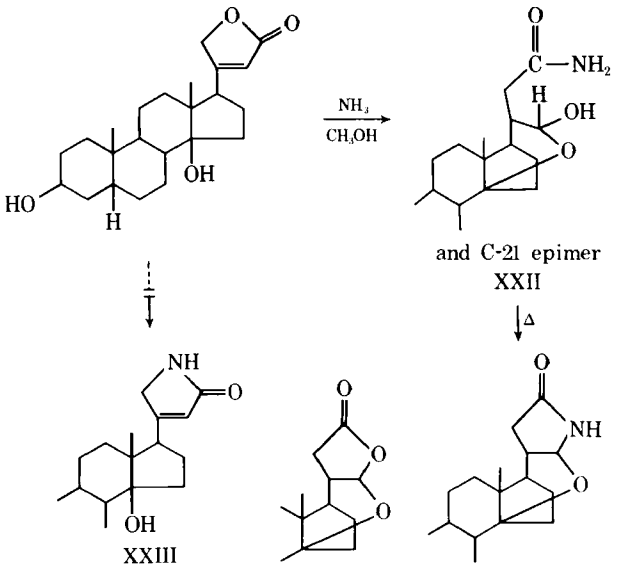
Nambara *et al.* (70) recently described the preparation of 17β-(5-isoxazolyl) and 17β-(3-pyrazolyl) derivatives of 14β,15β-oxidosteroids (XX and XXI, respectively) as potential cardiotonic compounds. In each case the lactone ring of the original cardenolide was replaced with an isosteric hetero ring. Both these compounds possess the 14β,17β-configuration essential for physiological activity in the natural cardenolides. For biological comparison, the 17α,14β-isomers were also prepared[1]. These workers felt that biological assay of the 14,17-*cis*-steroids (XX and XXI) may indicate the significance of the oxygen atom attached to C-21 of the cardioactive cardenolides.

A lactam analog (XXIV) of isodigitoxigenin has been described (71, 72). It was obtained by ammonolysis of digitoxigenin in methanol solution to give the lactol amide (XXII) which, on heating at 200° or on treatment with acid, cyclized to give the lactam (XXIV) (Scheme V). In no instance was the γ-crotonolactam (XXIII) obtained, probably due to the tendency of cardenolides to cyclize to the "iso" form in basic medium. Isodigitoxigenin (XXV) was considered to be an intermediate in the reaction.



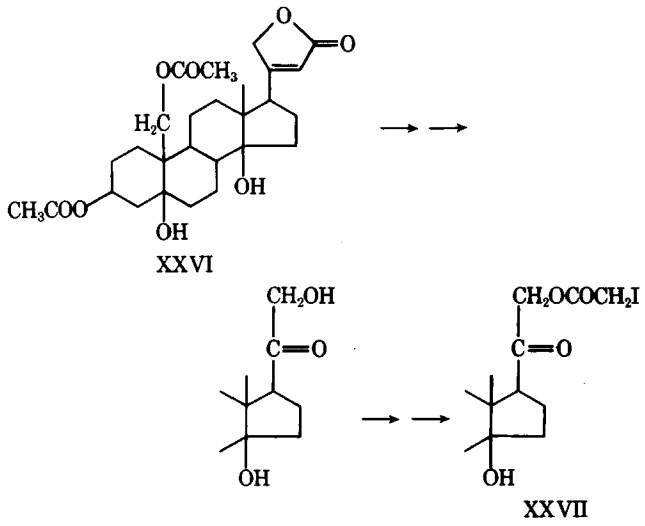
The influence of the lactam (XXIV) on the response elicited by physiologically active cardenolides on three different biological systems was examined. Although the lactam was devoid of cardiotonic activity, it inhibited the early inotropic response of acetyldigitoxigenin on isolated guinea pig atria. However, the effect of acetyldigitoxigenin on cat toxicity or on Na^+,K^+-dependent ATPase was not affected by the lactam. It was thought that the lactam may inhibit the mechanism responsible for transport of cardiotonic steroids across the cell membrane and, hence, increases the time required for the cardenolide to reach optimal concentrations at the inotropic receptor. Examination of acetylisodigitoxigenin (XXV) revealed that this compound also inhibited the positive inotropic response of guinea pig atria to cardenolides. Compounds of this nature (73) may prove useful in elucidating the mode of action of cardenolides.

Further studies relating to the role of the lactone ring in the biological activity of cardiac glycosides followed the discovery (74) that the 20-oxo-21-iodoacetate (XXVII) derived from the degradation of strophanthidol diacetate (XXVI) (Scheme VI) exhibited cardioactivity when tested on cats. The activity of



Scheme V

[1] The compounds prepared by Nambara *et al.* (70), described here and elsewhere in this review, were tested on the isolated frog heart preparation and found to be inactive (personal communication).



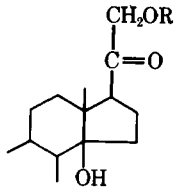
XXVI XXVII

Scheme VI

this compound was originally interpreted as a cardiotonic effect, resulting from the alkylating properties of the iodoacetate group. This finding led to the synthesis of a number of related compounds (75) in which the butenolide ring of strophanthidol and digitoxigenin was replaced with functional groups considered capable of reacting with sulfhydryl groups (XXVIII and XXIX). The basis of design of these compounds was the hypotheses (76) that alkylation of essential sulfhydryl groups on the receptor may be involved in drug action and that the unsaturated lactone or the iodoacetate group may perform this function.

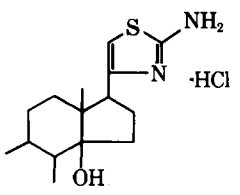
An analogous series of C-17α-compounds was prepared together with another series of steroids containing the same alkylating groups attached to different steroid moieties. Tests on isolated rabbit atria showed that all compounds prepared were devoid of cardiotonic activity. It was concluded that the observed cardioactivity, when tested on cats, was a cardiotoxic action unrelated to any cardiotonic effects. Such findings emphasize the care required when interpreting biological data on new compounds.

Other modified cardenolides capable of acting as alkylating agents have also been described (77). These compounds possess a maleimide function at

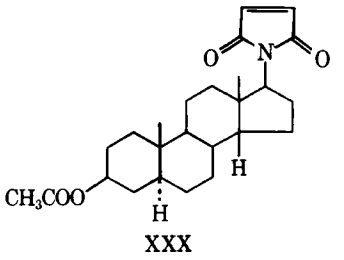


R_1 = $COCH_2Cl$, $COCH=CH_2$, COC_2H_5, or $CO(CH_2)_2CO_2H$

XXVIII



XXIX

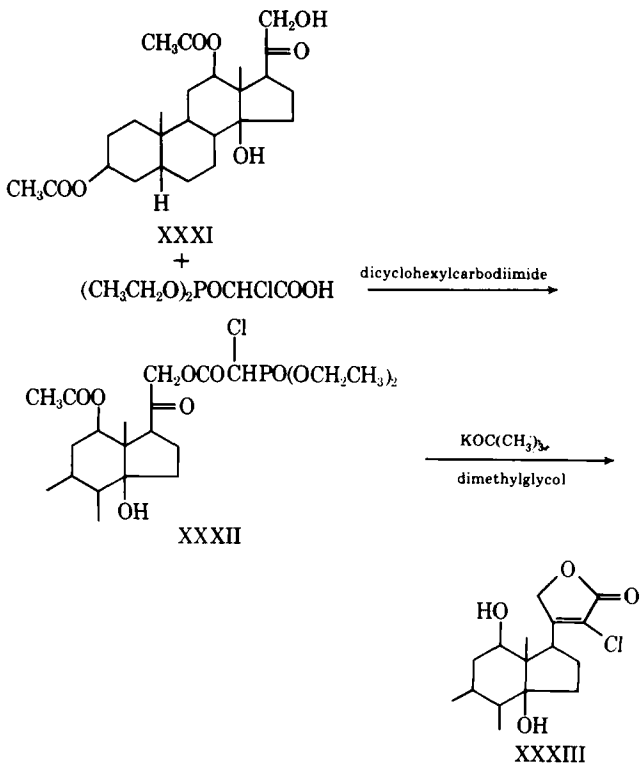


XXX

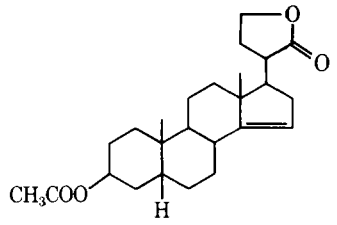
C-17, which is another functional group considered capable of reacting with sulfhydryl groups. Compound XXX fulfills the stereochemical requirements at C-14 and C-17 for cardiotonic activity. Biological evaluation has yet to be reported, but following the results obtained (75) it seems unlikely that alkylation of sulfhydryl groups is responsible for cardiotonic activity.

The introduction of halogen, alkyl, and alkoxy substituents into the 22-position of the lactone ring has been reported (78). The compounds were prepared by condensing the 21-hydroxy-5β-pregn-20-one derivatives (XXXI) of a series of cardiotonic steroids with appropriate phosphonate reagents followed by intramolecular PO-activated cyclization. A typical sequence is shown in Scheme VII. The authors briefly referred to the biological activity of these compounds, stating that contraction-promoting activity was increased by introduction of a fluoro group and decreased by introduction of a methoxy group. These observations are compatible with the model for the digitalis receptor proposed under *Structure–Activity Relationships.*

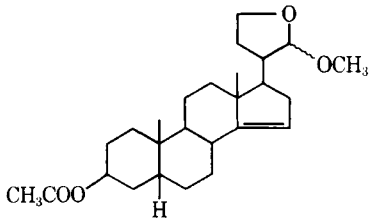
Isomeric cardanolides (XXXIV and XXXV) have



Scheme VII



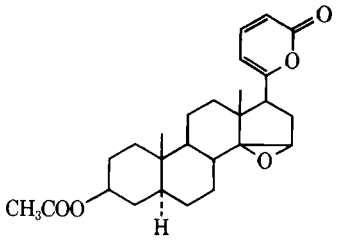
XXXIV



XXXV

been prepared and found to be weak inhibitors of Na^+,K^+-ATPase and Mg^{+2}-ATPase (79).

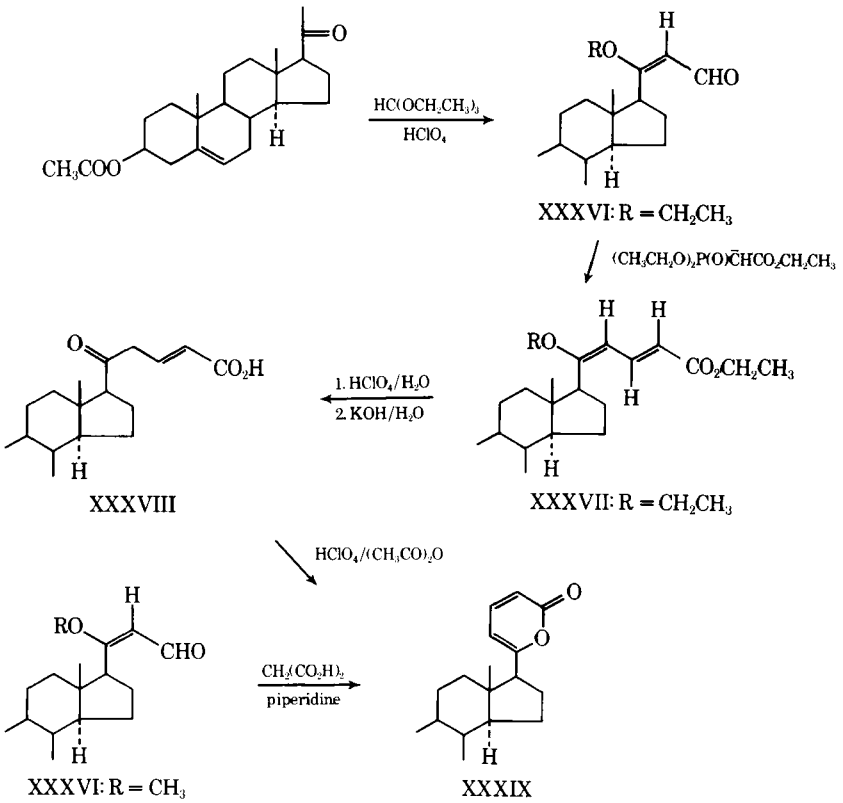
The 5′-substituted 2-pyrone ring of the naturally occurring bufadienolide genins has also received some attention, but few modifications of this group have been made. Their biological characteristics, including anticancer activity, have promoted the development of synthetic approaches to isomeric bufadienolides in which the 2-pyrone ring is linked at C-17 to one of three alternative positions (3′, 4′, or 6′). Details of the synthesis of 6′-isobufadienolides (XXXIX) were described (80) but no biological data on the compounds prepared were given. Condensation of the enol ether-aldehyde (XXXVI) with the ethoxycarbonylmethyldiethylphosphonate carbanion gave the *cis,trans*-ester (XXXVII) which, after hydrolysis to the unsaturated keto-acid (XXXVIII), readily cyclized to give the 6′-isobufadienolide (XXXIX) (Scheme VIII). Better yields of XXXIX were obtained directly from the enol ether-aldehyde (XXXVI) by condensation with malonic acid in the presence of a secondary amine (Scheme VIII). The 6′-isobufadienolides synthesized were of the C/D *trans*-series, and it is unlikely that they possess cardiotonic activity.



XL

The malonic acid condensation with steroidal enol ether-aldehydes such as XXXVI has been investigated (81–84) in attempts to prepare 6′-isobufadienolides with differing configurations at C-14 and C-17. Successful synthesis of 14β,15β-oxido-6′-isobufadienolide (XL) (82) gave a compound that was stereochemically related to the natural genins, but no biological data were included in the report.

Replacement of Lactone Ring with Open-Chain Structures—As part of a general study on the mode of action of cardiotonic steroids, Boutagy and Thomas recently replaced the lactone ring of digitoxigenin with a series of analogous open-chain structures (85–87). The purpose of this particular study was to define structure–activity relationships



Scheme VIII

and to generate a series of new compounds with potential value as therapeutic agents and as tools for exploring and correlating the biochemical and physiological manifestations of digitalis-like activity.

The general plan of the series of compounds synthesized is shown in Scheme IX. Digitoxigenin (II) was first converted to a 17β-formyl steroid (XLIV), which was the key intermediate for the series of compounds. This intermediate was then condensed with a series of phosphonate reagents or with various hydrazine derivatives, such as guanylhydrazine, to give the required compounds. The following types of changes were attempted:

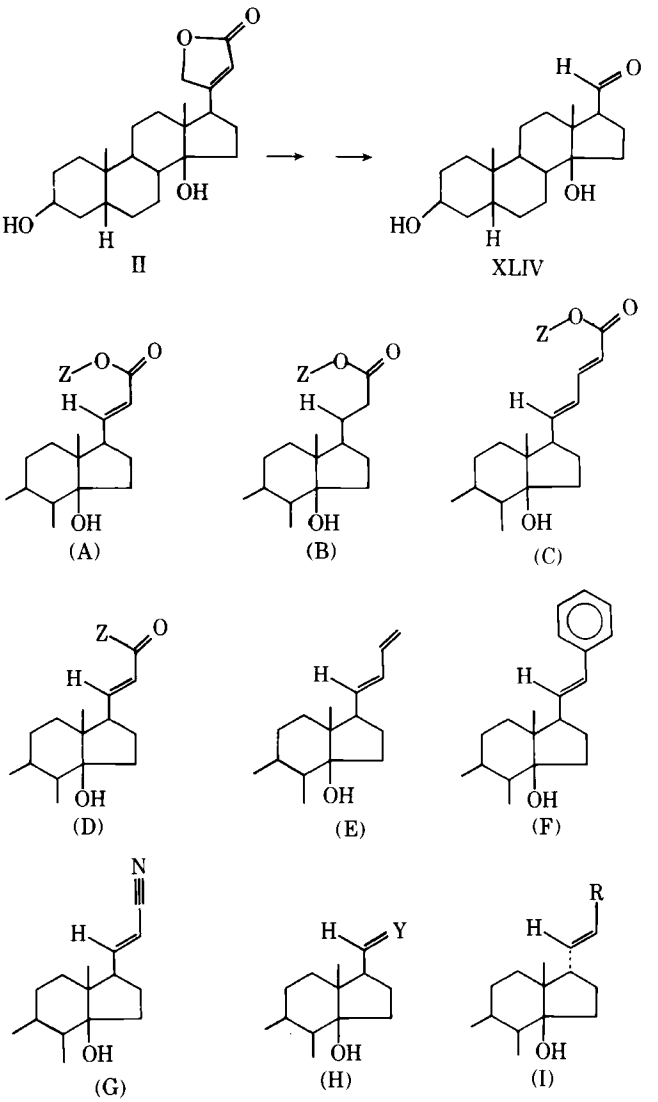
1. Replacement of the lactone by a *trans*-α,β-unsaturated ester (or acid) moiety (A), the bulk of which was varied by systematically increasing the size of group Z and by introducing substituents on the α-carbon atom.
2. Abolition of the conjugation of the carbonyl group (B).
3. Extension of the conjugation of the carbonyl group and expansion of the distance between the carbonyl oxygen and ring D (C). The bulk of the alkoxy moiety was changed by varying the size of group Z.
4. The role of the ether oxygen of (A) was studied by replacing the alkoxy group with an alkyl or aryl group or groups containing nitrogen (D).
5. Both the ether oxygen and carbonyl group were replaced by other electron dense groups such as C=C< (E), aryl (F), and C≡N (G).
6. The C-20 carbon atom was attached directly to a hetero atom (H), for example by preparing the hydrazone and related derivatives.
7. The importance of the 17β-configuration was studied by preparing a similar series of compounds possessing the 17α-configuration (I).

