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The Role of Na^+,K^+-ATPase in the Inotropic Action of Digitalis[1]

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I. Introduction

A. History of Studies on the Mechanism of Action of Digitalis

In 1785, William Withering introduced digitalis as a therapeutic agent in the treatment of certain forms of dropsy. Although the primary beneficial effect of the drug was believed to involve the heart, its direct inotropic action on cardiac muscle was not established until the experiments of Cattell and Gold in 1938 (31), who demonstrated that digitalis increases the force of contraction in the isolated cat papillary muscle. The cardiotonic drugs continue to be of intense interest and research, particularly with respect to the precise mechanism of action of the therapeutic and toxic manifestations of this class of compounds. These investigations have yielded several theories and hypotheses on how the cardiac glycosides act at the subcellular level to bring about their inotropic effect. Such studies have been described in the comprehensive review of Lee and Klaus (82).

Progress in attempting to explore the mechanism of the action of digitalis had been hindered by a persistent, ill-founded concept. Because the hemodynamic action of digitalis in the intact animal is observed only when cardiac function is compromised by an existing state or by an experimental procedure which reduces the force of contraction of the heart, it was thought that the increase in the force of contraction was confined to the failing heart. It is now recognized that the hemodynamic effects of digitalis are difficult to observe in the normal animal because of the extensive feedback control mechanisms inherent in the cardiovascular system. However, the contention that the cardiac glycosides are effective only in the decompensated heart led to the hypothesis that digitalis acts on a physiological or biochemical event which is somehow different in the failing heart. For several years, studies were undertaken with the view that the heart contains an intrinsic digitalis-like substance; that this substance decreases during cardiac failure and that digitalis substitutes for, or mimics the action of the substance. In the past two decades, it has been demonstrated many times that the digitalis drugs increase the force of contraction in normal, as well as in failing, hearts. As has been pointed out by Hajdu and Leonard (62), it is unlikely that the beneficial action of digitalis is a reversal of those changes which bring about heart failure. Thus, the basic defect, leading to the reduced force of myocardial contraction, is probably not influenced by the cardiac glycosides. In all likelihood, digitalis acts by influencing a mechanism or mechanisms which exist in normal hearts as well as in failing hearts.

B. Mechanisms by Which the Force of Contraction of Heart Muscle May Be Increased

The contraction of cardiac muscle is a complex event involving many steps. The process of cardiac contraction may be described as follows: the initial step is excitation of the muscle cell membrane. This membrane excitation is an electrical phenomenon characterized by rapid depolarization, followed by a relatively gradual repolarization. The physicochemical basis for this event is an initial rapid sodium influx, followed by an additional large, but slower influx. During the plateau phase of the action potential, a calcium influx is also prominent. The repolarization, or recovery phase of the action potential, is a result, in part, of potassium efflux. As a consequence of these events, there is an increase in the intracellular free calcium ion concentration. Free calcium combines with one of the modulating myocardial proteins, troponin C, to alter the conformation of the troponin complex, releasing the inhibition on the myofibrillar contractile mechanism, and resulting in contraction. The free calcium is removed from the sarcoplasm by an active calcium pump located within the sarcoplasmic reticulum of the cell and possibly within the sarcolemma. The sodium ions which enter the cell and the potassium ions

which are lost from the cell during membrane excitation are returned by an active sodium-potassium exchange mechanism mediated by a Na^+,K^+-ATPase activity.

Theoretically, any of the above steps might be altered to result in an increase in the force of contraction. Over the years, virtually all have been explored in a search for the mechanism of action of the cardiac glycosides. Thus, proposed hypotheses on the action of digitalis involved studies on energy metabolism, such as overall respiration, oxidative phosphorylation, Pasteur and Crabtree effects, glycogenolysis, the intracellular concentration of nucleotides, stimulation of protein synthesis, and on a more efficient use of cellular energy by some unknown mechanism. Effects of cardiac glycosides on the contractile proteins have also been investigated, including the actin-myosin interaction (superprecipitation), myosin and actomyosin ATPase, and actin polymerization. There is no substantive evidence to date for an interaction between the cardiac glycosides and the contractile or modulating proteins of the myocardial cell. Other areas that have been studied include effects of these agents on the sarcoplasmic reticulum, catecholamine uptake and release, the so-called intrinsic inotropic factor, Na^+,K^+-ATPase inhibition and stimulation, and calcium metabolism. By 1970, a number of the above had been ruled out as possible sites or mechanisms for the inotropic action of digitalis (82).

Because of the importance of calcium in the process of myocardial contraction, and because several laboratories had demonstrated an increase in exchangeable calcium as a result of exposure to therapeutic concentrations of the cardiac glycoside (81, 82), it has been assumed that calcium plays a fundamental role in the action of digitalis. However, because digitalis fails to affect any of the known steps which link increases in intracellular calcium ion concentration to cardiac contraction (82), and because the glycoside, at nontoxic concentrations, does not increase the resting tension of cardiac muscle, the critical consideration would appear to be the manner by which the intracellular calcium transient (the cyclic increase in intracellular free calcium ion concentration which follows each membrane excitation) is enhanced by digitalis.

Lee and Klaus (82) concluded their extensive review of the literature as follows: "At present, the singularly established effect of cardiac glycosides on a well-defined entity is their specific inhibitory action on Na^+,K^+-ATPase. It is possible that the effects of Na^+,K^+-ATPase inhibition by cardiac glycosides may indirectly influence calcium movements. There is an imposing degree of correlation between inotropic potency and Na^+,K^+-ATPase inhibitory potency of the glycosides, which appears to indicate an intimate relationship between these two actions of the cardiac glycosides." This conclusion is consistent with previous reviews which point out the close association of the pharmacological action of digitalis and alterations in ion transport (56, 62). Nevertheless, the relationship between Na^+,K^+-ATPase inhibition by digitalis and the enhancement of the calcium transient is not clear.

We have the firm conviction that Na^+,K^+-ATPase is the receptor for the inotropic action of digitalis and related cardiotonic agents, and that the inhibition of sodium pump activity resulting from this drug-receptor interaction results in the observed positive inotropic effect. While other publications have described the action of digitalis *in extenso* (2, 30, 80, 82, 88, 94, 121, 133) and there have been a number of monographs and reviews describing the biochemical and physiological roles of Na^+,K^+-ATPase (113, 119), the present exposition will be restricted to the perspectives of the above concept. No attempt was made to cite all of the publications in the literature which deal with this subject. Papers were selected and experiments cited which will allow the reader to assess the validity of the hypothesis. Every effort was made to represent opposing views. If we have inadvertently omitted a decisive paper, we apologize.

C. Na^+,K^+-ATPase: Its Role in Cell Function

Mammalian cardiac cells have a lower $[Na^+]_i$ and higher $[K^+]_i$ compared to the surrounding environment, or extracellular compartment. Although this is partly the result of the relative impermeability of the sarcolemma for these ions, ionic equilibrium would ultimately be achieved were it not for the existence of an active Na^+-K^+ exchange mechanism within the sarcolemma. Studies of the giant axon of squid, red cell ghosts, and other preparations have demonstrated that the active extrusion of Na^+, coupled with the active influx of K^+, both against chemical gradients, is performed at the expense of ATP which is hydrolyzed to ADP. After fragmentation of the cell membrane, this system (the sodium pump) may be identified as an ATPase (ATP phosphohydrolase) that is markedly activated by the simultaneous presence of Na^+ and K^+. This ATPase also requires Mg^{++} as a cofactor. Skou (117, 118) published the first significant papers linking the active ion transport of Na^+ and K^+ to an enzyme in a crab nerve particulate fraction. Figure 1, which has been redrawn from the study of Skou (118), illustrates the ability of Na^+ and K^+ to enhance the hydrolysis of ATP markedly. It may also be observed that the cardiac glycoside inhibits the stimulated portion in a dose-dependent manner and has no effect on the basal, Mg^{++}-stimulated ATP hydrolysis. The concentrations of glycoside required to inhibit the enzyme activity are high because of the relative insensitivity of the crab peripheral nerve preparation to this agent. These findings are consistent with the earlier work of Schatzmann (110) who had observed that the digitalis glycosides are potent inhibitors of the transport of Na^+ and K^+ in the red blood cell. Subsequently, Bonting *et al.* (27) demonstrated that Na^+,K^+-ATPase is found in all animal tissues in which a transmembrane Na^+ gradient may be observed. Further details on many of the properties of this enzyme system can be found in reviews by Skou (119), Post *et al.* (100), and Schwartz *et al.* (113).