Most compounds described were prepared by condensing the C-17 formyl intermediate (XLIV) with a variety of phosphonate reagents. These reagents, if they are of the appropriate type, will condense with carbonyl compounds under relatively mild conditions to form olefins in good yield. Under most conditions, the predominant product is the *trans*-olefin, a necessary property if the products are to be isosteric with the butenolide ring. The mechanism and scope of olefin synthesis using phosphonate carbanions were reviewed recently (88).

The reaction sequence used for the preparation of the intermediate etianaldehyde (XLIV) and for a typical α,β-unsaturated ester is shown in Scheme X (85).

Other reactions used for the preparation of C-17β-*trans*-(E)-unsaturated esters and derivatives are shown in Schemes XI and XII (86). Other compounds prepared in this study are shown in Schemes XIII–XVII (87).

The compounds shown in Schemes IX–XVII were tested for their ability to induce a positive inotropic effect in guinea pig hearts[2] and for their ability to inhibit guinea pig atrial Na^+,K^+-ATPase (89). A spectrum of activities was observed, and the results were



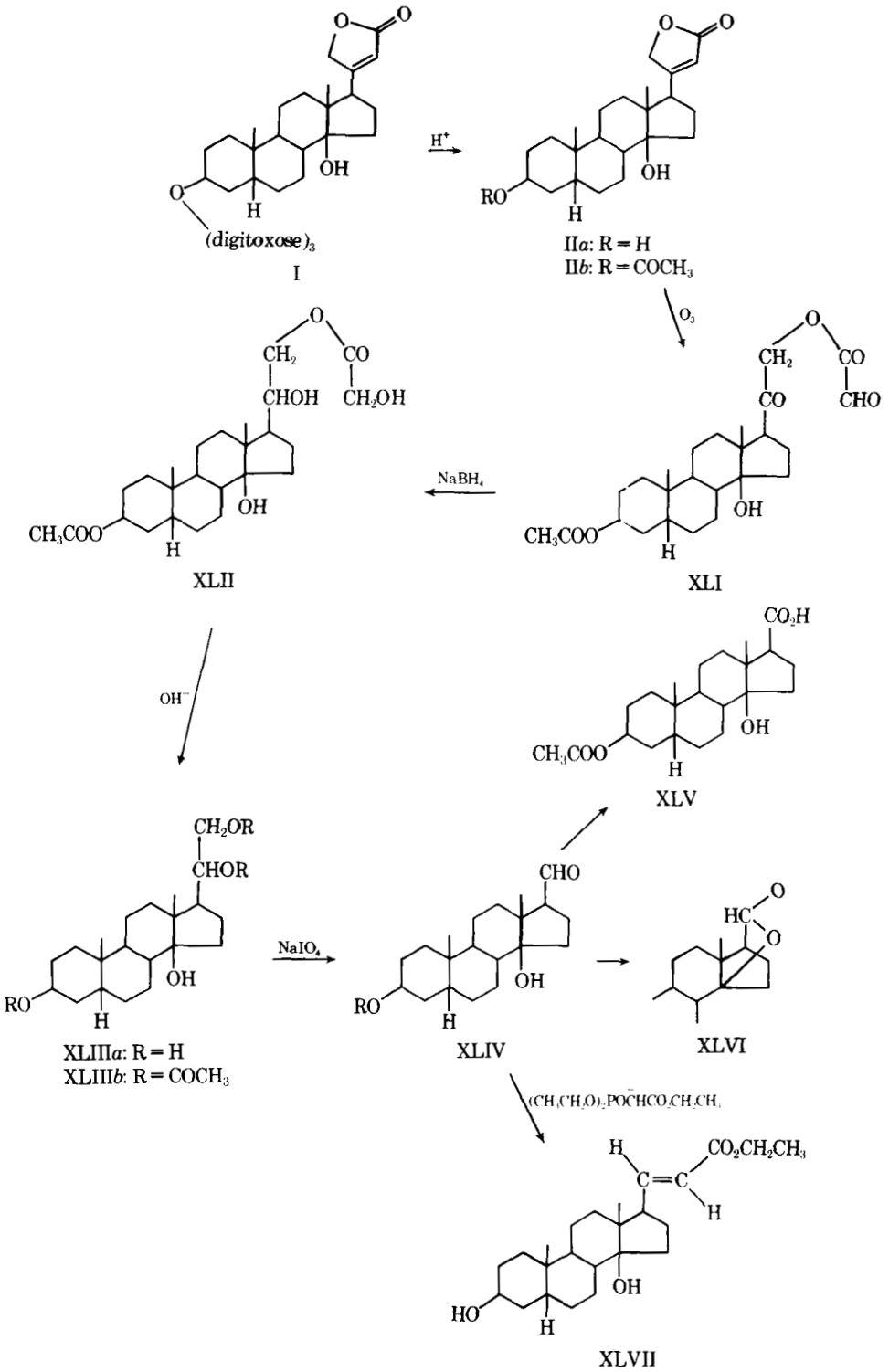
Scheme IX—Classification of digitoxigenin analogs prepared by Boutagy and Thomas (85–87)

used to infer a model of that part of the digitalis receptor that accommodates the C-17 side chain of cardiotonic steroids. These results are discussed further under *Structure–Activity Relationships.*

Modification of Steroid System—Analogs containing an unsaturated lactone attached to the C-17 position of a modified steroid system may be prepared either by attaching the lactone to an appropriate steroid or by modifying one of the many naturally occurring cardenolides or bufadienolides. Numerous such compounds have been synthesized, and many are described in the general reviews already cited. This review will deal only with recent developments in this field.

Attachment of Butenolide Ring—Two novel and relatively simple methods have emerged that were effective in the synthesis of the butenolide ring at C-17 of various steroid nuclei. The first method involves the use of a Wittig or phosphonate modification of the Wittig reaction with 20-oxo-21-hydroxy steroids (LXXX) followed by an intramolecular cyclization (90–93). A variation of this method involves a direct intramolecular Wittig-type condensation using 20-

[2] To be reported.



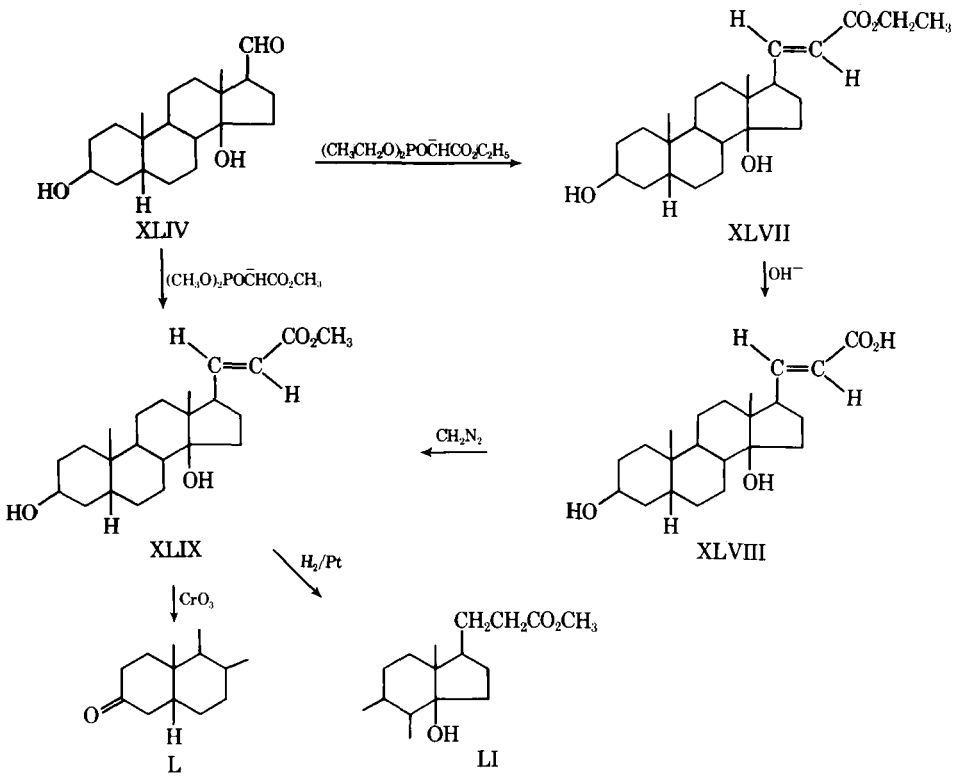
Scheme X—Synthetic sequence to the etianaldehyde (XLIV) and α,β-unsaturated ester (XLVII) (85)

oxo-21-acyl steroids such as LXXXI and LXXXV (94, 95) (Scheme XVIII).

The second method utilizes peracid oxidation of 17-furyl steroids (LXXXVI) (which are readily accessible from 17-oxo steroids) to form 21-hydroxycardenolides (LXXXVII) (53). Reduction of the latter with sodium borohydride, as previously described, gives the cardenolide (LXXXVIII) (Scheme XIX).

Introduction and Modification of Other Functional Groups—The introduction of various substituents into the steroid nucleus and modification at molecular centers associated with cardiac activity have produced numerous new compounds, some of which were discussed under *Properties of Naturally Occurring Cardiotonic Steroids*. Some recent work will now be reviewed.

The synthesis of a cardiotonic glycoside (XC) with an aromatic ring A has been described (96). The compound was prepared from the 3,14β-dihydroxy-19-norcarda-$\Delta^{1,3,5(10),20(22)}$-tetraenolide (LXXXIX) (97)



Scheme XI—Synthesis of C-17-trans-(E)-α,β-unsaturated esters and derivatives (86)

by glycosidation with acetobromoglucose and silver oxide (Scheme XX). No biological activity was reported.

Compound XC is of potential interest because of a previous report (98) that the $\Delta^{1,4}$-3-oxo derivative of digitoxigenin (XCI) may have an improved therapeutic ratio.

Danieli *et al.* (99) described the synthesis of a 17α-hydroxycardenolide (XCII) by selenium dioxide oxidation of digitoxigenin 3-acetate (Scheme XXI). The 17α-hydroxy group was shown not to influence the cardiotonic activity of digitoxigenin, although inversion of the two C-17 substituents abolished activity (33).

The biological consequences of inverting the C-16 hydroxy group of gitoxin to produce 16-epigitoxin has been studied (100). Compared with gitoxin (XCIII) and ouabain, the epi compound showed a greater difference between doses, producing minimum and maximum inotropic effects as well as having a greater influence upon myocardial contractility and a lesser effect on the pacemaker activity of the Purkinje conductive system. The authors made the important conclusion that the therapeutic and toxic properties of digitalis-like compounds may be partially dissociated by this type of chemical modification. 16-Epigitoxin was prepared by treatment of gitoxin with strong alkali and water (Scheme XXII) (100).

The pharmacological consequences of introducing oxygen functions at C-15 were recently reviewed (57). 15-Oxodigitoxigenin (XCV) was shown to be biologically active whereas 15α-hydroxydigitoxigenin (XCVI) was inactive (35, 36). One study (101) showed that 14α-artebufogenin (XCVII) was inactive whereas the 14β-isomer (XCVIII) was active. The corresponding butenolide analogs (XCIX and C) were both inactive. Chen and Henderson (102) made similar studies.

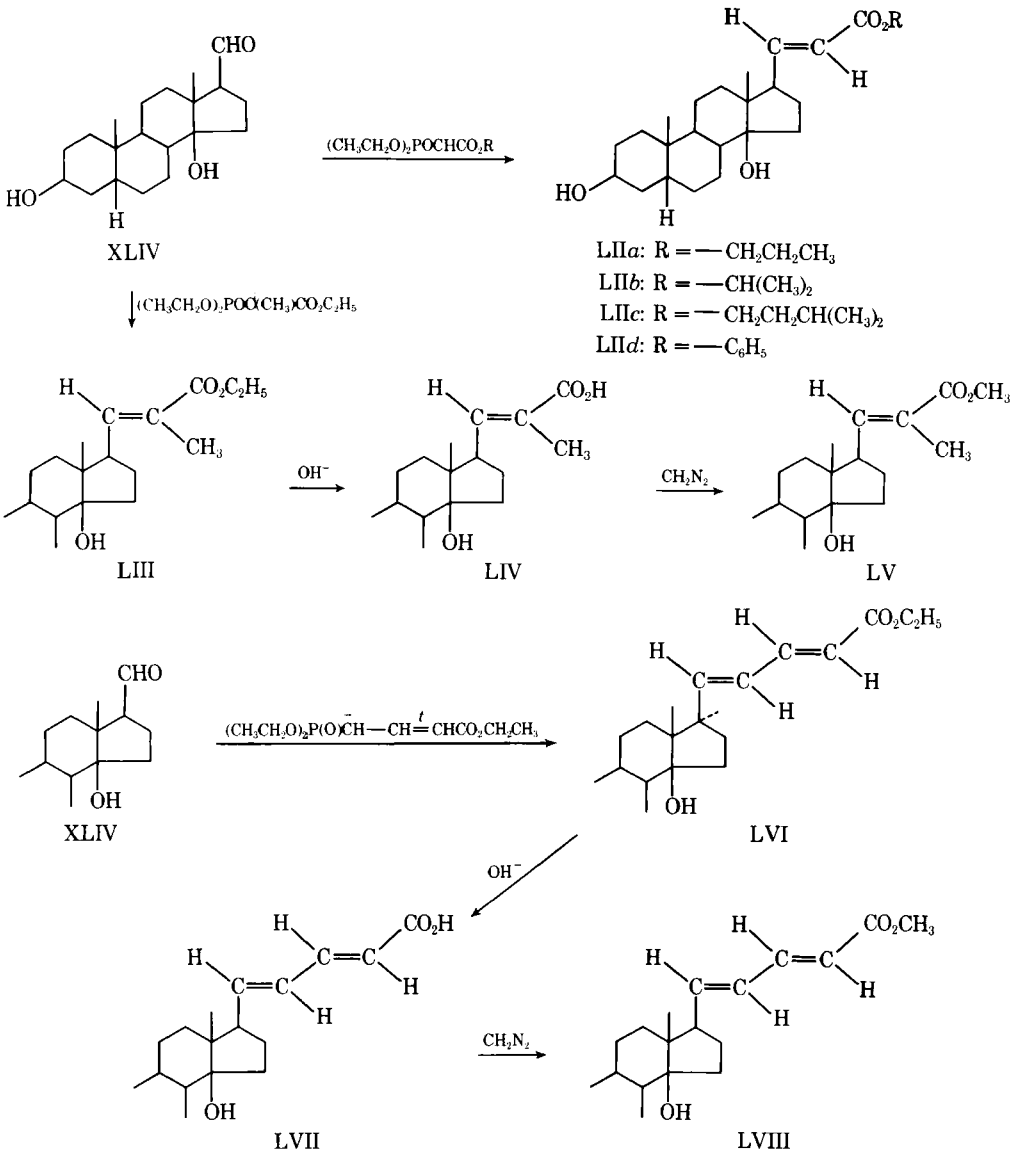
Recently, Okada and Saito (103) described the synthesis of a series of 15α- and 15β-hydroxycardenolides. The series included compounds with the usual digitalis-type stereochemistry, together with C-17 epimers and compounds with the *trans*-C/D junction. No biological activity was reported.

Wolff and Ho (104, 105) described the synthesis of 19-halo and 5,10-cyclo derivatives of strophanthidin. Several other derivatives of strophanthidin were synthesized and tested (106); the 3-haloacetate, 3-diazoacetate, and 19-oxime displayed considerable biological activity when tested on isolated cardiac muscle, Na^+,K^+-dependent ATPase, and tumor cells in tissue culture (cytotoxic activity).

Studies by Hokin *et al.* (107) showed that the 3-haloacetates of strophanthidin (CI) were biological alkylating agents and acted as active site-directed irreversible inhibitors of Na^+,K^+-ATPase.

Similar alkylating agents were prepared (108) from digitoxigenin, digoxigenin, 15α-hydroxydigoxigenin, and Δ^{14}-anhydrodigitoxigenin. All compounds were effective irreversible inhibitors of Na^+,K^+-ATPase. The fact that 15α-bromoacetoxydigitoxigenin 3-acetate was active whereas the parent 15α-hydroxy compound was inactive is interesting in view of the belief that α-substituents may interfere with drug–receptor interactions.

Significant cardiotonic activity was found in the 3,3-ethylenedioxy and the 3-tetrahydropyranyl ether derivatives of digitoxigenin and digoxigenin (12β-hydroxydigitoxigenin) (109). Another series of ketal



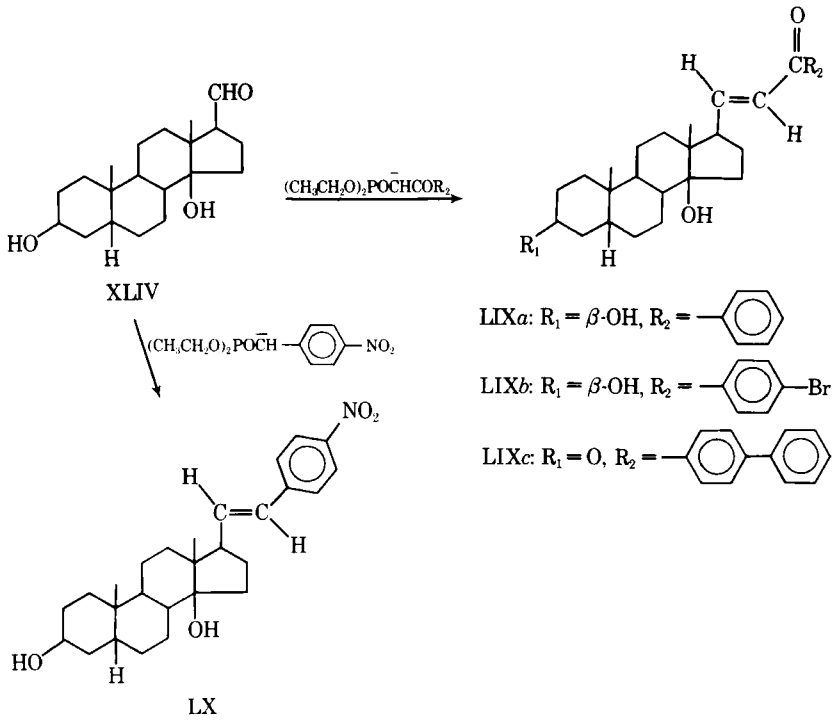
Scheme XII—Synthesis of C-17β-α,β-unsaturated esters and derivatives (86)

derivatives of 3-oxocardenolides was reported in the patent literature (110).

Other chemical modifications of cardiac genins which appeared recently include the 19-cyanomethylene derivative of strophanthidin and related 19-oxo genins (111); the thio analog of 14,15-epoxy genins (112); the 16-methyl- or 16-cyano-substituted cardenolides (32); the 14,15-*cis*-diol derivatives of digitoxigenin (113); the alkoxy and cyclocarbonate derivatives of gitoxin (114); the suberoxylalanine ester of digitoxigenin (115); the cardenolide nicotinates (116), nitrates (117), and glycerates (118); the 3-pyranyl ether derivatives of cardenolides (119), and some 4-chloro derivatives of cardenolides (120). The substitution of the 3-hydroxy group of digitoxigenin with a series of simple nitrogen-containing functions has been described (57), as has the preparation of glycosides of amino-sugars (121–123).

Modification of Pharmacokinetic Properties—Most compounds discussed in the preceding sections were synthesized with the objective of defining structure–activity relationships with respect to the pharmacodynamic properties of cardiotonic steroids. Recently, there has been much interest in the study of the pharmacokinetics[3] of digitalis (124–126) as a means of improving the therapeutic properties of these agents and reducing the incidence of toxicity. Part of the problem of digitalis toxicity arises because of the unsatisfactory nature of the physical properties of the commonly used cardiotonic steroids. Those glycosides, such as digoxin, that are sufficiently nonpolar to be effectively absorbed show a high degree of accumulation (127). The pharmacokinetic properties of the ideal cardiotonic steroid were recently defined (58) as being *(a)* complete absorption, *(b)* rapid onset of action, and *(c)* reduced accumulation as a result of an increased rate of elimination.

[3] The term pharmacokinetics refers to all factors (absorption, metabolism, excretion, *etc.*) that control the rate and the extent to which the drug reaches its site of action. The term pharmacodynamics refers to all processes involved in the interaction of the drug with the responding system and the subsequent manifestation of response.



Scheme XIII—Synthesis of C-17β-α,β-unsaturated keto and aryl derivatives (87)

Based on previous experience, these properties should be found in compounds that have been chemically modified so as to increase their hydrophobic character but which, after or during absorption, are converted to more polar derivatives which are rapidly excreted. Recently, there have been several attempts to synthesize such compounds.

One of the first attempts to modify deliberately the pharmacokinetic properties of cardiac glycosides was the work of Megges and Repke (128), who studied the penta-acetyl derivative of gitoxin (16β-hydroxydigitoxin). Because of its low lipid solubility, gitoxin is very poorly absorbed and is virtually inactive when given by the oral route. Conversion to the penta-acetyl derivative gave a compound whose rate and extent of absorption exceeded those of the highly lipid-soluble digitoxin. During and after absorption, the compound was rapidly deacetylated to yield the parent compound, gitoxin, which was then eliminated at a much faster rate than digitoxin. A similar rationale was applied in the design of two recently introduced derivatives of digoxin: β-acetyldigoxin (4‴-acetyldigoxin) (CII*b*) and β-methyldigoxin (4‴-methyldigoxin) (CII*c*).

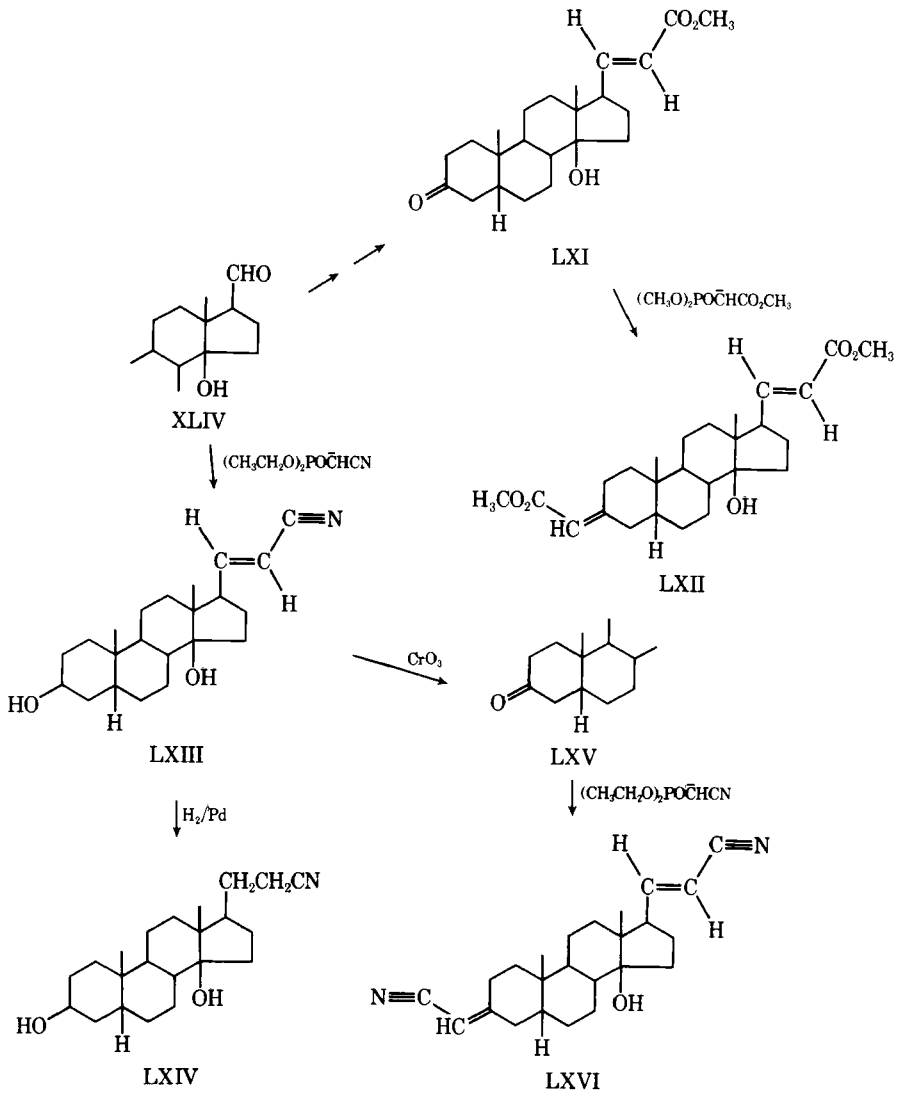
The development and biological evaluation of β-methyldigoxin, together with a series of related alkyl and acyl derivatives of the sugar portions of cardiac glycosides, were recently reviewed (58). It has been clearly demonstrated that the methylation of a single hydroxy group in digoxin leads to a pronounced increase in the rate at which the drug is absorbed. Several small clinical trials (129) indicated that β-methyldigoxin is effective and well tolerated. However, the observation that the drug is only slowly demethylated (130, 131) could mean that more extensive studies may reveal that the drug is more toxic than the parent digoxin because of an increased tendency to accumulate.

The pharmacokinetics of tritiated β-acetyldigoxin have been studied in humans (132); the drug was better absorbed than digoxin. Rapid deacetylation apparently occurred during or after absorption, since no trace of β-acetyldigoxin was found in plasma or urine following oral administration. By contrast, Rietbrock *et al.* (131) found no significant difference in the rat between the plasma levels of radioactivity following the intraduodenal administration of radioactive digoxin and β-acetyldigoxin. They did find that β-methyldigoxin was more rapidly and more extensively absorbed, but the plasma half-life of this drug was twice as long as that of digoxin (10 hr compared with 4.5 hr). The biliary clearance of β-methyldigoxin was only about one-third that of digoxin when observed over 12 hr.

The naturally occurring glycoside lanatoside C possesses an acetyl group which may be thought to endow the molecule with some of the desirable pharmacokinetic features described earlier. However, studies (133, 134) showed that lanatoside C is apparently converted to digoxin in the GI tract and that only the resulting digoxin is absorbed in appreciable quantities. These conclusions were subsequently confirmed (135).

STEROIDAL GUANYLHYDRAZONES AND OTHER CARDIOACTIVE COMPOUNDS

Cardiotonic activity has been claimed for compounds possessing none of the unique structural features associated with the biologically active cardiac glycosides. Of particular interest are the 3,20-bisgu-



Scheme XIV—Synthesis of monosubstituted and disubstituted unsaturated ester and nitrile derivatives (87)

anylhydrazone derivatives of prednisone (CIII) and prednisolone (CIV). These compounds were first reported in 1964 (136, 137) as a new class with digitalis-like activity. Both of these componds were subjected to various tests for digitalis-like activity and were shown to have high activity with respect to positive inotropic effects and inhibition of Na^+,K^+-ATPase. A systematic search of the patent literature since that time revealed a large number of steroidal guanylhydrazones and related molecules which have been synthesized as potential cardioactive compounds (138–146).

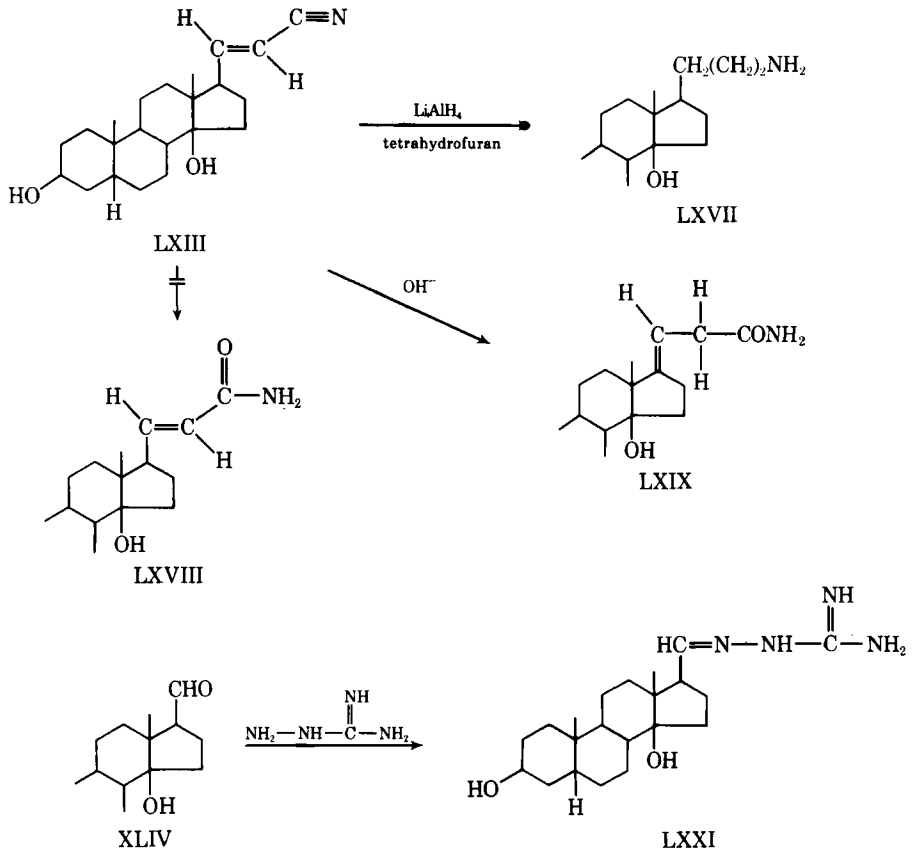
A review of the chemistry and biological activity of the guanylhydrazones was recently published (147). This review drew attention to several important features concerning the structure–activity relationships of these compounds. First, a wide range of steroidal 3,20-bisguanylhydrazones has been shown to exert a positive inotropic action on cardiac muscle; compared with the cardiac glycosides, their activity is not so significantly affected by changes in the stereochemistry of the steroid nucleus or of the substituents on the steroid. The bisguanylhydrazone derivatives of 5β- or 5α-pregnanedione do not differ significantly in activity.

Second, changing the distance between the two guanylhydrazone groups by changing the positions of their attachment to the steroid nucleus does not, within certain limits, alter the activity of the molecule to any great extent. Furthermore, some bisguanylhydrazones of nonsteroid molecules also show cardiotonic activity. For example, some diphenylbisguanylhydrazones and bisguanylhydrazone derivatives of di- and tricyclic molecules proved to be active.

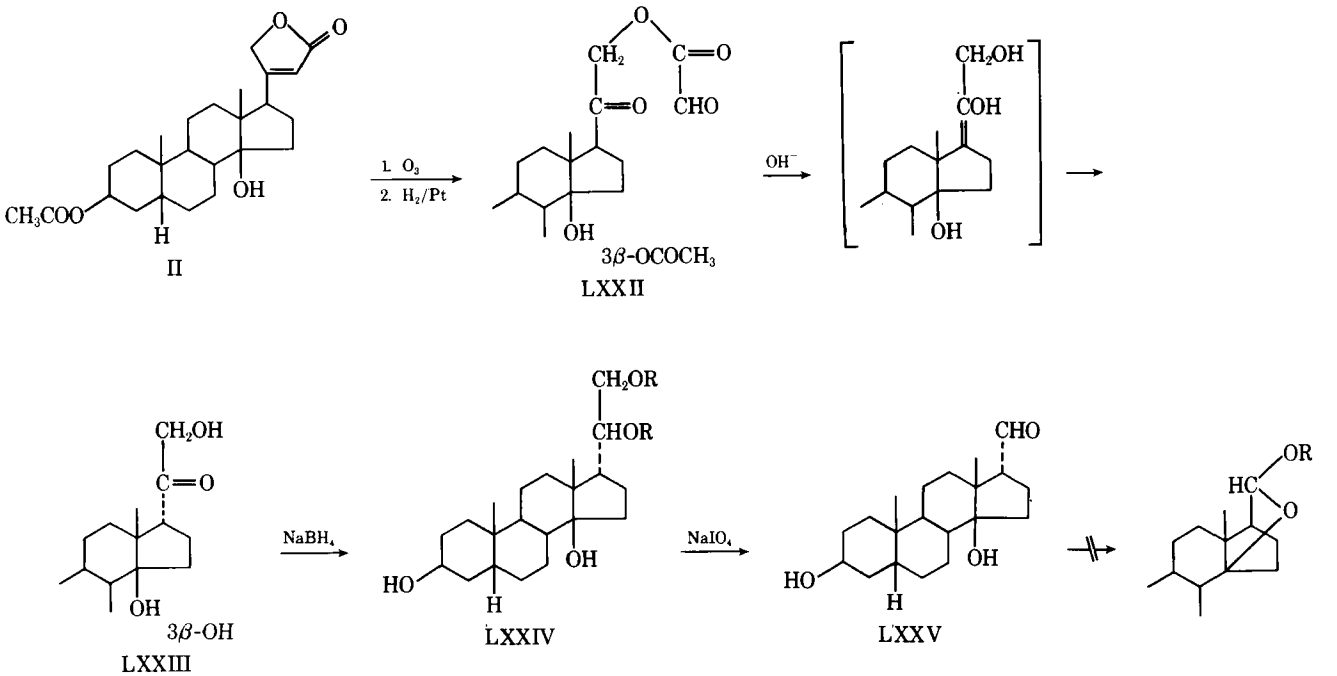
Third, certain steroidal mono- and trisguanylhydrazones are active. Of particular interest is the guanylhydrazone of 3-oxodigitoxigenin (CV) which is cardioactive whereas the guanylhydrazone derivative (CVI) of the corresponding 14-desoxy-14α-cardenolide is devoid of activity.

Fourth, hydrogenation of the guanylhydrazone group to give the diaminoguanidine derivative produced no significant loss in activity. However, the replacement of the guanylhydrazone moiety with other related groups, such as the hydrazine or the *S*-methylisothiosemicarbazone groups, gave inactive compounds.