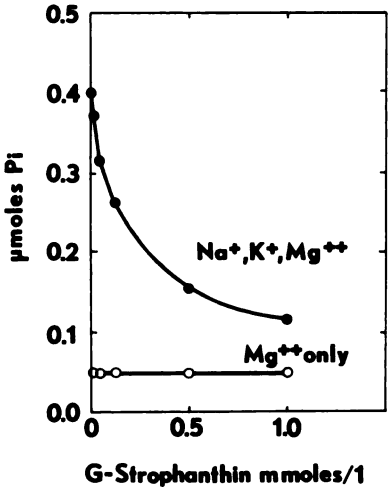


FIG. 1. Effects of ouabain (G-strophanthin) on ATPase activities. ATPase activity of microsomal fraction obtained from peripheral nerve of the seashore crab was assayed in the presence of 100 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$ and 3 mM ATP or in the presence of 3 mM $MgCl_2$ and 3 mM ATP at 30°C. The enzyme activity was estimated from the amount of inorganic phosphate released during a 30-min incubation period. (Redrawn by permission from Skou (118).)

1. Molecular mechanism of enzyme action. In tissue homogenates, or in partially purified Na^+,K^+-ATPase preparation, it is not possible to observe translocations of Na^+ and K^+ because of the destruction of cell membrane integrity and loss of compartmentalization. The enzyme, however, can be shown to undergo a cycle of conformational transitions in membrane fragments as it hydrolyzes ATP; the transitions presumably correspond to the transport of Na^+ and K^+ across the cytoplasmic membrane. In the presence of Na^+ and Mg^{++}, transphosphorylation of the terminal phosphate of ATP to the enzyme occurs (fig. 2). Ca^{++} may substitute for Mg^{++} in this step (127). Subsequently, a conformational transition of the phosphorylated protein takes place in the presence of Mg^{++}. Ca^{++} can compete with Mg^{++} and inhibits this conformational change. K^+ binds to the E_2-P form of the enzyme (fig. 2) and causes an immediate conformational transition to an E_x-P form. In this latter state, the phospho-

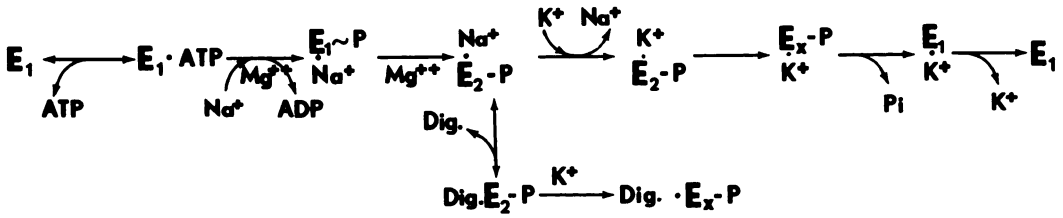


FIG. 2. Sequence of Na^+,K^+-ATPase reaction and digitalis binding. E_1, E_2 and E_x represents different conformations of the enzyme. E_x may be similar to E_1. Dig, digitalis. See text for details.

rylated enzyme is attacked by H_2O causing the release of inorganic phosphate. While the action of Na^+ in this cycle is not mimicked by other monovalent cations except Li^+ (see below), several monovalent cations, such as Rb^+, Cs^+, NH_4^+ and Tl^+, can substitute for K^+ (29, 117). Li^+ has interesting and unique properties; under certain conditions, it can partially substitute for either Na^+ or K^+ (63, 129). However, Li^+ by itself does not alter the basal ATPase activity. If Li^+ were capable of substituting for both Na^+ and K^+, then Li^+ alone should enhance ATPase activity, as does the simultaneous presence of Na^+ and K^+.

2. Cardiac glycoside-enzyme interaction. Digitaloids such as ouabain bind to the enzyme under specific conditions (114). The binding of these agents to the phosphorylated form of the enzyme, as represented by E_2-P in figure 2, appears to correspond to the binding that takes place in the beating heart (9). Since ATP, Mg^{++} and Na^+ are required for the formation of this form of the phosphoenzyme, and because K^+ decreases the E_2-P conformation, the binding of cardiac glycosides to Na^+,K^+-ATPase observed *in vitro* is enhanced by the presence of ATP, Mg^{++} and Na^+ and inhibited by K^+ (114). The concentration-dependent action of Na^+ to promote ATP-dependent [^{3}H] ouabain binding is shown in figure 3A and the effect of potassium to reduce that binding is illustrated in figure 3B. Li^+ can substitute for Na^+ to promote binding to a small extent while the action of K^+ to inhibit binding can be mimicked by Rb^+, Cs^+, NH_4^+ or Tl^+. The digitalis binding sites on the enzyme, therefore, appear to be more available when the enzyme is primarily in the E_2-P conformation, and less available when the enzyme assumes other conformations (14). When the glycoside is prebound to Na^+,K^+-ATPase, it can also be demonstrated that the release of the digitaloid from the enzyme is reduced in the presence of K^+ (6). Other cations are also capable of reducing the rate of release of digitalis from the enzyme. K^+, Rb^+, Cs^+, Tl^+ or NH_4^+, therefore, not only reduces the binding of the cardiac glycosides to the enzyme but also slows its release once the complex is formed.

When a cardiac glycoside such as ouabain is bound to Na^+,K^+-ATPase, the enzyme loses its ability to bind ATP (64, 100). This binding of ouabain to the enzyme also results in a loss of enzyme activity (17). The subsequent release of ouabain from the ouabain-enzyme complex results in a reactivation of enzyme activity (6). Thus, the interaction of digitalis with Na^+,K^+-ATPase should bring about a reduction in Na^+,K^+-ATPase and sodium pump activities.