The absorption, distribution, and other phar-



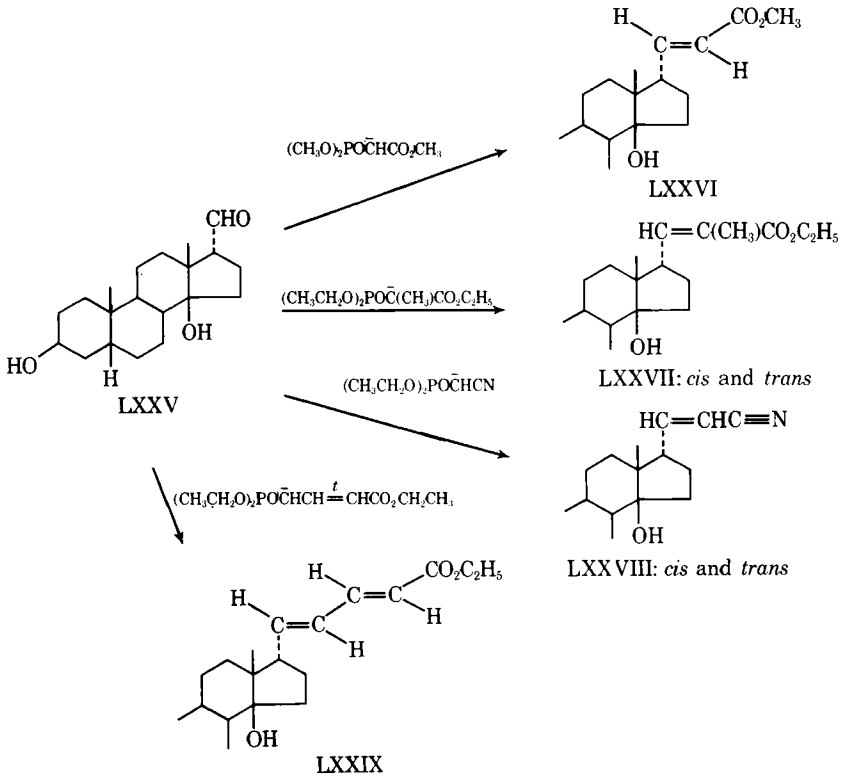
Scheme XV—Synthesis of C-17 amine, amide, and guanylhydrazone derivatives (87)



Scheme XVI—Synthetic sequence to the 17α-etianaldehyde (LXXV) (87)

macokinetic properties of the steroidal guanylhydrazones have been investigated *in vivo* (148) using ^{14}C-labeled molecules.

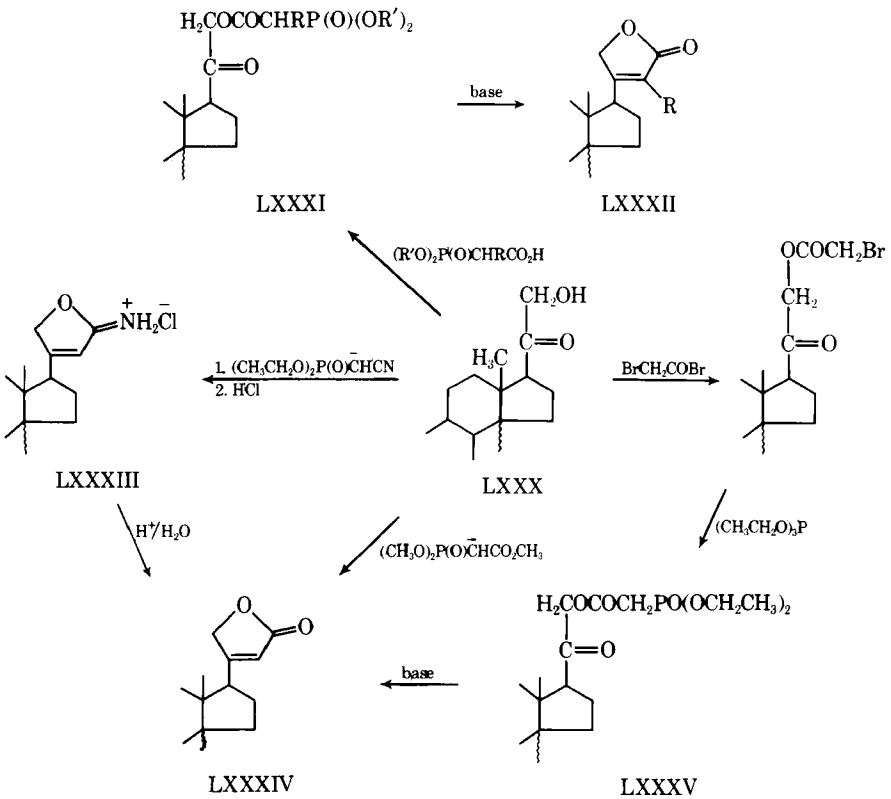
The pharmacology of bisguanylhydrazones in laboratory animals and in humans has been reviewed (149). The 3,20-bisguanylhydrazones of all steroids tested in animals were found to exert a powerful positive inotropic effect. The compounds also showed a marked ability to inhibit Na^+,K^+-ATPase, and the ranking order of the compounds in terms of the two



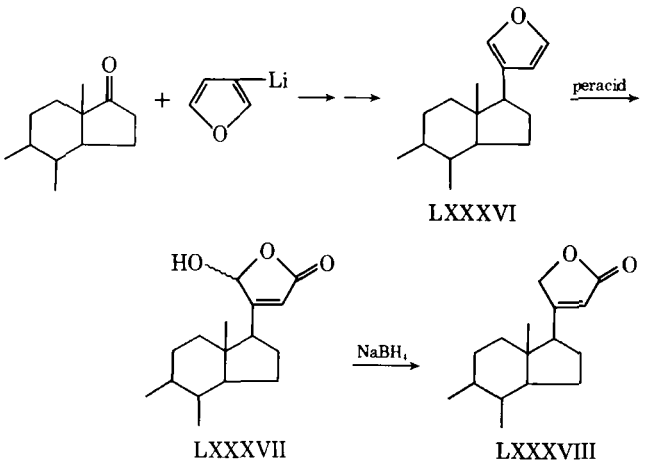
Scheme XVII—Synthesis of C-17α-analogs of digitoxigenin (87)

biological effects was the same. However, for individual compounds the ratio of the molar concentration required to produce a positive inotropic effect to that required to produce 50% inhibition of Na^+,K^+-ATPase varied from approximately 1:1 to 1:40. This may imply a separation of the two biological effects and will be discussed later.

The reader who refers to this review (149) should



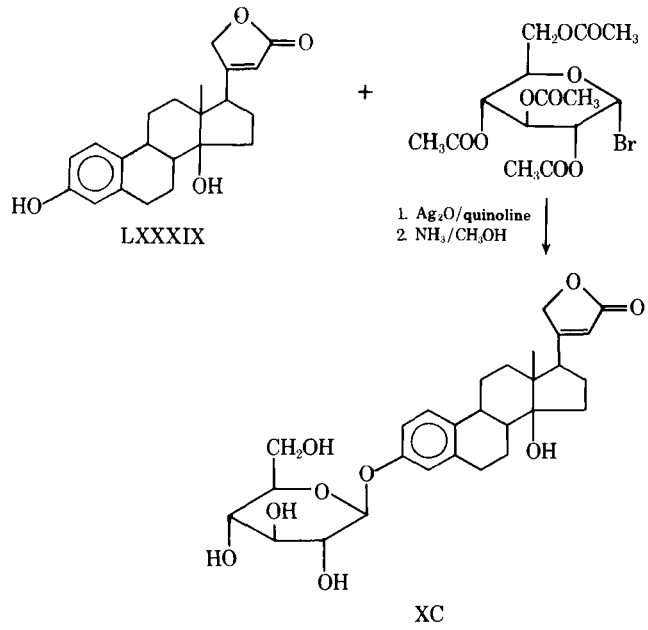
Scheme XVIII—Synthetic sequences to the C-17β-butenolide ring



LXXXVI LXXXVII LXXXVIII

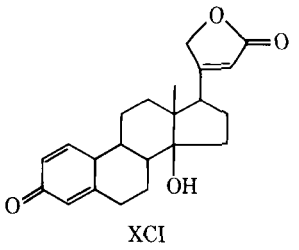
Scheme XIX

note that inotropic effects are related to concentrations expressed as grams per milliliter whereas inhibition of Na^+,K^+-ATPase is related to moles per liter (Ref. 149, Fig. 13). Furthermore, drug concentrations that produce positive inotropic effects refer to those at the foot of the log dose–response curves (threshold values). It seems also that maximum inotropic effects occur at doses approximately 100 times the concentrations given in the tables and that the maximum increase in the force of contraction was approximately 20–30% that of the predrug value. The therapeutic ratios of the 3,20-bisguanylhydrazones were approximately twice as favorable as those of the naturally occurring cardiac glycosides. The half-time for duration of contractility effects varied from less than 15 min in the case of progesterone-3,20-bisguanylhydrazone to 400 min with triamcinolone-3,20-bisguanylhydrazone. These differences were not related to the half-lives of elimination which were approximately 10–12 hr in all cases (based on elimination of total radioactivity following intravenous administration of radioactive drug).



LXXXIX XC

Scheme XX



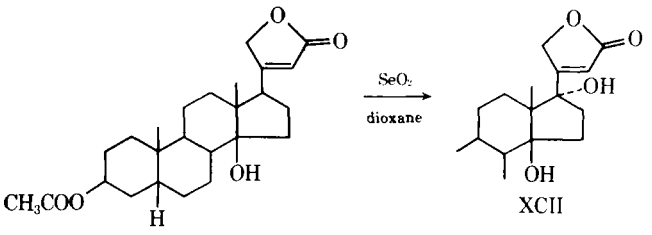
XCI

All effects described refer to studies in the guinea pig. On the basis of these studies, triamcinolone-3,20-bisguanylhydrazone was selected as the most promising drug for studies in humans. Ten patients with indications for digitalis were given 10 mg iv of the triamcinolone derivative. Cardiac output was increased by an average of 32%, and the rate of circulation was increased by an average of 16%. The heart rate was slowed but no bradycardia was observed. Unfortunately, the duration of action was too brief and the author concluded that the drug could not be regarded as a suitable substitute for digitalis.

Greeff and Schlieper (150) compared the biological effects of prednisolone-3,20-bisguanylhydrazone in humans, rats, rabbits, and guinea pigs. The authors found that the log dose–response curves for force of contraction in the isolated auricle of humans, rabbits, and rats were almost identical (half-maximum effects at approximately 6×10^{-5} *M*) whereas comparable effects in the guinea pig occurred at approximately 4×10^{-7} *M*. By contrast, the log dose–response curves for inhibition of erythrocyte Na^+,K^+-ATPase from humans, rabbits, and rats were distributed over a 100-fold range in concentration. Since Repke (23) showed that erythrocyte Na^+,K^+-ATPase is a suitable model for the myocardial enzyme, these results may signify that inhibition of Na^+,K^+-ATPase is not related to positive inotropic effects. (Compare Figs. 1–3.)

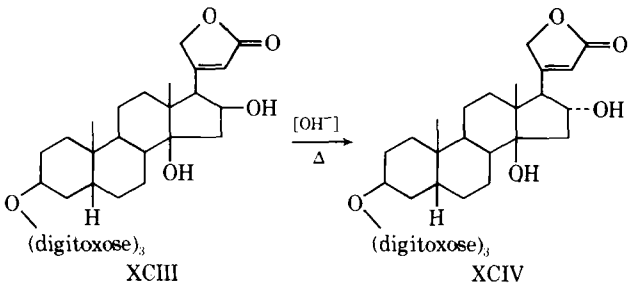
The *Erythrophleum* alkaloid cassaine (CVII) has been recognized for many years as having a powerful digitalis-like action on heart muscle (151). There appears to be little structural resemblance between cassaine and the cardiac steroids. While the unsaturated lactone ring is an important functional group for biological activity in the cardiac glycosides, the biological properties of cassaine depend on the presence of the unsaturated aminoethyl ester group. Hydrolysis of this group to give the corresponding acid destroys activity (152).

Clarke and associates (153–155) described the synthesis of simplified analogs of cassaine as part of a study to determine the role of the skeletal structure and the various substituents of cassaine in the pro-



XCII

Scheme XXI



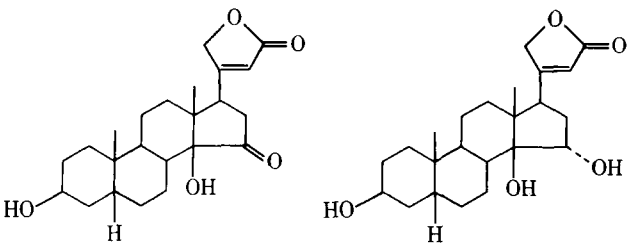
XCIII XCIV

Scheme XXII

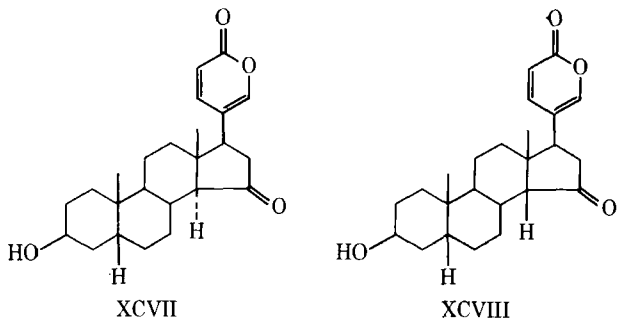
motion of cardiotonic activity. Some of the analogs prepared (CVIII, CIX, and CX) were shown to be active but less potent than cassaine when tested for cardiotonic activity on isolated cardiac muscle or in the intact dog.

There are several other groups of cardiotonic alkaloids. The biological properties of these compounds, along with those of the *Erythrophleum* group, have been reviewed (156) and are considered to be of no therapeutic interest because of their association with many other pharmacological effects.

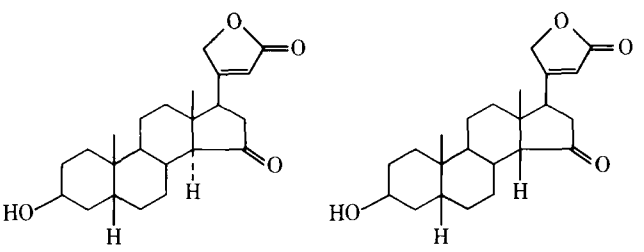
In view of the importance of the dimethylaminoethyl group in the activity of cassaine, it is interesting to note that esterification of bile acids with dimethylaminoethanol (157) imparts a slight digitalis-like activity in spite of the configurational differences between the bile acids and the cardiac genins. Recently, Brown *et al.* (158, 159) patented a new method to introduce the dimethylaminoethoxycarbonylmethylene function at different positions on the



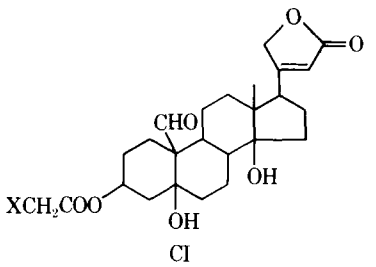
XCV XCVI



XCVII XCVIII



XCIX C



CI

steroid nuclei. On this basis, many such derivatives were prepared and assessed for cardiotonic activity. Compounds of the CXI type and other dimethylaminoethoxycarbonylmethylene steroids were claimed to be cardioactive, but no biological details were given in the abstract.

Digitalis-like activity has been claimed (160) for some adrenocorticoid steroids. However, there is no general acceptance of this idea because it appears that the effects of these compounds are slight and of doubtful significance.

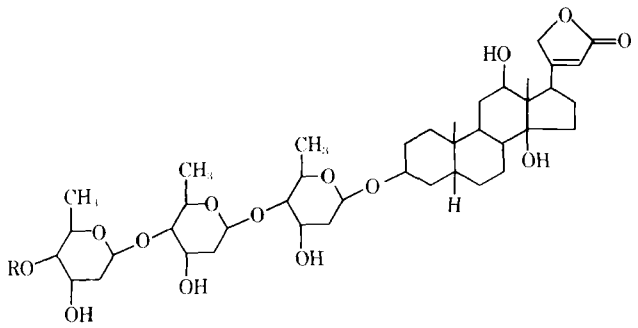
The studies described indicate that digitalis-like activity is not limited to compounds possessing the specific requirements deduced by the classical studies described previously. A logical extension of this conclusion is that cardiotonic activity may be found in nonrigid molecules of the appropriate type. Recently, Inhoffen *et al.* (161) described the synthesis of a "pseudosteroid" (CXII) which, on testing, was found to have a negative inotropic effect.

A variety of aromatic and aliphatic bisguanylhydrazones (*e.g.*, CXIII, CXIV, and CXV) were reported (149) to have a weak inotropic effect on guinea pig heart.

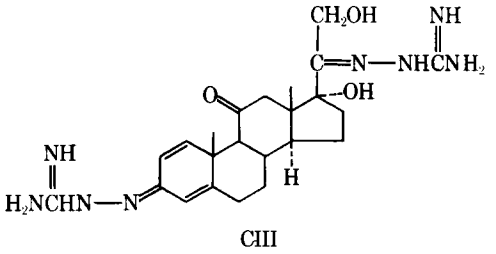
To conclude this section, mention should be made of the wide variety of nonsteroidal compounds known to inhibit Na^+,K^+-ATPase. Discussion of these compounds is beyond the scope of this review and the reader is referred to a recent comprehensive review (162) for an account of this subject.