As indicated earlier, in intact cells, the Na^+,K^+-ATPase system represents the functional sodium pump. In mammalian cells, it is recognized that Na^+ binds to the enzyme from the inside of the cell membrane and is transported to the outside. Conversely, the binding of K^+ to Na^+,K^+-ATPase occurs with that part of the membrane in contact with the extracellular environment. Thus, the $[Na^+]_i$ and $[K^+]_o$ should be the primary determinants of enzyme activity. However, the half-maximal activation (K_m) of Na^+,K^+-ATPase by K^+ occurs at less than 1 mM, which is substantially lower than the $[K^+]$ found in the extracellular fluid (5–6 mM). It is apparent that in the intact cell, Na^+,K^+-ATPase ac-

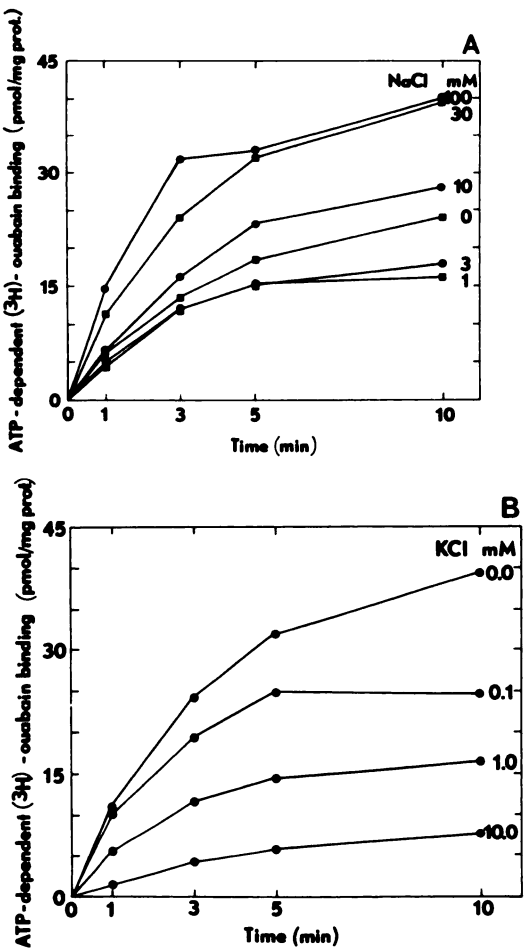


FIG. 3. Effects of Na^+ (A) and K^+ (B) on the ATP-dependent binding of [^{3}H]ouabain to Na^+,K^+-ATPase. Partially purified rat brain Na^+,K^+-ATPase preparations (20 μg of protein per ml) are incubated with 10 nM [^{3}H]ouabain in the presence of 5 mM Tris-ATP, 5 mM $MgCl_2$ and various concentrations of NaCl (A) or 5 mM Tris-ATP, 5 mM $MgCl_2$, 100 mM NaCl and various concentrations of KCl at 37°C (B). Millipore filters are used to separate bound from unbound [^{3}H] ouabain. Values observed in the absence of ATP are subtracted to estimate ATP-dependent binding. Similar data can be observed with cardiac muscle enzyme.

tivity is primarily regulated by the $[Na^+]_i$. In brief, the concentration of the ion to be transported controls the sodium pump activity (126).

The binding of digitalis to Na^+,K^+-ATPase in intact cells exhibits "sidedness." It has been demonstrated for many intact preparations that the glycosides bind to Na^+,K^+-ATPase from the outer aspect of the cell membrane (113). As was pointed out above, K^+ binding sites are also localized on the outside of the membrane, but the glycosides and K^+ appear to bind to two distinct sites on the enzyme at the outer surface of the membrane. When K^+ reduces ouabain binding, it does so by lowering the concentration of that conformation of the protein which most avidly binds the glycoside. In the past, this phenomenon has been interpreted to indicate that there is a competitive relationship between K^+ and ouabain as a result of binding to the same site. K^+, however, affects the characteristics of the glycoside-enzyme complex and, therefore, can combine with the enzyme when the glycoside binding sites are already occupied (6, 14).

Because K^+ is transported from the outside to the inside of the cell after binding, conjecture arose with respect to the fate of ouabain after binding to the enzyme. Is it possible that ouabain might be transported into the cell? As has been depicted above (fig. 2), K^+ induces a conformational transition of the ouabain-phosphoenzyme complex. Whether or not this conformational transition brings about an internalization of the ouabain binding site and its subsequent release into the cytoplasm of the cell is not known.

Because[^{3}H]ouabain binds more avidly to isolated cardiac Na^+,K^+-ATPase in the presence of high $[Na^+]$, and to a lesser extent when $[Na^+]$ is low, it was suggested that the glycoside binds to the enzyme at an outer surface of the cell membrane, is translocated across the membrane, and then released to an inner compartment of the cell (48) where $[Na^+]$ is lower. When the ouabain-enzyme complex is formed in the presence of Na^+, Mg^{++} and ATP, the removal of Na^+ from the incubation medium increases the rate of release of the bound ouabain (9). The potassium-induced conformational change of such a complex, however, reduces rather than facilitates the release of bound ouabain (6, 14).

How does Ca^{++} affect Na^+,K^+-ATPase activity? As discussed above, in the intact

cell, enzyme activity is influenced by the intracellular Na^+, Mg^{++} and ATP concentrations and the extracellular K^+ concentration. In isolated enzyme preparations, Ca^{++} completely inhibits Na^+,K^+-ATPase activity by blocking a transition of the enzyme to its E_2-P form (fig. 2) (127). However, this occurs only at concentrations that would normally be found in the extracellular milieu. In the beating myocardial cell, if the sodium pump was regulated by $[Ca^{++}]_o$ the pump would be inhibited at all times. No demonstrable effect on Na^+,K^+-ATPase activity is apparent at $[Ca^{++}]$ which normally exist in the intracellular compartment (10^{-7} M).

II. Action of Digitalis on Na^+,K^+-ATPase *In Vitro*

A. Stimulation of Enzyme Activity

Several investigators have reported that the cardiac glycosides are capable of increasing Na^+,K^+-ATPase activity (82). Stimulation is usually observed with fresh, untreated enzyme preparations exposed to extremely low concentrations of the drug. In certain studies, using a single concentration of the cardiac glycosides, an initial brief stimulatory period has been observed, which becomes progressively inhibitory. Lee and Yu (83) have reported that the concentration of digitalis necessary to produce significant stimulation is approximately $\frac{1}{300}$ of that required to cause half-maximal inhibition of enzyme activity. The stimulation of enzyme activity at very low concentrations of digitalis and the inhibition at higher concentrations of the drug superficially resemble the positive inotropic action at therapeutic concentrations and the toxic action in the presence of higher concentrations of the glycoside. Therefore, it is tempting to speculate that the cardiotonic action of digitalis results from a stimulation of Na^+,K^+-ATPase, while inhibition of the enzyme brings about the toxic effect.

Several electrophysiological studies support the contention that the sodium pump is stimulated by the glycoside under certain conditions. Most interesting are the studies of Cohen *et al.* (33) that provide evidence to show that the cardiac glycosides stimulate the sodium pump in sheep Purkinje fibers. They estimated changes in the K^+ gradient by measuring the reversal potential for a K^+-specific current and monitoring steady state current-voltage relationships. In the presence of 8 mM extracellular K^+, the addition of low concentrations of ouabain shifted the reversal potential in a negative direction, suggesting that ouabain may stimulate the Na^+,K^+-exchange pump. When the $[K^+]_o$ was 5.4 mM, the K^+ gradient could change in either direction, reflecting either an inhibition or stimulation of the ion pump. Finally, with low $[K^+]_o$ in the range of 2.7 to 4.0 mM, and with low concentrations of ouabain, the Na^+,K^+-exchange pump was always inhibited. The force of contraction was not simultaneously recorded in these preparations, and, therefore, it is not possible to know whether the inotropic event was related to either stimulation or inhibition of the sodium pump. Generally, the inotropic action of digitalis is more easily observed when $[K^+]_o$ is low (58). In certain studies, it has also been demonstrated that low concentrations of the cardiac glycosides increase ion transport, but no attempt was made to correlate the stimulation with the force of contraction in these muscle preparations (82).