BIOLOGICAL ACTIVITY

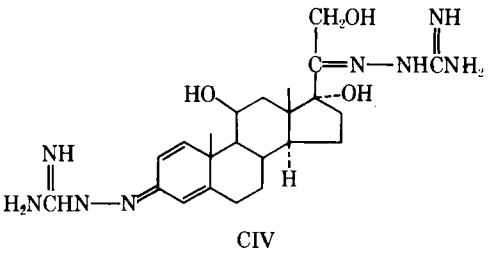
The interpretation of drug action in terms of biochemical effects arising from interactions with specific receptors has much philosophical appeal and, in some cases, is of practical value in the design of new drugs. Therefore, it is appropriate to outline current concepts of the mode of action of cardiotonic ste-



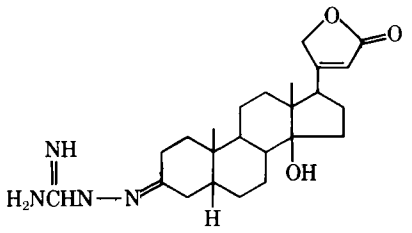
CII*a*: R = H (digoxin)
CII*b*: R = $COCH_3$ (β-acetyldigoxin)
CII*c*: R = CH_3 (β-methyldigoxin)



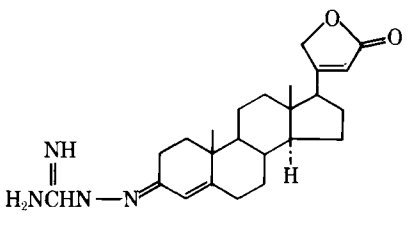
CIII



CIV



CV



CVI

roids, particularly as these relate to the interpretation of structure–activity relationships and to the biological evaluation of new cardioactive substances.

The basic problem is one that always applies in the study of the biochemical pharmacology of drug action, namely the difficulty of correlating events at different levels of biological organization. Cardiotonic steroids are known to cause effects at three biological levels: at the enzyme or subcellular level, at the cellular level, and at the organ or tissue level.

The biochemical effects of cardiotonic steroids have been the subject of considerable study. In 1961, at the First International Pharmacological Meeting, the effects of cardiotonic steroids on a wide range of biochemical systems were described. In many cases, reasonable circumstantial evidence was presented to link these biochemical effects with effects on heart muscle contractility (163). Recent accounts of the subcellular effects of cardiotonic steroids were presented (14, 17), and these reviews suggested that the effects of cardiotonic steroids on myocardial contractility arise as a result of one of the following mechanisms: *(a)* inhibition of Na^+,K^+-ATPase, *(b)* increase in intracellular free Ca^{+2} unrelated to effects on Na^+,K^+-ATPase, and *(c)* increase in intracellular free Ca^{+2} as a consequence of inhibition of Na^+,K^+-ATPase.

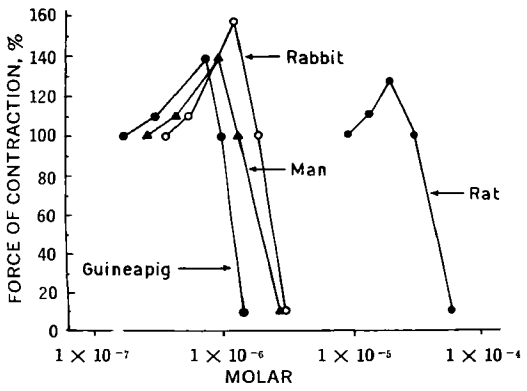


Figure 1—*Influence of K-strophanthin on the force of contraction of the myocardium of different species. (After Greef and Schlieper, Ref. 150.)*

Other reported subcellular effects of cardiotonic steroids, including direct effects on contractile proteins and modification of myocardial energy metabolism, seem to have no *in vivo* significance or are secondary to effects on contractility (17).

At the cellular level, cardiotonic steroids directly or indirectly affect the transport and distribution of a

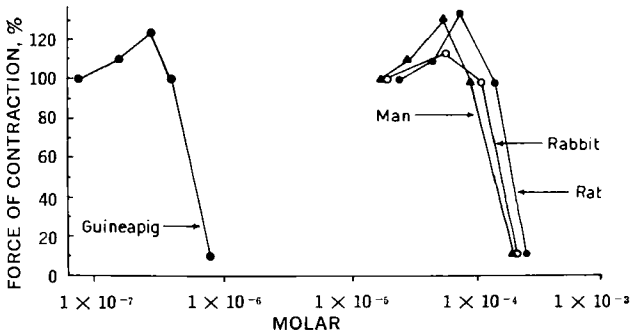


Figure 2—*Influence of prednisolone-3,20-bisguanylhydrazone on the force of contraction of the myocardium of different species. (After Greef and Schlieper, Ref. 150.)*

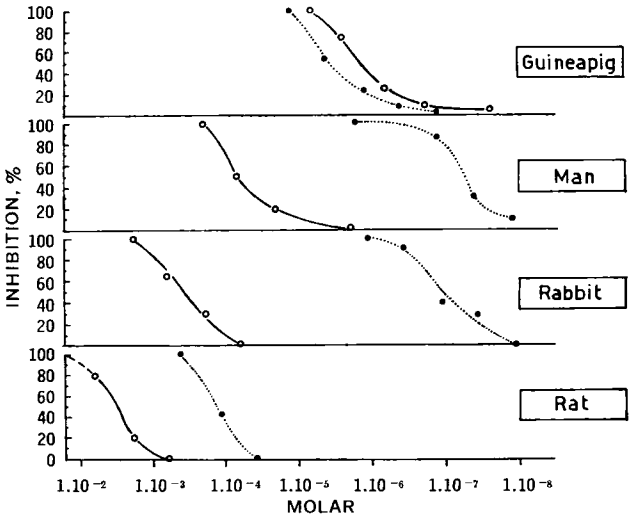
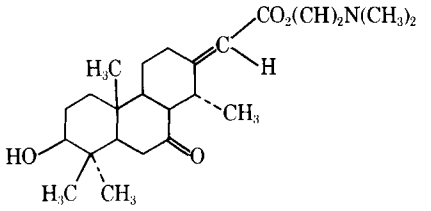
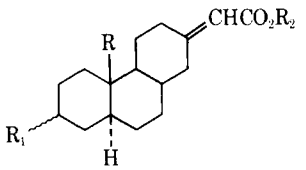


Figure 3—*Influence of K-strophanthin (. . .) and prednisolone-3,20-bisguanylhydrazone (—) on the active transport of Na^+ and K^+ in cooled erythrocytes of different species. (After Greef and Schlieper, Ref. 150.)*

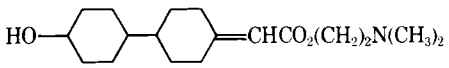


CVII

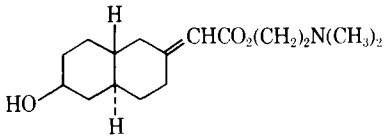


CVIII: R = H or CH_3

R_1	R_2
H	$(CH_2)_2N(CH_3)_2$
$NNH(C{=}NH)NH_2$	$(CH_2)_2N(CH_3)_2$
O	$(CH_2)_nN(CH_3)_2$, $n = 2$-4
α-OH or β-OH	$(CH_2)_nN(CH_3)_2$, $n = 2$-4
β-OH	$(CH_2)_2N(R'_3)_2$, $R' = H, CH_3, C_2H_5$



CIX

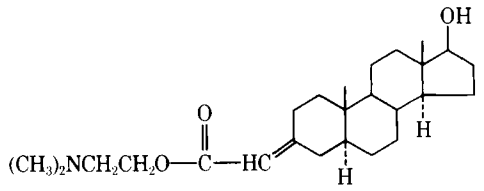


CX

wide range of substances including monovalent and divalent cations and possibly carbohydrates, amino acids, biogenic amines, and lipids. The reader is referred to a recent review for an account of these aspects (14).

At the organ level, the most striking effect is on the heart. The complex nature of the pharmacological effects of cardiotonic steroids on the failing and nonfailing heart has been documented (17, 18, 164). Recent studies emphasized that the rate of onset and intensity of the effects of cardiotonic steroids on myocardial contractility may be significantly affected by various factors such as temperature, frequency of stimulation, and concentrations of Na^+,K^+ and Ca^{+2}. (See Refs. 17 and 164 for a critical discussion of these effects.) Furthermore, it is now apparent that the older view which held that the positive inotropic effects of cardiotonic steroids occurred *in situ* only in failing hearts is no longer tenable (17). Failure to recognize this fact may have been due to the development of compensatory effects in the normal heart. All of these observations have obvious significance in the determination of structure–activity relationships. In addition to effects on the heart, cardiotonic steroids also have a pharmacodynamic action in the central nervous system and in the kidney.

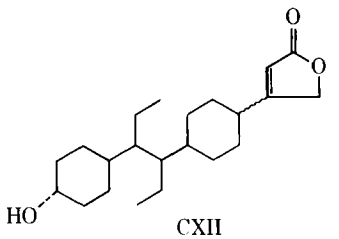
Interrelation of Biological Effects—The possible links between the effects of cardiotonic steroids at the various levels of biological organization will now be considered. At the outset, it must be emphasized that there is much controversy in the literature. This arises partly because of variations in experimental parameters and partly because of the inherent difficulties that arise when the intact functioning system is disturbed by the experimental probe. To quote Lee and Klaus "... to date, the enormous information about the effects of cardiac glycosides has not provided a clear mechanism of positive inotropism on a reasonably firm experimental basis" (17). What follows is the present authors' selection of what appears to be the most adequately established conclusions. Almost every point, however, is in dispute, and the reader is referred to the previously cited reviews and to others to be cited for a critical appraisal.



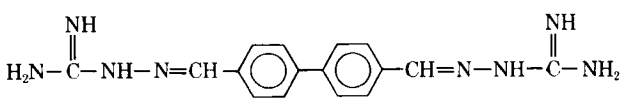
CXI

As previously mentioned, the purpose of this review is to direct the medicinal chemist to relevant sections of the voluminous literature on the mode of action of cardiotonic steroids. It is advisable to start with an appreciation of the morphology and physiology of the heart and of the events involved in excitation–contraction phenomena. This should then be followed by an appreciation of what is meant by positive inotropism and of the methods used to measure this phenomenon. The possible mode of action of cardiotonic steroids should then be considered against this background. The present authors found a number of reviews useful with respect to these concepts (165, 166, 164, 167–169, 14, 17, 170; the reviews are probably best read in the order given).

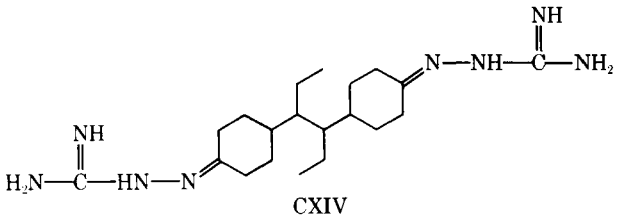
With the hope that it would be a further guide to the reader, a "systems analysis" for the coupling of myocardial excitation and contraction was prepared (Scheme XXIII). The scheme was prepared by incorporating the conclusions of many authors, and it includes much that is hypothetical and controversial.



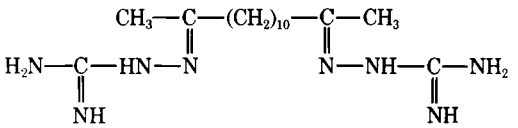
CXII



CXIII



CXIV



CXV

The scheme can thus be regarded as an eclective model and should be considered more as a guide to the literature than a guide to the heart.

Some aspects of the proposed model can be regarded as well established. These include:

1. Excitation leads to an influx of Na^+ and an efflux of K^+, and this in turn leads to depolarization of the plasma membrane of the sarcolemma. (The sarcolemma is a double-membrane structure that surrounds and invaginates the myoplasm. It consists of an inner or plasma membrane and an outer or basement "membrane" or external lamina composed of a dense mat of fine filaments.)

2. As a consequence of 1, there is an increase in the concentration of free or "activator" Ca^{+2} in the vicinity of the myofibrils (the contractile elements within the cell).

3. When the level of the free Ca^{+2} exceeds a critical value, it begins to antagonize the inhibitory action of troponin. (In the resting state, the existence of an actin–troponin–tropomyosin–myosin complex constitutes a barrier against the formation of actomyosin.)

4. Antagonism of troponin by Ca^{+2} permits the formation of the energetically stable actomyosin complex, with resulting contraction of the myocardium, using energy supplied by ATP.

5. Relaxation (uncoupling of actin and myosin) requires an input of energy (supplied by ATP) and sequestration of activator Ca^{+2} by some internal store, which is probably located in the sarcoplasmic reticulum. [The sarcoplasmic reticulum is a longitudinal system of fine interconnecting tubules that permeate the myoplasm and come in close contact with the myofibrils, T-tubules, and sarcolemma. The T-tubules are invaginations (mainly lateral) of the sarcolemma.]

6. Repolarization results from activation of Na^+,K^+-ATPase. The latter is located in the plasma membrane of the sarcolemma and is activated when the internal concentration of Na^+ (Na_i^+) and the external concentrations of K^+ (K_o^+) rise above a critical level. The system thus constitutes a feedback control.

7. The biological effects of cardiac glycosides result from interaction with some component on the external face of the plasma membrane. Since the plasma membrane invaginates (as T-tubules), it is thus possible for cardiac glycosides to interact with structures deep within the myocardial cell.

8. Cardiac glycosides combine with the external face of the Na^+,K^+-ATPase macromolecule (or complex of macromolecules) and inhibit enzyme activity by an allosteric mechanism.

9. The toxic effects of cardiac glycosides are a direct consequence of extensive inhibition of Na^+,K^+-ATPase.

The following aspects of the analysis shown in Scheme XXIII are disputed.

1. There is no general agreement on the nature of the link, if any, between Na^+ influx and Ca^{+2} uptake.

2. The origin of activator Ca^{+2} is probably the most controversial aspect of the link between excitation and contraction. Some investigators believe that activator Ca^{+2} is derived primarily from the "internal stores" located probably in the sarcoplasmic reticulum, whereas others believe that the prime or sole source is either from some superficial store located in or close to the plasma membrane or from the extracellular fluid. The interpretation of data is complicated by the fact that there seems to be no simple relationship between the concentrations of free intracellular Ca^{+2} and the development of muscle tension.

3. The interconnection between Ca^{+2} influx and the various stores of Ca^{+2} is poorly understood.

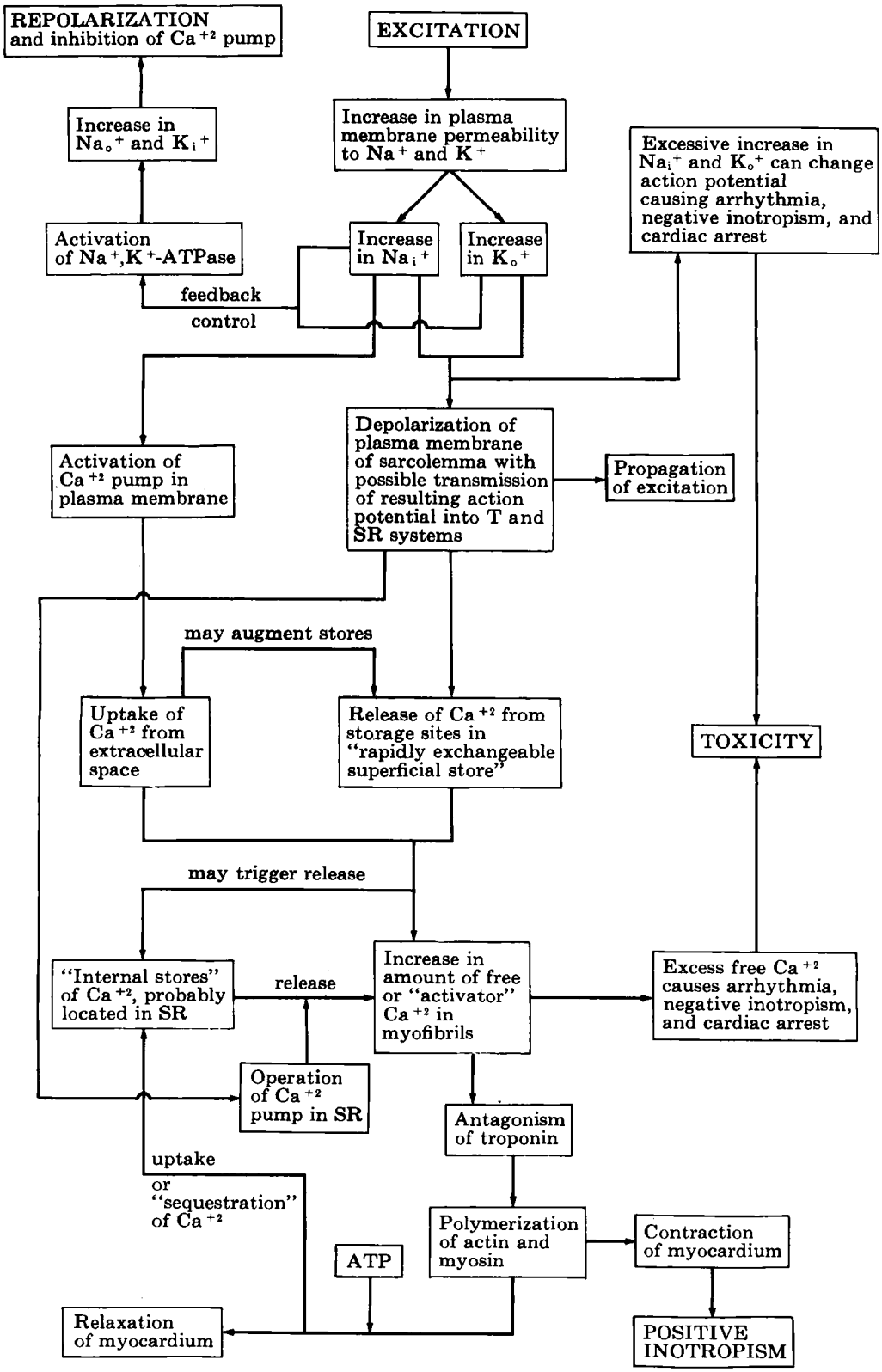
4. The mechanism of the inotropic action of cardiac glycosides is the subject of much dispute. While there is general agreement that the toxic effects of cardiac glycosides result from inhibition of Na^+,K^+-ATPase and are accompanied by a net influx of Na^+ and efflux of K^+, no such agreement exists with respect to the effects of therapeutic doses of digitalis.