There are several difficulties with the theory that stimulation of the enzyme is related to the inotropic event. One is that the observed stimulation of enzyme activity by the cardiac glycosides varies between 10 and 20% above control (82) and, hence, its biological significance is questionable, particularly when $[Na^+]_i$, rather than the enzyme activity itself, is the primary determinant of sodium pump activity (126). More pertinent, however, is the observation that myocardial Na^+,K^+-ATPase is inhibited, and not stimulated, at the time of the inotropic action of digitalis (see below). The magnitude of this inhibition is generally between 30 to 40%. Recently, low concentrations of ouabain (1–3 nM) were demonstrated to cause a slight increase in cel-

lular K^+ and a decrease in Na^+ in isolated guinea pig atria; findings which would appear to be consistent with a stimulation of sodium pump activity (57). Higher concentrations of the drug (10 nM and above) caused the opposite changes. Changes in force of contraction were not simultaneously estimated. It is generally recognized in the guinea pig, that the lowest concentration of ouabain which increases the force of myocardial contraction is approximately 50 nM, a concentration which would cause K^+ loss and Na^+ accumulation in atrial tissue (57). What happens to the function of the myocardium when the heart is exposed to concentrations of digitalis 2 orders of magnitude lower (or 1/300) than the concentration which produces 40% enzyme inhibition is not known.

There are several mechanisms based upon electrophysiological observations that might result in a cardiac glycoside-induced stimulation of sodium pump activity. In the red blood cell, under ideal conditions, Na^+,K^+-ATPase exchanges 3 Na^+ for 2 K^+ with each molecule of ATP consumed. This would occur most readily when Na^+ and K^+ are abundant at sites from which these cations are transported (53). The Hill coefficient for Na^+, however, is usually less than 2 when determined using isolated enzyme preparations (122). This indicates that even when fewer than three Na^+ binding sites are occupied, the enzyme is still capable of completing its cycle or changing its conformation. Let us assume that under normal conditions, the enzyme can change its conformation with less than 3 binding sites occupied by Na^+. If the cardiac glycosides now induce a slight increase in Na^+ concentration at the site where Na^+ is transported, the full occupancy of the 3 Na^+ binding sites could occur before the same conformational change. This would alter the electrogenicity of the sodium pump, and the electrophysiological parameters would be altered in a manner which could be interpreted as stimulation of sodium pump activity. Cohen *et al.* (33) conclude from their studies that pump stimulation might occur at some sites even when overall inhibition is observed.

B. Inhibition of Enzyme Activity

Repke (102) predicted, based upon plasma concentrations of digitalis in patients and the glycoside-sensitivity studies in isolated Na^+,K^+-ATPase preparations, that the enzyme would be inhibited 40% in patients receiving therapeutic doses of the drug. Subsequently, similar magnitudes of Na^+,K^+-ATPase inhibition were demonstrated in anesthetized dogs during the intravenous administration of ouabain, but before the onset of arrhythmias (11, 12). This same degree of inhibition has been found in the dog heart perfused *in situ* with blood (24) and in isolated perfused hearts (73) treated with concentrations of the glycosides adequate to produce inotropic effects. Thus, the relationship between the inotropic action of digitalis and inhibition rather than stimulation of Na^+,K^+-ATPase, should be explored for causality.

1. Species differences. Among the unique features of the action of digitalis are the marked differences in sensitivity to the drugs among various species. Some of the species-dependent diversity may be explained by qualitative and quantitative differences in drug disposition (71). This cannot be the sole factor, since the same remarkable variations in sensitivity are observed in isolated hearts from various species. For example, adult rat hearts are particularly insensitive to digitalis. Because it was noted that in the rat myocardium an increase in stimulation frequency results in a decrease in the force of cardiac contraction, it was postulated that the underlying mechanism for the glycoside insensitivity of rat hearts and the negative frequency-force relationships are related, and further, that a step in the excitation-contraction coupling mechanism which is responsible for the "staircase" phenomenon, is also the site of action of digitalis (26, 77). This possibility was weakened by the finding that

the "ascending staircase" phenomenon may be converted to a "descending staircase" by treatment of the rabbit heart with verapamil, an antagonist of Ca^{++} conductance. In addition, verapamil fails to decrease the magnitude of the positive inotropic action of ouabain (89). Therefore, at a time when ouabain increases the force of cardiac contraction, the rabbit heart, like the rat heart, shows a "descending staircase" response to frequency stimulation.

Although some other factors may also be involved (7), rat hearts seem to be insensitive to digitalis primarily because their Na^+,K^+-ATPase has a low affinity for the glycoside (18, 76). One can observe a positive inotropic action of digitalis in the rat by exposing the heart to concentrations of digitalis which are high enough to cause a moderate inhibition of Na^+,K^+-ATPase. In various animal species, it has been shown that the ability of digitalis to produce its positive inotropic action is related to the affinity of the glycoside for the cardiac enzyme (11, 18, 103, 130).

2. Time course of the inhibition and inotropic action. Another unique feature of the inotropic action of digitalis is its slow onset, which is observed even after an intravenous injection of the drug or when the drug is placed in direct contact with isolated perfused heart preparations and may be explained by the slow rate of interaction between the glycoside and the enzyme (16). This slow rate of interaction is observed *in vitro* at low or therapeutic digitalis concentrations, particularly in the presence of K^+ (3).

Others have postulated that the slow onset of inotropic action of digitalis is a result of the time required for digitalis to enter the cell and gain access to the receptor for inotropic action (41, 97). Dutta *et al.* (41) have speculated that this receptor is inside the cell and have advanced the concept of a carrier mechanism for the cardiac glycosides that is linked to cation flux. According to this theory, Na^+,K^+-ATPase is the mechanism by which the cardiac glycosides are transported to their site of action and, hence, the binding of digitalis to this enzyme is related to, but is not essential for, its action. The dependence of the development of the positive inotropic action of digitalis on the number of contractions (91, 97, 109) is used as supportive evidence, because the transport of cardiac glycoside may be facilitated by membrane depolarization.

This concept was further sustained by the finding that the onset of the inotropic action of the aglycones, which are much more lipid-soluble than the corresponding glycosides, are not dependent upon the number of contractions (97). Park and Vincenzi (97) concluded that the receptor for the inotropic action of digitalis is located in a membrane-limited compartment to which aglycones may gain access *via* passive diffusion but to which glycosides gain access *via* a carrier mechanism. Differences in affinity of cardiac Na^+,K^+-ATPase for ouabain in the presence of low and high concentrations of Na^+ were cited as evidence for the digitalis transport system (48). Such results, however, do not indicate whether digitalis should move from the high affinity sites to the low affinity sites, or *vice versa,* or whether the system is capable of transporting digitalis at all. These studies and others do not allow one to decide whether the binding of the glycoside to Na^+,K^+-ATPase is merely to transport these agents to the ultimate site of action, or whether the binding to the enzyme is the first step in a sequence of events leading to the increase in the force of contraction. For cardiac muscle, there is substantial support for the interaction of digitalis with the sarcolemma. Intracellular organelles appear to be unaffected by the glycosides and only in Na^+,K^+-ATPase-enriched preparations does the glycoside bind in a specific, saturable manner (15). There is further evidence indicating a superficial location of the receptor for the inotropic action of digitalis. When isolated hearts are perfused with ^3H-labeled glycosides and then washed out, the

inotropic action disappears long before the bulk of the glycoside is washed out (84, 106, 107).