5. Except for the activation of Na^+,K^+-ATPase, the various feedback, augmenting, and triggering mechanisms shown in Scheme XXIII are largely conjecture.

References supporting these statements may be found in the previously cited reviews (14, 17, 164–170).

While the biochemical basis of the inotropic effects of cardiotonic steroids is still unresolved, the bulk of recent work indicates that therapeutic doses of digitalis-like compounds affect cation fluxes in a manner consistent with the conclusion that inhibition of Na^+,K^+-ATPase is the primary event that leads, through an increase in activator Ca^{+2}, to the positive inotropic effect. Two important qualifications must now be applied to this statement.

First, the methods used to isolate Na^+,K^+-ATPase discard most of the enzyme activity during the process of partial purification. Therefore, it is possible to hypothesize that there exists a small subfraction of "Na^+,K^+-ATPase" which has acquired some specialized role and which may be the entity mediating the therapeutic effects of digitalis. This "fraction," which may be discarded during purification, may even have lost its ability to transport Na^+ and K^+. It may, for example, be located in the T-tubules where these



Scheme XXIII—Eclectic model for coupling of excitation and contraction in myocardium (14, 17, 164–170)

come in close contact with the sarcoplasmic reticulum and hence may play some role in triggering the release of the internal stores of Ca^{+2}.

The second qualification is really a corollary of the first. It is possible that the apparent correlation between the therapeutic effects of cardiac glycosides and inhibition of Na^+,K^+-ATPase may simply reflect the possibility that the enzyme is acting as a "model" receptor. This problem is common in biochemical pharmacology. For example, it may be found that a group of substances stabilizes lysosomal membranes and that this effect reflects structure–activity rela-

tionships as these apply to some therapeutic effect. This does not necessarily mean that the therapeutic effect results from stabilization of lysosomal membranes. It is equally possible that the therapeutic effect results from the stabilization of some other membrane structure and that the lysosomal membranes are simply a suitable model for this effect. Likewise, the effects of cardiotonic steroids on Na^+,K^+-ATPase may simply be reflecting some other (therapeutically related) drug–receptor interaction.

As mentioned earlier, Na^+,K^+-ATPase was probably elaborated very early in the evolution of life and it would not be surprising if the heart had adapted a portion of this enzyme for some specialized role. If so, it might explain the unique effects of cardiac glycosides on the heart. Digitalis-sensitive Na^+,K^+-ATPase is found in most, if not all, of the body's cells. In addition, the basic essentials of excitation–contraction coupling depicted in Scheme XXIII apply equally to skeletal muscle. What then is the basis of the unique effects of digitalis on the heart? It is inadequate, in fact it is tautological, to simply say that the unique effects of digitalis on the heart reflect the fact that the heart is different from other organs. What needs to be known is the nature of the difference that forms the basis of the selective action of digitalis.

Studies of the microstructure of cardiac cells have led to the suggestion [discussed by Langer (170)] that the source of activator Ca^{+2} is different from that in skeletal muscle. This could well be the basis of the selective action. Besch and Schwartz (171) hypothesized that the inotropic effects of digitalis-like compounds may not be consequential to changes in Na^+ and K^+ flux but may arise because the interaction of cardiotonic steroids with the Na^+,K^+-ATPase macromolecule may induce conformational changes in neighboring regions of the plasma membrane, with consequential release of a hypothetical store of Ca^{+2}.

Much significance has been given to the studies of Baker *et al.* (172) who used the perfused squid axon (from which the axoplasm had been previously extruded) to obtain direct evidence that Ca^{+2} influx was increased when the concentration of Na^+ was decreased on the outside of the membrane or increased on the inside. These results, while supporting the proposition that inhibition of Na^+,K^+-ATPase promotes the uptake of Ca^{+2}, do not, for obvious reasons, explain the unique effects of digitalis on the heart.

Any comprehensive theory of digitalis action must also incorporate an explanation of the fact that insulin potentiates the action of cardiotonic steroids (173) whereas thyroxine raises the threshold for digitalis toxicity (174). All of these comments focus attention on the important role played by the cell membrane in regulating intracellular events. Langer (170) has emphasized this point and his quotation from Whaley and associates (175) is worth repeating: "... there is a growing awareness that many cellular functions are directly influenced or 'controlled' by macromolecules 'outside' the cell, either as components of the plasma membrane, or as cell surface-associated materials, or as components of intercellular matrices." The manner by which these mechanisms operate is largely unknown.

Knowledge of the interior of the cell is equally limited. In 1972, Schwartz *et al.* (14) wrote: "We have practically no concept of the inside of a living cell." In 1945, Needham (176) wrote: "How, for instance, does an egg know which of its ends is which; which end is to be the front of the future animal and which end the back? If a transparent egg of a sea-urchin, for example, is centrifuged ... the contents of the egg are largely stratified, different sorts of fat, granules, *etc.*, coming together in layers. Yet the further development is not in the least affected. The egg will shortly afterwards bud forth its new small cells in exactly the same place as it would if nothing had been done to it. It 'knows' which end is which, and your throwing things about inside it has not in the least confused it. It looks as if it had a 'crystal lattice' inside it, like one of those rigid cat's cradles of coloured balls which you can see in the South Kensington museums." Modern research has confirmed that that which we call the "soluble fraction" of the cytoplasm is, in fact, a highly structured system, but we are still at the very threshold of comprehending its intricate nature.

The study of the biological effects of cardiac glycosides should thus be seen to have a much broader significance than the mere elucidation of a therapeutic effect. Increasingly, biologists are using cardiac glycosides as tools to study membrane phenomena in a wide variety of tissues. Further discussion of this fascinating subject is beyond the scope of the present review, but the medicinal chemist has played an invaluable role in providing the biologist with chemical probes with which to study the molecular basis of life. Our present knowledge of protein synthesis, for example, would not have been gained without such drugs as puromycin and other antibiotics, antimetabolites, and biological alkylating agents.

Na^+,K^+-ATPase as Digitalis Receptor—Irrespective of the role of Na^+,K^+-ATPase in the mediation of effects on myocardial contractility, two conclusions are beyond dispute: the enzyme contains a receptor for digitalis-like compounds, and the enzyme plays a vital role in most, if not all, of the body's cells. It is appropriate then that medicinal chemists should develop new series of compounds with selective effects on Na^+,K^+-ATPase. Such studies may yield new drugs with effects other than on the heart and may further assist the biologist in elucidating the role of this enzyme in controlling cell ecology. As an aid to the medicinal chemist, some of the important properties of Na^+,K^+-ATPase are listed here. But this is not a critical appraisal, and the reader is referred to the various specialist reviews [particularly that of Schwartz *et al.* (14)] for a comprehensive review of the literature.

The Na^+,K^+-ATPase referred to in this review has already been defined as a membrane-bound, Mg^{+2}-dependent ATPase which is activated by Na^+ and K^+ and specifically inhibited by ouabain and other cardiotonically active cardiac glycosides. At least

three other membrane-bound ATPases have been identified in heart muscle cells (177), but only Na^+,K^+-ATPase (sometimes referred to as transport-ATPase) shows all of the listed properties. Cytochemical studies (177, 178) indicate that Na^+,K^+-ATPase is located in the plasma membrane of the sarcolemma. Biochemical techniques used to isolate and measure Na^+,K^+-ATPase activity have been described in detail (179).

The enzyme appears to be an intrinsic part of the cell membrane, and no enzyme activity passes into solution when cells are extensively homogenized. Treatment of the enzyme suspension to remove lipids produces a soluble protein preparation with reduced ability to bind cardiac glycosides and no Na^+,K^+-ATPase activity. The addition of phosphatidylserine to the solubilized protein produces a soluble complex which binds ouabain and splits ATP in a manner comparable to the particulate preparation (180). The similarity between the properties of the reconstituted soluble enzyme complex and those of membrane fragment preparations led Chipperfield and Whittam (180) to conclude that phosphatidylserine may be closely involved in the *in situ* operation of Na^+,K^+-ATPase. These results are in accord with other work (*e.g.*, Ref. 181), indicating that the enzyme is lipoprotein in nature.

Estimates of the molecular weight of Na^+,K^+-ATPase range from 250,000 to 1,000,000 daltons. These values were obtained using the method of radiation inactivation (182, 183) and by various techniques of gel filtration and centrifugation (184, 185). Divergence in estimates reflects the limitations of the various techniques and the fact that the enzyme probably consists of subunits. What is more important is that these estimates show that the enzyme is of sufficient size to penetrate the full width of the plasma membrane and thus has the necessary dimensions to act as the cation carrier in the active transport of Na^+ and K^+. There is much evidence to suggest that hydrolysis of ATP (which provides the energy for active cation transport) takes place on the internal face of the enzyme (186) and that the sodium and potassium binding sites are located, respectively, on the inner and outer surfaces of the enzyme (187). Various models have been suggested to depict the mechanical transfer of Na^+ and K^+, but these generally lack experimental verification (188). There is, however, abundant evidence that the enzyme can and does undergo controlled conformational changes and that these changes form the basis of active transport (14).

There have been extensive studies of the kinetics of ATP hydrolysis by Na^+,K^+-ATPase, including studies of the effects of cardiac glycosides. The interpretation of the data from these studies is by no means straightforward, and the relevance of some work is questionable since there is good reason to believe that the properties of the cation pump are significantly influenced by many features of the *in situ* environment. The following sequence may be applicable to the intact system.

Rising concentrations of Na^+ on the inside of the cell membrane result in the binding of this ligand to specific cation-binding sites on the inner face of the enzyme. The energy changes consequent to the uptake of Na^+ ions could be manifested in several ways including changes in charge distribution, changes in enzyme conformation, and changes in the structure of bound water. Whatever the mechanism, the uptake of Na^+ leads to the binding of one molecule of ATP to an active site on the inner face of the enzyme. Stage one is thus the formation of an enzyme–ATP complex. This is followed very rapidly by Mg^{+2}-catalyzed hydrolysis of ATP to produce a phosphorylated enzyme (stage two). What happens next is unknown, but it is assumed that the high energy released by the hydrolysis of ATP results in a major conformation change or series of changes, the net result of which is the transfer of Na^+ across the membrane and subsequent release into the "extracellular fluid" (in the heart this would be into the space between the plasma and basement membranes of the sarcolemma). The release of Na^+ terminates stage three.

The energy or conformational changes consequent to stage three result in the generation of specific K^+ binding sites on the outer face of the enzyme. These sites may be the same or different from those that release Na^+. The uptake of K^+ by the "new" cation-binding sites (stage four) results in further changes, which culminate in dephosphorylation of the enzyme (stage five). The energy changes consequent to dephosphorylation induce another series of major conformational changes in the enzyme with the result that K^+ is transferred across the membrane and released into the cytoplasm (stage six).

This sequence incorporates suggestions by Albers *et al.* (189) and Schönfeld *et al.* (190). The former workers proposed that the conformational changes occurring in the cycle are of the *cis/trans*-variety but there seems to be no experimental justification for this suggestion. The controversial role of Mg^{+2} ions seems to have been settled by Schönfeld *et al.* (190) who showed that an enzyme–ATP complex will form in the presence of Na^+ in Mg^{+2}-free medium containing cyclohexyldiaminetetraacetate to chelate traces of contaminating Mg^{+2} ions. Addition of Mg^{+2} was found to be necessary for phosphorylation of the enzyme (stage two in the sequence). Furthermore, studies in some tissues, such as erythrocytes, indicate that the hydrolysis of one molecule of ATP is coupled with an inward movement of two ions of K^+ and an outward movement of three ions of Na^+ (191). Studies of the stoichiometry of ion transport in the myocardium have yielded conflicting results (14).

Na^+,K^+-ATPase is inhibited by cardiac glycosides in concentrations comparable to those associated with the therapeutic and toxic effects of these substances. Inhibition appears to result from interaction of the drug with the external face of the enzyme (192). This is of particular interest in view of recent direct evidence that the inotropic effects of cardiac glycosides are mediated by drug acting on the outer surface of the membrane. This evidence was obtained using albumin-bound glycoside and glycoside specific antibodies (170). Since a substantial body of evidence

(*e.g.*, Ref. 186) indicates that hydrolysis of ATP takes place on the inner surface of the membrane, it would appear that inhibition of hydrolysis by cardiac glycosides is the result of an allosteric mechanism. Using a variety of techniques, Schwartz *et al.* (14) obtained good evidence that the interaction of ouabain with membrane fragments containing Na^+,K^+-ATPase leads to conformational changes of gross magnitude.

Matsui and Schwartz (193) also demonstrated that only physiologically active cardiotonic steroids can compete with ^3H-digoxin for receptor sites on the enzyme. The conditions that determine maximum binding of cardiac glycosides to the enzyme have been examined by many groups and have been shown to parallel both *in vivo* and *in vitro* inhibition of enzyme activity (189, 194). This evidence, together with a large volume of similar studies (see previously cited reviews for references), has firmly established the existence of a "digitalis receptor" as part of the macromolecular complex constituting the Na^+,K^+-pump.

Mathematical models (rate equations) have been formulated to describe the complex interaction of Na^+,K^+-ATPase with its many ligands (Na^+,K^+, ATP, Mg^{+2}, and ouabain) (190, 195, 196). It is clear from these studies that binding affinities are interdependent and vary with varying ligand concentrations. The system is further complicated by the fact that Na^+ inhibits K^+ binding at the K^+ binding site and vice versa. In the *in vivo* situation, the existence of an intact membrane modifies this situation. In spite of the complexities of interpretation, several conclusions seem applicable to the *in vivo* state:

1. It has been corroborated that conformational changes accompany the binding of ligands.

2. It is clear that the digitalis receptor is generated during the cyclical series of conformational changes constituting the normal operation of the pump. The receptor is probably not part of any of the actual cation-carrying moieties but is so constituted that its occupation by a cardiotonic steroid stabilizes one of the intermediate phosphorylated states of the enzyme.

3. There is some uncertainty as to the actual stage in the pump cycle at which the digitalis receptor appears, but the bulk of evidence favors its appearance at the stage where ATP is bound but before phosphorylation has occurred. The receptor may persist through several intermediate stages prior to the release of Na^+. If so, its occupation at any of the various stages could lead to different forms of the drug–enzyme complex. It is also possible that two quite distinct digitalis-binding sites may be generated during the pump cycle. Lindenmayer and Schwartz (196) believed that there is essentially only one species of ouabain–receptor complex. Schönfeld and associates (190) believed that there are least four distinct states of the binding conformation for ouabain, and Taniguchi and Iida (197) used Scatchard plot analysis to detect two distinct ouabain-binding sites in Na^+,K^+-ATPase.

Albers *et al.* (189) showed that the rate at which cardiotonic steroids are bound to the enzyme is inversely proportional to the number of sugars and hydroxy groups, and this was interpreted as inferring that the receptor may be hydrophobic in character. The same workers also showed (189) that the enzyme–ouabain complex can be phosphorylated by orthophosphate whereas the native enzyme requires the presence of a more effective leaving group (the adenine dinucleotide) for what is presumably a nucleophilic substitution reaction with phosphate.

The following scheme has thus emerged. Cardiotonic steroids inhibit Na^+,K^+-ATPase by combining with a hydrophobic patch on the extracellular face of the enzyme. This combination induces conformational changes which are relayed across the full width of the plasma membrane. These changes profoundly affect the reactivity of the cardinal site on the intracellular surface so that the energy barrier for phosphorylation is lowered and the stability of the resulting phosphorylated enzyme is increased to such a level that the pump is "frozen" at this stage in its cycle. The original hydrophobic patch is itself not a consistent component of the enzyme surface but is generated as a result of the binding of Na^+ and ATP to the inner surface of the membrane.

Since this review is intended primarily for chemists, it is appropriate to point out that conformational interactions of the type depicted here are fully in accord with modern concepts of molecular biology. As far back as 1945, Needham (176) wrote of the "ceaseless interchange [by which] the pattern of the body is fully maintained." A fascinating example is that of the amoeba which moves by continuously tearing down and building up its plasma membrane, the "old" membrane material "disappearing" into the cytoplasm.

Dissociation of Inotropic Effects from Inhibition of Na^+,K^+-ATPase—An apparent contradiction in the literature concerns the widely different reports of the stability of the drug–enzyme complex. According to Lindenmayer and Schwartz (196) and Albers *et al.* (189), the complex is virtually irreversible. In fact, the series of binding studies carried out by Schwartz and associates is based on the assumption that no dissociation of drug occurs under nonequilibrium conditions. In one such study (198), ouabain was administered to dogs in sufficient dosage to produce maximum inotropic effect. The hearts were then excised and Na^+,K^+-ATPase was extracted and subjected to partial purification under nonequilibrium conditions. The enzyme was found to possess only 40% of the activity of control enzyme, presumably because of bound ouabain. The authors stated that qualitatively similar results were obtained when the enzyme was subjected to more extensive purification procedures involving treatment with deoxycholate and prolonged extraction with concentrated sodium iodide solutions. By contrast, Yoda (199) reported that when ouabain–Na^+,K^+-ATPase complexes, which had been preincubated for 10 min, were subjected to 10-fold dilution, the apparent first-order dissociation rate constant was 0.42 hr^{-1}. This value was associated with an initial enzyme inhibition of 72%. Somewhat similar results were obtained for a selection of other monoglycosides.

Yoda's results were obtained under conditions in

Table I—Composite Table of Results Selected from Okita *et al.* (207)

Cardiotonic Steroid	Approximate Mean Increase in Force of Contraction, % of Control	Period Required to Washout Positive Inotropic Effect, min	Mean Percent Inhibition of Na^+,K^+-ATPase Isolated *Ex Vivo* after Washout Period
Strophanthidin-3-bromoacetate	30	30–45	29
Strophanthidin	30	40–60	38
Ouabain	30	60–90	39

which binding was promoted in the presence of Mg^{+2} and inorganic phosphate (P_i) as the only ligands. This is in accord with studies (200) indicating that significant ouabain binding occurs in the presence of either Mg^{+2} + ATP + Na^+ or Mg^{+2} + P_i or Mn^{+2} and that similar conformational changes may be associated with all three sets of binding conditions. Studies similar to those of Yoda were described in the previously cited comprehensive report (190) and are in substantial agreement with Yoda's results. The latter authors claimed that the release of ouabain from its complex with Na^+,K^+-ATPase is independent of whether phosphorylation was produced with ATP + Mg^{+2} + Na^+ or with P_i + Na^+. Schönfeld *et al.* (190) acknowledged that these conclusions are not supported by some other workers (201, 202) but cited different experimental techniques as the basis for lack of agreement. In another study (203), it was suggested that ouabain-induced enzyme inhibition was directly proportional to binding in the presence of K^+ and that the interaction of digitalis with the enzyme under optimal conditions may proceed through an intermediate state in which the drug was loosely bound and then to a more stable tightly bound complex. However, the same report suggested that the stable complex may also form as a result of binding in the presence of only Mg^{+2} and P_i.

In spite of this controversy, the authors of this present review accept the general conclusion reached by Allen *et al.* (203) that inhibition of Na^+,K^+-ATPase under *in vivo* conditions is associated with the formation of an extremely stable, relatively irreversible, drug–enzyme complex. This conclusion is important in view of the relative ease with which the inotropic effects of cardiac glycosides, particularly the more water-soluble types such as ouabain, can be washed out of organ bath preparations of myocardium (including whole heart).

In 1966–1967, one of the present authors worked for 10 months in the laboratory of Dr. George T. Okita. During that period, studies (204, 205) were initiated which are of considerable relevance to the issues raised. The experiments were based on the observations of Hokin *et al.* (206) that strophanthidin 3-haloacetates acted as active site-directed irreversible inhibitors of brain Na^+,K^+-ATPase. Hokin *et al.* found that these alkylating agents rapidly combined with the receptor to form a reversible complex, which was then more slowly converted to an irreversible complex, the conversion being complete within 3 hr. In the study of Okita and associates, guinea pig and rabbit atria were driven for 3 hr in a medium containing sufficient strophanthidol 3-bromoacetate-19-^3H to cause a 30–40% increase in the force of contraction. The atrial preparations were then washed, and it was found that the drug effect on contractility was rapidly removed (half-life for loss of drug effect from rabbit atria was 7 min compared with a half-life for drug loss of 240 min). To confirm that all contractility receptors had been cleared of drug, the ouabain dose–response curve was determined for the washed preparation and found to be identical to that of a properly constituted control.

The following explanation was offered to account for the dissociation of drug effect and drug half-life. Since the drug must have been associated with the contractility receptors for 3 hr (a 30–40% increase in force of contraction was recorded for all of this period) and since this drug effect was rapidly and completely reversed by washout, the "contractility" receptor, unlike Na^+,K^+-ATPase, was incapable of being alkylated and was different from Na^+,K^+-ATPase. This conclusion was subject to the following limitations:

1. The concentrations that Hokin *et al.* (206) found necessary for irreversible alkylation were at least one magnitude greater than those used in Okita's work.
2. The mode of action of alkylating cardiotonic steroids may be different from that of nonalkylating steroids.
3. The atrium may not be representative of the whole myocardium.

These limitations were overcome in a recent study (207). In addition to using an alkylating agent, the effects of strophanthidin and ouabain were also examined. The experiments were carried out using the Langendorff heart preparation, and Na^+,K^+-ATPase activity was assayed both before and after the washout period. The main features of Okita's work are shown in Table I, which is based on Ref. 207.

The results shown in Table I clearly infer a separation of positive inotropic effects from inhibition of Na^+,K^+-ATPase. However, recent studies similar to those described by Okita *et al.* have failed to demonstrate the separation of effects observed by the latter (A. Schwartz, private communication). Since both groups of workers apparently commenced washout procedures before the phase of drug equilibration was complete and also used isolation techniques (K^+-free media) that were not optimum for minimizing dissociation of the enzyme-drug complex, more work in this area is needed.

In 1973, Goldman *et al.* (208) published the results of an *in vivo* study which they claimed supported Repke's hypothesis. These authors studied the effects of digoxin in normokalemic and hyperkalemic dogs and found that both the positive inotropic effects and the degree of inhibition of Na^+,K^+-ATPase were significantly reduced in the hyperkalemic dog as compared with the normokalemic animal (K^+ is known to inhibit the ability of cardiotonic steroids to

inhibit Na^+,K^+-ATPase; see Refs. 14 and 17 for a discussion of this effect). The main features of the Goldman *et al.* study are shown in Table II.

Table II—Composite Table of Results Selected from Goldman *et al.* (208)

Parameter	Approximate Percent by which Parameter Was Reduced in Hyperkalemic Dogs as Compared with Normokalemic Dogs
Positive inotropic effect (*dp/dt*)	82
Drug concentration in left ventricle, ng/mg	23
Drug bound to microsomal fraction, ng/mg	30
Percent inhibition of Na^+,K^+-ATPase	37

STRUCTURE–ACTIVITY RELATIONSHIPS

Structure–activity relationships are studied for various reasons: to provide empirical guidelines for the synthesis of new compounds; to seek evidence for the existence of a receptor; and to establish degrees of correlation between biochemical, physiological, therapeutic, and toxic effects associated with the drug. For all of these purposes, structure–activity relationship analyses can prove both useful and misleading and should always be interpreted with caution. Most correlations between biological effects are based on circumstantial evidence; the danger is that once a theory, which seems reasonable, has become widely accepted, all new evidence is conceptualized in terms of the theory and alternative explanations are not sought. Even such a well-established concept as the role of the cholinergic system in nerve transmission can be turned "on its head," and one group of workers (209, 210) rejected the theory that acetylcholine is involved in synaptic transmission.

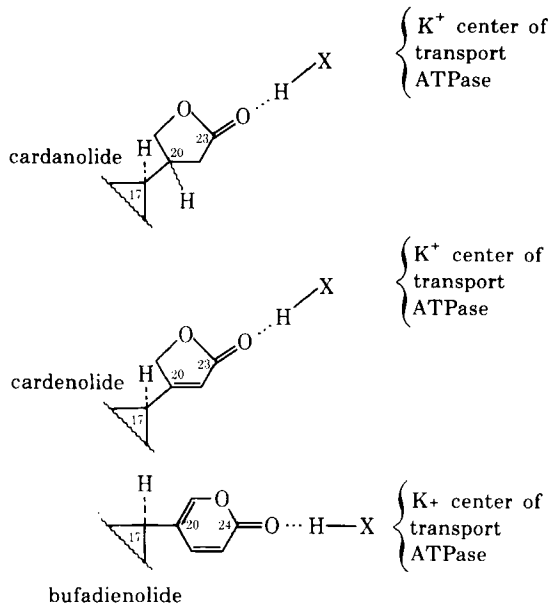
The first step in any structure–activity relationship analysis is to decide, if possible, whether the drug is acting by a specific (receptor-mediated) mechanism or by a nonspecific mechanism (such as membrane swelling). Drugs that act by a nonspecific mechanism may still be selective in action if their physical properties cause them to concentrate in particular parts of the body. The barbiturates, for example, are thought by some workers to act nonspecifically by selective effects (probably membrane swelling) which result in depression of the arousal center in the brain (211). Slight modifications to the structure of barbiturate molecules can subtly alter the physical properties of these drugs so that their selective effect is manifested elsewhere in the brain and the body convulses.

The activity of drugs that act by a receptor-mediated mechanism usually show great sensitivity to minor changes in structure. This sensitivity to structural changes applies in the case of the phenothiazine antipsychotics; yet there is great controversy as to whether these drugs act by a specific or nonspecific mechanism (212). In fact, the phenothiazine antipsychotics are a good group of drugs with which to compare the cardiac glycosides. There is much dispute as to the molecular and biochemical basis of antipsychotic activity. Many properly established, but conflicting, correlations have been made, including one that is often used in the screening of antipsychotics, namely the good correlation that exists between blocking of the conditioned-avoidance response in the rat and antipsychotic activity in humans. Here at least it is obvious that one is not dealing with a cause-and-effect relationship but with an associative relationship that reflects some feature common to both species but separately connected with the two phenomena. This is not an unusual situation and, in philosophical terms, it reflects one of the most important scientific concepts of the 20th century, namely the quantum theory which states, in general terms, that nature has achieved the vast complexity of the universe by utilizing a relatively small number of basic building blocks. It is the *organization* of the building blocks that constitutes the essential features of different systems, but it is often only the building blocks that the probes detect.

Model for Digitalis Receptor—It is generally accepted, and it is probably true, that the physiological effects of the cardiac glycosides are mediated by an interaction with a specific receptor. This conclusion is based on the high potency, high selectivity, and apparently specific structural requirements of digitalis. The idea is supported by the existence of at least one type of identifiable digitalis receptor, Na^+,K^+-ATPase. One reliable indicator of a receptor-mediated mechanism, the existence of a structually related competitive antagonist, has not been found for digitalis. Nevertheless, it will be assumed that the biological effects of cardiotonic steroids result from the interaction of these drugs with one or more closely related receptors.

According to the classical studies of the structure–activity relationships of digitalis, the entire molecule seems to take part in the drug–receptor interaction. Thus, the 17β-lactone ring, the unique stereochemistry of the digitalis steroid moiety, and the nature of the substituent at C-3 were all shown to be important for activity. It was also known that the inactive 17β-H isomers of cardiac glycosides were not effective as inhibitors of the active 17α-H isomers, so the determining factors in structure requirements were probably concerned with what is now called affinity (binding) rather than intrinsic activity (factors that affect the nature of the drug–receptor complex).

In 1963, Repke (213) showed that similar relationships applied to the binding of active cardiotonic steroids to Na^+,K^+-ATPase. Concentrations of 17β-H isomers up to 10,000 times those of the active 17α-H isomers were without effect on the latters' log dose–response curves for enzyme inhibition. Repke concluded that the lactone ring played a key role in the drug–receptor interaction and that unless the ring was correctly orientated with respect to the rest of the molecule, no binding at all took place. Repke fur-



Scheme XXIV—Representation of hypothetical one-point attachment of digitalis molecule to receptor enzyme by hydrogen bridge binding (16)

ther hypothesized that the lactone ring was bound to the receptor by a single-point attachment comprising a hydrogen bond, the strength of which was proportional to the fractional negative charge on the carbonyl group. Thus, the increasing capacity of the cardanolide, cardenolide, and bufadienolide systems to form hydrogen bonds (Scheme XXIV), was shown to parallel ability to inhibit Na^+,K^+-ATPase (213).

Repke recognized that a single-point attachment involving a hydrogen bond would have negligible binding force. He proposed (16) that the β-face of the steroid system interacted with a complementary patch on the receptor surface and that this interaction, through an accumulation of van der Waals forces, provided most of the binding energy. The role of the α,β-unsaturated lactone was thus envisaged as one that directed the molecule toward the receptor by the formation of a hydrogen bond acting over a relatively long distance; but once the molecule was correctly in position, the shorter range van der Waals bonds became the chief binding force.

The role that Repke assigned to the steroid ring is almost certainly correct since lactone rings *per se* are inactive. Forces that bind the steroid, however, may be other than van der Waals forces and could involve conformational changes in the receptor surface of the type discussed by Belleau (214), in which binding results as a consequence of a shift by a macromolecule to a more thermodynamically stable state with the drug molecule becoming locked within a fold of the receptor.

The role that Repke described for the lactone also seems reasonable, but consideration needs to be given to whether a single hydrogen bond is adequate to initiate the formation of the drug–receptor complex. After all, the possibilities for hydrogen bond formation in the body are immense, and hydrogen bonding with water must first be disrupted before the lactone can react with the receptor. Moreover, the difference in the ability of cardenolides and cardanolides to form hydrogen bonds is not an adequate explanation, in terms of Repke's theory, to explain the observed differences in biological potency. It is therefore hypothesized (89, 215) that the α,β-unsaturated lactone binds by a reinforced hydrogen bond and that such a possibility is not available for the cardanolides. Some evidence for this proposal will now be examined.

To study further the role of the lactone in controlling the biological activity of cardiotonic steroids, we have replaced the lactone ring of digitoxigenin (II) with a series of open-chain structures of varying electronic and steric similarity to the lactone (XLVII–LXXIX). These compounds were tested for their ability to inhibit guinea pig myocardial Na^+,K^+-ATPase (89) and for their ability to affect guinea pig myocardial contractility (215). A selection of the results is shown in Table III.

The results in Table III show that a range of potency was observed, varying from compounds with activities comparable to those of the parent molecule, digitoxigenin, to compounds that were inactive or that produced a negative inotropic effect. We have shown that it is possible to replace the butenolide ring with certain open-chain structures, namely, $-CH=CHCOOCH_3$ (XLIX), $-CH=CH-CN$ (LXIII), and $-CH=N-NH-C(NH)NH_2$ (LXXI), and still retain considerable potency (Table III). All of these compounds increased the force of myocardial contraction by approximately 120% at maximum therapeutic doses (Table IV). Compounds of the 17α-series (*e.g.*, LXXVI) were inactive or virtually inactive, confirming Repke's hypothesis. Reduction of the Δ^{20}-double bond (*e.g.*, LI) abolished activity.

Table III—Comparison of Effects on Guinea Pig Atrial Contractility and Guinea Pig Myocardial Na^+,K^+-ATPase (89, 215)

Compound	Positive Inotropic Effect (Potency Relative to Digitoxigenin)[a]	Inhibition of Na^+,K^+-ATPase: Potency Relative to Digitoxigenin[a]	Inhibition of Na^+,K^+-ATPase: Inhibition at 10^{-4} M[a], %
Digitoxigenin (II*a*)	1.0	1.0	100
Methyl ester (XLIX)	0.49	1.3	100
Nitrile (LXIII)	0.66	1.1	100
Guanylhydrazone (LXXI)	0.20	0.14	76
Branched ester (LV)	0.04	0.09	47[b]
Ethyl ester (XLVII)	0.03	0.09	44[b]
Isopropyl ester (LII*b*)	0.005	0.06	55
Phenyl ester (LIX*a*)	0.005	0.12	50[b]
Diene ester (LVIII)	0.004	0.03	33
17α-Methyl ester (LXXVI)	0.003	Inactive	Inactive
Reduced methyl ester (LI)	Inactive	0.03	24[b]
Acid (XLVIII)	Inactive	Inactive	Inactive
Semicarbazone	Inactive	Inactive	Inactive
Phenyl ketone (LIX*a*)	Negative inotropic effect	0.06	30[b]

[a] Calculated from the log dose–response curve. [b] Percent inhibition at 10^{-5} M. Inhibition at 10^{-4} M was not read from the dose–response curve because of suspected insolubility at or above this concentration.

Increasing the bulk of the alkoxy group (*e.g.*, XLVII and LII*b*) greatly reduced activity. Increasing the distance between the electronegative carbonyl group and the steroid system by means of an extended (diene) chain (*e.g.*, LVIII) was also associated with marked loss in potency. The presence of the carbonyl group was shown not to be an essential requirement for activity since the α,β-unsaturated nitrile (LXIII) and the guanylhydrazone (LXXI) were both potent substances.

Any interpretation of these results must include an evaluation of the relative importance of both the steric and electronic consequences of the changes described as well as the possibility that reduction in activity may be associated with unfavorable interactions with the rest of the molecule or with the receptor surface. These factors were considered in a detailed analysis of the results published elsewhere (215). If, for the moment, consideration of the guanylhydrazone (LXXI) is excluded, then the results indicate that all that is required for activity is the following *coplanar* arrangement of atoms in the side chain —CH=CH—C(—)=A (where A = a hetero atom).

Our analysis also indicated that this side chain probably interacted with the receptor by means of a two-point attachment, involving perhaps a hydrogen bond at one of the points as shown in Fig. 4.

The model shown in Fig. 4 is compatible with our observation that reduction of the C-20 double bond led to total or almost total loss in activity since the reduced compound lacked both the fractional positive charge at C-20 and the coplanar arrangement of atoms. The model would also account for the great loss in activity observed when a methyl group was substituted at C-21 (LV), since it may be presumed that the methyl group would physically prevent the close association needed to establish an effective two-point attachment. This explanation is also compatible with the results of Eberlein *et al.* (78) who found that the introduction of fluorine into the C-21 position of the lactone ring gave an active compound whereas introduction of a methoxy group at C-21 (XXXIII) greatly reduced activity. Fluorine has approximately the same size as hydrogen.