The species from which the enzyme preparation is obtained determine the stability of the digitalis-enzyme complex (3, 130, 131). If the rate of release of the glycoside from an enzyme prepared from the heart of a moderately digitalis-sensitive species, such as a guinea pig or rabbit, is followed, the half-time for the dissociation of the ouabain-enzyme complex formed *in vitro* is much shorter than that observed in the enzyme prepared from a highly glycoside-sensitive species such as the cat or dog (fig. 4). In a similar fashion, using isolated perfused hearts, the decay of the positive inotropic action of ouabain is also considerably

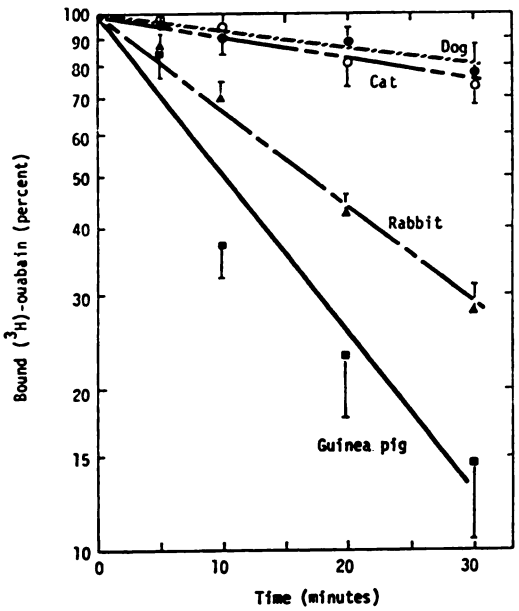


FIG. 4. Release of [^{3}H]ouabain from cardiac Na^+,K^+-ATPase at 27°C. Partially purified enzyme preparations obtained from ventricular muscle of various species are incubated with 0.6 μM [^{3}H]ouabain in the presence of 10 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.5) at 27°C for 10 min. At this time, nonradioactive ouabain (final concentration, 0.3 mM) is added to prevent further binding of labeled ouabain. KCl (final concentration, 7.5 mM) is also added and the subsequent release of [^{3}H]ouabain monitored. Concentrations of NaCl and KCl are chosen to simulate the effective ion concentrations at the ion-activating sites of Na^+,K^+-ATPase *in vivo*. Mean ± S.E.M. of five experiments. (Reprinted by permission from Akera *et al.* (3).)

faster in guinea pigs and rabbits than in cats and dogs. Thus, the digitalis-Na^+,K^+-ATPase complex formed *in vitro* has a stability similar to a complex of the glycoside with the receptor for its inotropic action, and one can predict the rate of offset of the positive inotropic action of digitalis during drug washout by the rate of release of the drug from the cardiac enzyme of that species (3).

3. Digitalis derivatives: force of contraction and enzyme inhibition. The potency of various digitaloid derivatives to inhibit cardiac Na^+,K^+-ATPase activity *in vitro* correlates with their ability to increase the force of contraction (65, 102). Certain derivatives which fail to produce a positive inotropic effect also fail to inhibit Na^+,K^+-ATPase activity *in vitro*. The differences in the ability of various compounds to cause enzyme inhibition appear to result from variations in dissociation rate constants (stability) of the digitalis-enzyme complex (130). This would predict that the washout of the inotropic action of the digitaloids would be faster with the weaker inhibitors (those having higher dissociation rate constants) and slower with the more potent compounds, when the force of contraction is enhanced by the same magnitude. Such results have been reported (47). Washout of inotropic action and reversibility of enzyme inhibition *in vitro* are compared in Fig. 5. Thus, it appears that among the various digitalis derivatives, their ability to produce positive inotropic effects can be correlated with their effectiveness as Na^+,K^+-ATPase inhibitors.

Katzung *et al.* (70) suggested the possibility that digitalis may produce Na^+,K^+-ATPase inhibition in the isolated myocardium without a concurrent positive inotropic action. Their suggestion was based upon the observation that the inotropic action of 3-acetyldigitoxigenin is delayed by the addition of its corresponding lactam, when these compounds are studied in isolated atrial preparations. When the compounds were similarly assessed in rat cardiac microsomal preparations, no delay in inhibition of Na^+,K^+-ATPase activity was

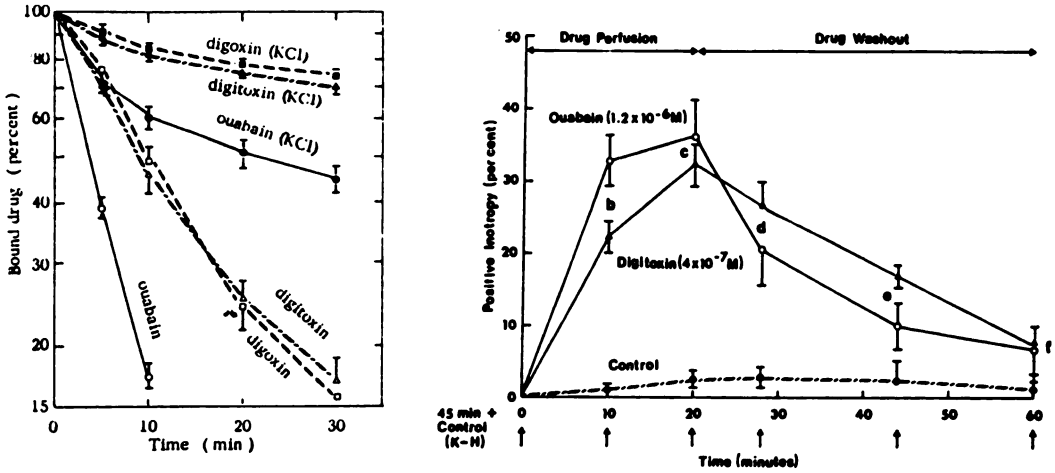


FIG. 5. Release of ouabain, digoxin and digitoxin from Na^+,K^+-ATPase (left) and the loss of inotropic effect (right). Left: the cardiac glycoside-enzyme complex is formed with ATP, NaCl and $MgCl_2$, washed to remove ATP, cations and free glycosides, and allowed to dissociate at 37°C in the presence of 10 mM Tris-HCl buffer (pH 7.5) with or without 5 mM KCl. Mean ± S.E.M. of four experiments. (Reprinted by permission from Akera *et al.*, Ann. N.Y. Acad. Sci. **242**: 617, 1974.) Right: Langendorff preparations of guinea pig hearts are perfused at 30°C with 0.4 μM digitoxin or 1.2 μM ouabain for 20 min (perfusion rate, 4 ml/min), and subsequently perfused with drug-free solution to monitor the loss of inotropic response. Mean ± S.E.M. of five experiments. (Reprinted by permission from Ku *et al.* (73).)

observed. As discussed earlier, the enzyme system in intact cells and in isolated preparations is under the influence of different ligand conditions. It is essential that Na^+,K^+-ATPase inhibition (or some estimate of that activity) be determined in the same heart in which the change in force of contraction is measured. It is interesting that modification of a lactone ring to a lactam resulted in a compound which neither produced a positive inotropic effect nor inhibited Na^+,K^+-ATPase; a finding which extends the earlier observation of Repke (102).

In 1969, Fricke and Klaus (46) claimed that strophanthidin-3-bromoacetate (SinBA) produced an irreversible inhibition of Na^+, K^+-ATPase, whereas its inotropic action was rapidly reversed. Again, Na^+,K^+-ATPase activity of an isolated enzyme preparation observed *in vitro* was compared with the inotropic action occurring in the beating heart. The irreversible interaction of Na^+,K^+-ATPase by an alkylating agent such as SinBA, observed in microsomal Na^+,K^+-ATPase preparations, may occur at sites not normally accessible in the intact cell. In the studies reported by Fricke and Klaus, Na^+,K^+-ATPase activity was estimated after preincubation of the enzyme preparation with the compound and subsequent centrifugation to test reversibility. Because specific binding of digitalis to the enzyme does not occur in the absence of ATP, it is apparent that the observed inhibition is not the result of the interaction of SinBA with the cardiotonic steroid binding site. It is not known whether SinBA produces irreversible inhibition of Na^+,K^+-ATPase in intact cardiac cells. These findings again emphasize the importance that attempts to correlate enzyme inhibition with inotropic action should be performed in the same heart.

III. Action of Digitalis on Na^+,K^+-ATPase *in Vivo* and in Isolated Hearts

A. Direct Estimates of Na^+,K^+-ATPase Activity

The first observation that changes in Na^+,K^+-ATPase activity could be detected in the heart of an intact animal after the administration of the cardiac glycosides was by Akera *et al.* (11). In the intact anesthetized dog, the cardiac glycosides can

be administered intravenously until a change in the electrocardiogram occurs, whereupon the heart is rapidly removed for preparation of the enzyme. At the time of the physiological response, as monitored by changes in the EKG and the blood pressure but before the onset of arrhythmias, there is a marked reduction in Na^+,K^+-ATPase activity. The use of the dog as the test species was rather fortuitous; if another species which was less sensitive to the cardiac glycosides had been used, a change in Na^+,K^+-ATPase activity would probably not have been observed (112).

These initial studies led to experiments, also performed in the dog, in which the correlation between cardiac Na^+,K^+-ATPase activity with ouabain-induced positive inotropic action was explored in greater detail (12). When the effect of ouabain infusion on the force of cardiac contraction, EKG, blood pressure, Na^+,K^+-ATPase activity and Mg^{++}-dependent ATPase activity are determined in the open chest, anesthetized dog, a positive correlation is observed between Na^+,K^+-ATPase activity and the force of contraction (fig. 6). Short-term exposure to ouabain, which produces a 26% increase in the force of contraction, results in Na^+,K^+-ATPase activity which is 28% lower than that found in the hearts of control animals. When the drug is infused for a longer period of time, there is a greater increase in the force of contraction and a greater reduction of Na^+,K^+-ATPase activity. Thus, there appears to be a correlation between cardiac Na^+,K^+-ATPase activity and the increase in the force of myocardial contraction. Mg^{++}-ATPase activity was unaffected by ouabain and Na^+,K^+-ATPase activity was unaffected by isoproterenol or by continuous stimulation of the inferior cardiac nerve, both of which increased the force of cardiac contraction.

At this time, Besch *et al.* (24) also found a correlation between Na^+,K^+-ATPase inhibition and the inotropic action of digitalis. They used a modified dog heart Langen-

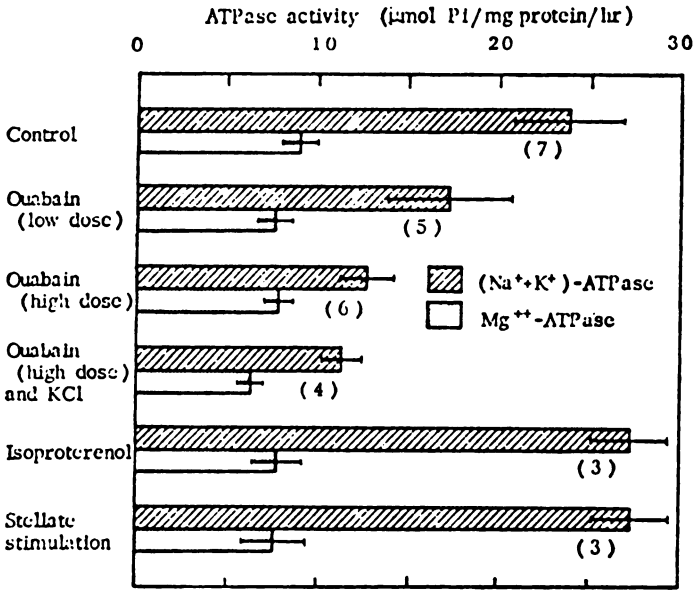


FIG. 6. Cardiac ATPase activity after the perfusion with ouabain or isoproterenol, or after prolonged stimulation of the inferior cardiac nerve. Anesthetized dogs are infused with ouabain or isoproterenol to produce a sustained positive inotropic response as monitored by a strain-gauge arch attached to the left ventricle. In some dogs, the inferior cardiac nerve is electrically stimulated to produce a sustained cardiotonic response. At predetermined times, the hearts are quickly removed. Enzyme preparations obtained from these tissues are assayed for ATPase activity. Numbers in parentheses indicate the number of animals. Horizontal lines, S.E.M. (Reprinted by permission from Akera *et al.* (12).)

dorff preparation perfused with a solution containing 1 μM ouabain and measured the force of contraction of the ventricular muscle with a strain-gauge arch and intraventricular balloon. At the maximal inotropic effect, the heart was removed and the tissue assayed for Na^+,K^+-ATPase activity. Hearts were exposed to the glycoside for different time periods, the shorter time period producing a 15% and the longer time period a 45% increase in the force of contraction. The Na^+,K^+-ATPase isolated from these hearts were inhibited 31 and 59%, respectively. Thus, this study, as well as that described above, provided evidence of a correlation between the inotropic action of the glycoside and Na^+,K^+-ATPase inhibition, although not demonstrating that these events were necessarily causally related.

B. Alterations in the Ionic Environment

One method of assessing causality indirectly is to examine and compare the effects of changing the ionic environment on the binding of digitalis to Na^+,K^+-ATPase and observing how such ionic manipulations influence the inotropic action of digitalis. As noted before, the binding of digitalis to Na^+,K^+-ATPase in the intact cell is facilitated by intracellular Na^+ and reduced by extracellular K^+. The effect of K^+ to delay the onset of the inotropic action of digoxin has been reported (101). Drugs or maneuvers which increase transmembrane Na^+ influx enhance the development of the positive inotropic action of ouabain (13). Conversely, conditions which decrease transmembrane Na^+ influx delay the development of the positive inotropic action of the glycoside (13, 134). Therefore, the binding of digitalis to Na^+,K^+-ATPase appears to be related to the inotropic action observed in the heart. It is also well known that when digitalis is bound to the enzyme, the latter is no longer able to interact with ATP and, thus, enzyme activity is inhibited (64, 100). These observations, however, do not allow one to decide whether the binding is merely required for the transport of digitalis to its ultimate site of action or whether binding is causally related to the inhibition of enzyme activity and the increase in the force of contraction.

Several investigators have altered plasma $[K^+]$ and under these conditions, measured the inotropic and toxic actions of digitalis (58, 87). Similar to findings in isolated hearts, elevating plasma $[K^+]$ in the intact animal delays, and lowering the plasma $[K^+]$ enhances, the development of the inotropic action of digitalis. It would appear to be more than coincidental that the degrees of cardiac Na^+,K^+-ATPase inhibition at the time of identical increases in force of cardiac contraction are quite similar, regardless of the particular plasma $[K^+]$ or the time of exposure to the cardiac glycoside (12, 24, 58). Goldman *et al.* (58) found about a 40% inhibition of Na^+,K^+-ATPase activity in animals given digoxin which induced a 50% increase in left ventricular dP/dt. In animals that were made hyperkalemic, it was necessary to administer twice as much drug to achieve the same increase in the force of cardiac contraction and the same degree of inhibition of Na^+,K^+-ATPase activity. Thus, at an equivalent level of inotropic effect, Na^+,K^+-ATPase activity seems to be inhibited to the same extent.