If it may be further supposed that the whole of the C-17 side chain fits into a "cleft" in the enzyme surface, then it is possible to explain the influence of R′ (Fig. 4) on biological activity. When R′ does not exceed a critical size, no unfavorable steric interactions occur and activity reaches a maximum. This condition operates in the case of the nitrile (LXIII), where R′ is absent, and in the case of the methyl ester (XLIX), where R′ is methoxy. It also applies to digitoxigenin (II*a*), where R′ is equivalent to methoxy. When R′ is ethoxy (XLVII), steric interactions occur with the edge of the cleft and activity is greatly reduced. Further increases in the size of R′ lead to no further appreciable fall in activity, indicating that steric interactions reach an unfavorable maximum once the size of R′ exceeds methoxy. The concept of drug molecules fitting into and interacting with clefts in biological macromolecules is well established. Actinomycin D, for example, is believed to fit into the lesser groove of DNA.

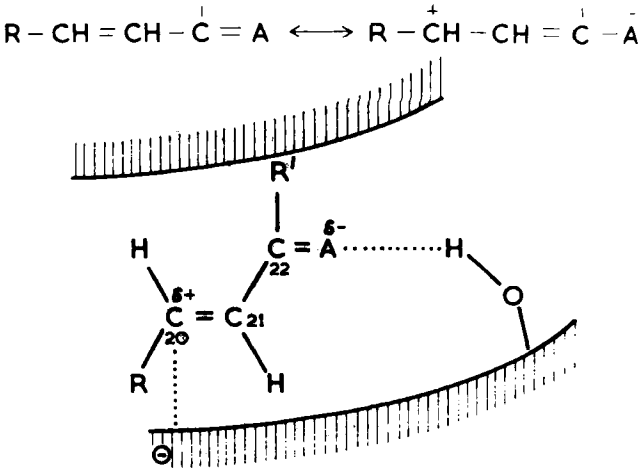


Figure 4—*Proposed interaction of the C-17 side chain of active cardenolide analogs with the "digitalis" receptor. The side chain is shown as lying within a cleft on the enzyme surface. Binding is depicted as involving two points on the side chain, the electron-rich hetero atom (A) and the electron-deficient C-20. The origin of the charge distribution in the side chain is shown by the resonance structures drawn above the receptor model. The scheme is compatible with structure–activity relationship analyses based on both inhibition of Na^+,K^+-ATPase (89) and positive inotropic effects (215).*

Finally, the model incorporates the observation that the ester group, as present in the lactone ring of digitoxigenin (II*a*) and in the methyl ester (XLIX), is not essential for activity since the nitrile (LXIII) has comparable activity.

The side chain of the acid (XLVIII) has some features common to those of the active compounds:

methyl ester (XLIX): —CH=CH—C(OCH$_3$)=O (active)

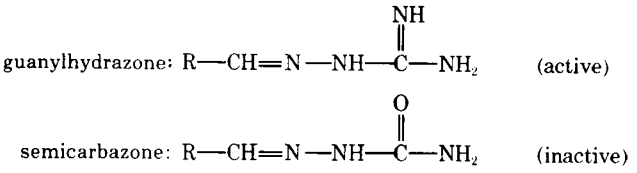
nitrile (LXIII): —CH=CH—C≡N (active)

acid (XLVIII): —CH=CH—C(=O)—O$^-$ (inactive)

The acid meets the stereochemical requirements of the model but, under physiological conditions, it lacks a fractional positive charge at C-20 and has a full negative charge distributed over the terminal oxygens. The model thus predicts the inactivity of the acid since, although the acid can very effectively hydrogen bond, the full negative charge would be repelled by the negative charge proposed for the receptor. The fact that compounds with electron-rich groups, such as the α,β-unsaturated nitrile (LXIII) or ester (XLIX), can interact with the receptor is not incompatible with this reasoning since, in the case of the ester and nitrile, the fractional negative charge is neutralized by the formation of a hydrogen bond and as such contributes to binding.

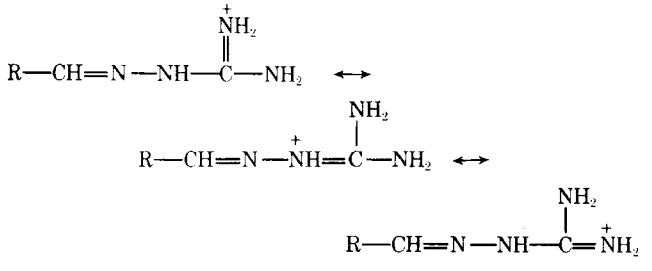
Further indirect evidence for the existence of a substantial negative charge on the receptor surface was obtained from a consideration of the activity of the guanylhydrazone (LXXI), which was approxi-

mately one-fifth as potent as digitoxigenin. Under physiological conditions, the guanylhydrazone group would exist as a cation with the positive charge dispersed over most of the side chain. The electron-rich group thought to be necessary in the case of the other active compounds is thus absent in the guanylhydrazone cation. Since we found that the log dose–response curve of the guanylhydrazone was parallel to those of the other active compounds, including digitoxigenin, we suggested that the guanylhydrazone cation interacted with the proposed receptor by the formation of a single-point attachment involving an ion-pair interaction. The fact that the nonionized semicarbazone analog was inactive supports the suggestion that electronic interactions between the side chain and the receptor are essential to the activity of cardiotonic steroids. The close similarity between the two side chains is shown below:



The guanylhydrazone group is a strong base because it can accept a proton to produce a cation with the positive charge distributed over all three nitrogens of the terminal guanidine portion of the side chain (Scheme XXV).

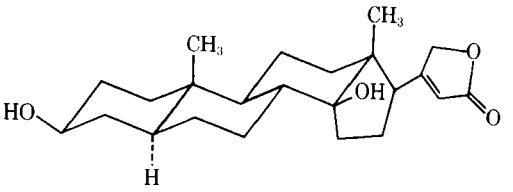
This line of reasoning can be extended to provide an explanation for the cardiotonic activity of the bisguanylhydrazones of prednisolone and related steroids (see *Steroidal Guanylhydrazones and Other Cardioactive Compounds*). The steroid systems of these compounds do not possess the properties predicted by Repke for the formation of effective van der Waals forces, so the main source of binding energy proposed by Repke for digitalis-like steroids is greatly diminished or absent. If we now propose that there exists a second anionic site on the receptor surface in the vicinity of the area that binds the C-3 region of the steroid, then the binding of the prednisolone-type derivatives can be accounted for in terms of two ion-pair interactions. As discussed previously, the stereochemistry of the steroid system is less critical for the 3,20-bisguanylhydrazones than for digitalis-like compounds. Also, since the positive charge of the guanylhydrazone group is distributed over three atoms, it is possible to allow a considerable latitude in the distance between the two guanylhydrazone groups and still align a positive charge with the fixed



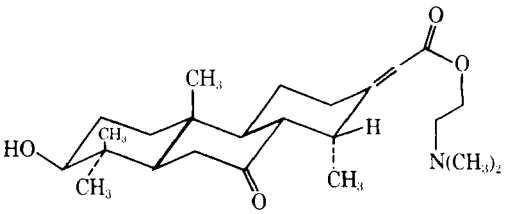
Scheme XXV

anionic sites on the receptor. Our proposal that there is a strong nucleophilic group (perhaps a full anion) in the region of the receptor which corresponds to the C-3 portion of the steroid is supported by the fact that this region can be alkylated by 3-haloacetyl derivatives of cardiotonic steroids (107).

It remains now to account for the activity of the other main group of cardiotonic substances, the *Erythropleum* alkaloids. The structural formula of the principal member of this group, cassaine (CVII), is shown here together with that of the cardiotonic steroid uzarigenin (CXVI).



CXVI: uzarigenin



CVII: cassaine

The structure–activity relationships of cassaine and related semisynthetic analogs have been studied (153–155) and are compatible with the proposed model for the digitalis receptor (Fig. 4). Cassaine and its active analogs possess the required >C=CH—C(—)=A grouping (in this case an ester). Activity is lost when the ester is hydrolyzed to the free acid or if the double bond is reduced. Activity is greatly reduced when a methyl group is attached to the double-bonded carbon atom (analogous to C-21 in the cardiotonic steroids). It will be recalled that we accounted for a similar fall in the activity of the digitoxigenin analog (LV) by postulating that the methyl branch at the double bond interfered with the formation of the two-point attachment to the receptor.

There is one significant way in which the structure–activity relationships of the cassaine analogs differ from those of digitoxigenin, namely that the length of the alkoxy group (R′, Fig. 4) is critical for the digitoxigenin series but not for the cassaine series. However, if the width of the cassaine alkoxy groups is increased by replacing the terminal *N*-methyl groups with ethyl groups, almost all activity is lost. Thus, steric features of the receptor seem to be limiting the scope of molecular modification for both series, but the limitation is expressed in a different way. It is possible to still retain the idea of the side chain fitting into a cleft as depicted in Fig. 4 by assuming that the alkoxy group of cassaine lies along the axis of the cleft rather than across it. This assumption is not unreasonable in view of the difference in the manner of attachment of the cassaine side chain to the ring system.

In attempting to correlate structure with activity, we have made the simplest assumption, namely that all of the three groups of cardiotonic substances (digitalis-like steroids, bisguanylhydrazones, and cassaine-like alkaloids) interact with the same receptor. While we have no definite proof for this assumption, the proposal is consistent with structure–activity relationship analyses for the three groups. Our proposals for the nature of the receptor were based on correlations with cardiotonic activity but, in the case of our own work at least, apply equally well to inhibition of Na^+,K^+-ATPase (Table III).

We have shown that cardiotonic activity is associated with certain steric and electronic features of the C-17 side chain (or its equivalent in the cassaine alkaloids). These features suggest that the side chain may fit within a cleft or depression in the receptor surface and interact with an anionic site. This interaction may involve a single ion-pair association or a two-point attachment consisting of a weak electrostatic interaction reinforced by a hydrogen bond. It is possible that the electronic interaction promotes a redistribution of charge density and that this may play some role in the allosteric mechanism suggested for digitalis-like activity (see *Biological Activity*). It would not be possible for the binding forces depicted in our model (Fig. 4) to account for the pseudo irreversible binding associated with at least some aspects of digitalis-like activity. The remaining portions of the molecule must thus play some highly significant role in binding. It is possible that the receptor itself undergoes "accommodating perturbations" (214) when it interacts with cardiotonic substances, and this may provide part of the binding energy.

Relationship between Cardiotonic Activity and Inhibition of Na^+,K^+-ATPase—In 1961, at the First International Pharmacological Conference, Repke (23) proposed that inhibition of Na^+,K^+-ATPase may be the biochemical basis for the effects of digitalis on myocardial contractility. This theory was further developed at the Second International Conference (213) when Repke showed that there were many parallels between the two biological effects of digitalis. The ouabain log dose–response curve for inhibition of guinea pig myocardial Na^+,K^+-ATPase was shown to encompass the range of concentrations producing inotropic and toxic effects. It was also shown that both biological responses were affected in a similar way by hypothermia and by changes in the concentrations of K^+, H^+, Ca^{+2}, Na^+, and Li^+.

While these effects are significant, they are too general to establish firmly a cause-and-effect relationship. Repke, therefore, compared the concentration of drug required to inhibit guinea pig Na^+,K^+-ATPase with the dose (micromoles per kilogram) required to produce lethal effects in the intact cat (see Ref. 216 for a recent presentation of these data). This comparison was carried out for 21 digitalis-like compounds. The results showed in a general way that compounds highly toxic in the cat were powerful inhibitors of myocardial Na^+,K^+-ATPase in the guinea pig. Since the therapeutic ratios of most cardiac glycosides are similar, it may also be inferred that a similar parallelism exists between inhibition of the enzyme and positive inotropic effects.

Table IV—Comparison of Na^+,K^+-ATPase Inhibition with Stimulant Activity on Guinea Pig Atrial Muscle (All Values Were Read from the Log Dose–Response Curve)

Compound	Concentration, $\times 10^{-7}$ *M*	Inhibition of Na^+,K^+-ATPase, % ± Mean Deviation	Increase in Force of Contraction of Atria, ± *SE*
Digitoxigenin (II*a*)	3.2	10 ± 5	25 ± 5
	9.0	20 ± 3	75 ± 12
	21	30 ± 2	130 ± 21
	33	40 ± 4	Toxicity
	65	50 ± 2	Toxicity
Methyl ester (XLIX)	3.2	10 ± 5	10 ± 3
	10	20 ± 3	40 ± 8
	20	30 ± 4	80 ± 10
	31	40 ± 3	115 ± 15
	65	50 ± 2	Toxicity
Nitrile (LXIII)	4.0	10 ± 2	15 ± 3
	10	20 ± 2	55 ± 10
	21	30 ± 2	105 ± 12
	33	40 ± 2	Toxicity
	63	50 ± 2	Toxicity
Guanylhydrazone (LXXI)	50	10 ± 1	75 ± 7
	100	20 ± 2	120 ± 12
	200	30 ± 2	Toxicity
	310	40 ± 2	Toxicity

This conclusion is supported by our own studies (Table III) in which we compared the positive inotropic effects of a series of analogs of digitoxigenin with ability to inhibit Na^+,K^+-ATPase. The ranking order and relative potencies are in good overall agreement for the two biological effects but a strict quantitative relationship was not observed (Table IV).

Since the results in Table IV were obtained using two different biological preparations, the differences in relative potency may reflect differences in transport and distribution phenomena for the two systems. Alternatively, our results could be taken at "face value" and the conclusion drawn that the same percent of enzyme inhibition is associated with different degrees of positive inotropism and hence that the two effects were not causally related.

Repke *et al.* (217) studied species differences with respect to response to ouabain. They compared inhibition of Na^+,K^+-ATPase with positive inotropic and lethal effects in eight species. With one exception, the frog, each species showed the same rank order of sensitivity to ouabain for the three parameters. Quantitatively, the sensitivity scales were similar for each parameter measured for a particular species, but strict ratios were not preserved. Nevertheless, the study represents one of the most significant pieces of evidence in support of a cause-and-effect relationship between inhibition of the enzyme and effects on contractility.

Repke (23) also showed that the cardiotoxic effects of cardiac glycosides in humans and rats parallel inhibition of Na^+,K^+-ATPase from erythrocytes of the respective species. He also found that the sensitivity of human erythrocyte Na^+,K^+-ATPase reflects the changes in cardiotoxic effects that accompany modification of the structures of various cardiotonic ste-

roids. In other words, erythrocyte Na^+,K^+-ATPase reflects the properties of the digitalis receptor in the heart. The question that we have not been able to answer satisfactorily is whether myocardial Na^+,K^+-ATPase is also just a suitable model for the "contractility" receptor or whether it is the actual receptor.

CONCLUSIONS

In spite of considerable effort, it has not been possible to explain satisfactorily the mode of action of cardiac glycosides or to find a suitable, less toxic substitute. There is some evidence that structural modification may improve the therapeutic index, but clinical evidence has yet to be obtained. There have been several attempts to modify toxicity by changing the pharmacokinetic properties of digitalis, but adequate clinical data to substantiate these claims have yet to be published.

The question of whether inhibition of Na^+,K^+-ATPase is the biochemical basis for the effects of digitalis on myocardial contractility has yet to be resolved. There is no doubt that there is a remarkable series of parallelisms between the effects of cardiotonic steroids on the two biological resposes. Dose ranges, structure–activity relationship analyses, and species differences all give general support for a cause-and-effect relationship. This is further substantiated by a large body of biochemical data, which shows that a digitalis receptor is present in the enzyme complex and that the conditions for binding to this receptor and for inhibiting the enzyme parallel those that determine the magnitude of effects on contractility.

On the other hand, it is known that a number of substances will inhibit the enzyme without affecting myocardial contractility. It has also been claimed that it is possible to wash out the inotropic effect and still leave the drug bound to the enzyme receptor. Finally, there is evidence that a strict quantitative relationship between ability to inhibit the enzyme and to produce a positive inotropic effect may not apply.

It seems then that the data can be interpreted in four different ways:

1. Na^+,K^+-ATPase is the receptor for contractility but an inotropic response only occurs when the enzyme is inhibited in a particular way (for example, by digoxin and not by oligomycin). The digitalis-like mode of inhibition may induce a particular type of conformational change which triggers some mechanism (such as the release of Ca^{+2}) which then mediates the inotropic response.

2. The contractility receptor is contained by a subfraction of the cell's Na^+,K^+-ATPase content. This specialized form of the enzyme has an explicit role in controlling myocardial contractility and forms readily reversible complexes with cardiotonic steroids. The enzyme comprises only a minute proportion of the cell's total Na^+,K^+-ATPase content.

3. The contractility receptor is not Na^+,K^+-ATPase but has evolved from it and closely resembles it in many ways. Na^+,K^+-ATPase is thus a suitable model for this receptor.

4. Cardiotonic steroids act by combining with a receptor present within the myoplasm. The drugs enter the cell by a process of active transport which requires Na^+,K^+-ATPase. Combination with the transport receptor is the rate-determining step. Under *in vivo* conditions, therapeutic doses of digitalis-like substances do not inhibit the enzyme, although this is shown under *in vitro* conditions. There is some evidence (218) that the action of cardiac glycosides depends upon active transport into the myocardium.

The present authors believe that the second proposal is the most likely, but it is recognized that the stage at which the study of cardiotonic steroids has reached is not, to paraphrase Churchill, at the beginning of the end but simply at the end of the beginning.

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