Attempts to demonstrate the effect of altering plasma $[K^+]$ on the myocardial uptake of labeled cardiac glycosides are more difficult to interpret. For example, Francis *et al.* (44) reported that hypokalemia in dogs does not cause an increase in total digoxin content of the heart. Their data, however, show that plasma digoxin concentrations are decreased in hypokalemic animals. Since the digoxin content of the heart was similar to that of normokalemic animals, it may be concluded that the tissue/plasma digoxin concentration ratios are increased at low plasma K^+. One would hardly expect the myocardial digoxin content to increase during hypokalemia, since Na^+,K^+-ATPase is ubiquitously distributed and the enhanced binding of digoxin during hypokalemia should tend to increase di-

goxin uptake in all tissues. Furthermore, the effect of potassium on the nonspecific or nonsaturable uptake of the glycoside is unknown. In hyperkalemic dogs, the higher plasma [K^+] results in a lower myocardial digoxin concentration (86, 90). Even though there is an apparent decrease in the binding of digoxin to the microsomal fraction of the heart in the hyperkalemic animal, these studies are open to the same uncertainties as those performed in the hypokalemic animals.

While the action of K^+ to delay the binding of the glycoside to the enzyme *in vitro* and to lengthen the time required for the development of the positive inotropic effect in the isolated heart has been confirmed many times, the effect of K^+ on the equilibrium level of digitalis binding *in vitro* is controversial. Also unsettled is the role of K^+ in changing the magnitude of the inotropic action of digitalis once a steady state has been achieved. Allen and Schwartz (19) reported that K^+ delays the binding of ouabain to isolated Na^+,K^+-ATPase preparations *in vitro* but fails to influence the ultimate level of that binding. Prindle *et al.* (101) have indicated that the presence of higher [K^+] in the incubation medium delays the development of the positive inotropic action of digoxin, but does not affect the ultimate magnitude of that action. As has been discussed earlier, K^+ influences both the association and the dissociation of the glycoside with Na^+,K^+-ATPase. Recent studies from our laboratory on kinetic parameters of the digitalis-enzyme interaction indicate that K^+ reduces the association rate constant to a greater extent than the dissociation rate constant (32). This difference is greater with the aglycones than with the glycosides. The affinity of the glycosides for the enzyme is moderately reduced by K^+, but is greatly reduced for the aglycone (135). Consequently, K^+ slightly reduces the magnitude of the positive inotropic action of digoxin at steady state, but markedly reduces the extent of the inotropic effect if the heart is exposed to digoxigenin. In these studies, however, Na^+,K^+-ATPase activity was not determined in isolated heart preparations at the time of the inotropic action of the cardiotonic substances with varying concentrations of K^+. Thus, it cannot be concluded that the effects of K^+ on the force of contraction and on the digitalis-enzyme interaction are causally related.

C. Time Course of Events

There has been a more direct experimental approach to test whether the inhibition of Na^+,K^+-ATPase activity is responsible for the increase in the force of cardiac contraction induced by digitalis. Generally, enzyme activity has been estimated at various times, before the addition of the cardiac glycoside, at the peak of the maximal inotropic effect and after drug washout, at a time when the physiological response has dissipated. Some of these studies support the contention that Na^+,K^+-ATPase inhibition is related to the inotropic effect while others appear to report results in which an apparent dissociation of the two phenomena are observed. Several studies are discussed below.

Among the most frequently cited studies opposing the hypothesis that a causal relationship exists between enzyme inhibition and the digitalis-induced increase in cardiac contraction are those of Okita and co-workers (95, 106, 124). Isolated, perfused rabbit hearts were perfused with solutions containing 10^{-5} M SinBA resulting in a 50 to 80% increase in the force of contraction during the 3-hr perfusion period. When Na^+,K^+-ATPase activity (note LiBr treatment for 18 hr) from this group of animals was compared with control, solvent-perfused hearts, there was no significant difference between the two groups (106). The authors concluded that the lack of demonstration of inhibition of Na^+,K^+-ATPase activity was probably the result of dissociation of the drug-enzyme bond during isolation of the enzyme. In a later study (95), the same method for the determination of the force of contraction was used and cardiac membrane Na^+,K^+-ATPase was also

estimated by the method used earlier, except that enzyme preparations were exposed to LiBr treatment for 1 hr. It was found that perfusion with SinBA results in a positive inotropic effect and a 27% reduction of Na^+,K^+-ATPase activity. In a group of hearts exposed first to SinBA and subsequently to a drug-free solution for 30 min, inhibition of the enzyme still persisted. In similar experiments using ouabain or strophanthidin, inhibition of enzyme activity was detected as long as 6 hr after the disappearance of the inotropic effect. A summary of the findings of Okita and co-workers is shown in table 1. Because there is no recovery from the Na^+,K^+-ATPase inhibition after washout they concluded that there is a dissociation between the drug-induced inotropic effect and inhibition of the enzyme. Similar conclusions were also reached by Bentfeld *et al.* (23).

Unfortunately, there has been no confirmation of the data produced by Okita and co-workers, although attempts have been made by several other groups to reproduce this work. Under conditions used by Okita and co-workers, it is not possible to show that the enzyme is inhibited at the time of cardiac glycoside-induced inotropic effect either in the rabbit or in the guinea pig (the guinea pig and rabbit have equivalent sensitivity to the cardiac glycosides and therefore equivalent instability of the glycoside-enzyme complex). Schwartz *et al.* (112) reported that when the peak inotropic effect is obtained in the rabbit heart (Langendorff preparation) and enzyme activity assessed, no detectable inhibition of that activity is observed. However, when the same studies are performed in a glycoside-sensitive species such as the cat, a marked reduction in cardiac enzyme activity is observed at the time of the increase in the force of contraction. In a similar manner, studies by Akera *et al.* (4) in the isolated guinea pig heart verified that, using standard methods for assessing Na^+,K^+-ATPase activity, inhibition of enzyme activity could not be detected at peak inotropic action in that species. These observations are consistent with

TABLE 1

Effect of various digitaloids on Na^+,K^+-ATPase activity in rabbit Langendorff preparations

		N	Na^+,K^+-ATPase	Remarks
			μmol/mg protein/hr	
I.	Control	5	6.1 ± 1.0*	Roth-Schecter *et al.* (107)
	SinBA-treated (inotropy, 3 hr)	7	5.9 ± 0.52	LiBr treatment, 18 hr
II.	Controls			
	A. Zero time after 90 min equilibration	5	9.3 ± 0.3	Okita *et al.* (95)
	B. 30–45 min after equilibration	5	8.6 ± 0.4	LiBr treatment, 1 hr Otherwise method as above
	C. 90–120 min after equilibration	5	8.9 ± 0.3	
		15	($\bar{X}$ = 8.9 ± 0.3)	
III	Control	15	8.9 ± 0.3	Okita *et al.* (95)
	SinBA inotropy	6	6.5 ± 0.6	
	SinBA inotropy and 30 min washout	5	6.3 ± 0.6	Control from II (?)
IV.	Control	15	8.9 ± 0.3	Okita *et al.* (95)
	Ouabain inotropy and 60–90 min washout	7	5.4 ± 0.2	Control from II (?)
	Strophanthidin inotropy and 40–60 min washout	3	5.5 ± 0.4	

* Mean ± S.E.

earlier investigations demonstrating that the half-life of dissociation of the drug from the enzyme closely parallels the half-time for offset of the inotropic effect in a number of species (3). This does not imply that the enzyme is not inhibited at the time of the inotropic action, but rather indicates that in some species because of the conditions of enzyme isolation, it is not possible to detect such an inhibition.

There are several aspects of the studies by Okita and his co-workers that need to be clarified. It is unclear from the several publications whether or not the day-to-day variability in the assay for enzyme activity was controlled. Were untreated animals assayed for Na^+,K^+-ATPase activity on the same day as those animals exposed to the cardiac glycosides? (table 1). Some comment should also be made relative to the methodology utilized for the Langendorff perfusion. The studies by these workers were performed by perfusing the hearts under constant pressure, while others have employed the constant flow rate perfusion technique. It is a common experience that digitalis frequently fails to produce a positive inotropic effect in isolated heart preparations perfused under constant pressure. This is probably because the glycosides have the ability to induce a marked vasoconstriction. As a result of an increased oxygen demand resulting from the glycoside-induced increase in the force of contraction and the reduced flow rate as a consequence of the vasoconstriction, the digitalis-treated myocardium under these conditions may become hypoxic. It has been shown that ischemia reduces myocardial Na^+,K^+-ATPase activity (21). Thus, the data of Okita and co-workers may not reflect an irreversible inhibition of cardiac Na^+,K^+-ATPase by digitalis after washout of the inotropic action.

The irreversible inhibition of sodium pump activity after the exposure of isolated guinea pig atria to ouabain and subsequent washout has recently been examined (23). Atrial tissue continued to accumulate Na^+ and lose K^+ after washout of ouabain when the atria are stimulated at high frequency (4 Hz) but not at lower frequencies. Bentfeld *et al.* (23) suggest that the intracellular ionic milieu is disturbed when ouabain is present during high frequency stimulation because of a larger Na^+ influx rate. As the result of Na^+-Ca^{++} exchange, Na^+,K^+-ATPase is now inhibited by Ca^{++}. It should also be considered that an increase in $[Na^+]_i$ can impair the function of the mitochondrial oxidative phosphorylation mechanism (60). The above considerations and the work of others would tend to indicate that Na^+,K^+-ATPase inhibition produced by the *glycosides* during drug perfusion is normally readily reversible.

Our own studies (8) using isolated guinea pig hearts (Langendorff preparations) may be more pertinent. In these studies (fig. 7), we determined the concentration of Na^+,K^+-ATPase interacting with digitoxin and resulting in an inotropic effect during perfusion by estimating the initial velocity of the specific binding of [^{3}H]ouabain to a ventricular homogenate of a heart exposed to the drug. This was carried out rapidly and for a short incubation time, so that there could be no significant dissociation of digitoxin from the enzyme during tissue manipulation (73). If the enzyme was uninhibited, one should be able to label the enzyme fully with [^{3}H]ouabain. On the other hand, if Na^+,K^+-ATPase was inhibited by digitoxin, the ATP-dependent [^{3}H]ouabain binding would be significantly less than that seen in control hearts. The data substantiate the conclusion that digitoxin is bound to Na^+,K^+-ATPase at the time of the inotropic effect. It is well recognized that the binding of the drug to the enzyme results in inhibition (1, 17, 64). It should be noted that these experiments were performed with homogenates in the absence of detergents and, therefore, it is unlikely that rupture of the drug-enzyme bond occurred during the isolation and assay procedures. We were able to demonstrate conclusively that there was a reduction of enzyme activity during the inotropic action and a recovery of that enzyme activ-

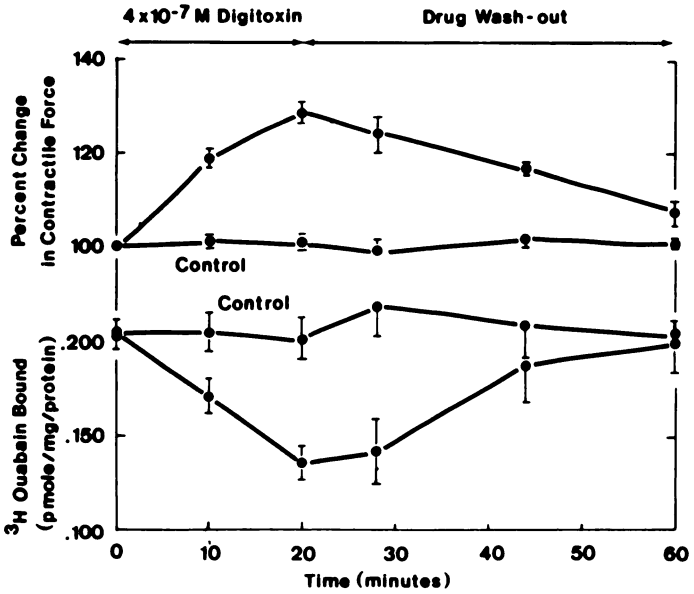


FIG. 7. Digitoxin perfusion: Inotropic effect and the binding of digitoxin to Na^+,K^+-ATPase. Langendorff preparations of guinea pig hearts are perfused at 30°C with 0.4 μM digitoxin (perfusion rate, 4 ml/min). After a 20-min perfusion with digitoxin, hearts are perfused with drug-free solution to monitor the loss of inotropic response. At a predetermined time, hearts are removed and homogenized immediately to assay the initial velocity of [^{3}H]ouabain binding (an estimate of unoccupied or uninhibited Na^+,K^+-ATPase concentration). Homogenates are incubated at 37°C with 10 nM [^{3}H]ouabain in the presence of 100 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tris-HCl buffer (pH 7.5), for 3 min with or without 5 mM Tris-ATP. Values obtained in the absence of ATP are subtracted from corresponding values obtained in the presence of ATP. Decreases in [^{3}H]ouabain binding indicate occupancy of Na^+,K^+-ATPase by digitoxin during perfusion. Mean ± S.E.M. of five experiments. Unbound digitoxin in the tissue is diluted 250-fold and thus does not interfere with [^{3}H]ouabain binding assay. (Reprinted by permission from Akera *et al.* (8).)

ity after washout (fig. 7). This study, therefore, established the quantitative relationship between digitalis binding to Na^+,K^+-ATPase and the inotropic effect during onset and offset of drug action.

Other studies have also purported to demonstrate a dissociation between inotropic action and Na^+,K^+-ATPase inhibition. One of these is that of Peters *et al.* (98). Their conclusion is based upon the observation that in the isolated guinea pig atrium, the half-time of washout of the inotropic effect is extremely rapid for ouabain, but when the enzyme is inhibited with cardiac glycosides, the rate of reversibility is extremely slow. Studies on reversibility of Na^+,K^+-ATPase inhibition *in vitro,* however, were performed with an enzyme obtained from calf heart, a tissue which is extremely sensitive to the cardiac glycosides. Studies on the inotropic effect were done on isolated guinea pig atria, a species only moderately sensitive to the cardiotonic steroids. Not only did the authors neglect to use the same hearts in attempting to dissociate these effects, but they even failed to use the same species! Species differences in stability of the digitalis-enzyme complex are well known (see above).

The studies of Rhee *et al.* (104) are also worthy of mention (table 2). These investigators infused ouabain in anesthetized dogs over a 6-hr period and observed a significant inhibition of myocardial Na^+,K^+-ATPase after the infusion of toxic doses, but not after the infusion of nontoxic doses of the glycoside. Because the lower dose did produce an increase in the force of myocardial contraction, these investigators concluded that Na^+,K^+-ATPase inhibition could not be the cause of the inotropic action of digitalis. It should be noted, how-

TABLE 2

*Effect of the "inotropic" and "arrhythmic" doses of ouabain on cardiac Na^+, K^+-ATPase in anesthetized dogs**

Ouabain concentration in plasma was maintained at approximately 6 ng/ml (inotropic dose) or 18 ng/ml (arrhythmic dose) by intravenous administration of ^3H-labeled ouabain. At indicated time, hearts were removed and partially purified Na^+,K^+-ATPase preparations were obtained. Activities of Na^+,K^+-ATPase and Mg^{++}-ATPase and the amount of [^3H] ouabain bound to the enzyme preparation were estimated. Values are mean ± S.E.M. *N*, number of experiments.

Treatment	*N*	Ouabain Binding to Na^+,K^+-ATPase Preparation	Mg^{++}-ATPase	Na^+,K^+-ATPase
		pmol/mg protein	*μmol P_i/mg protein/hr*	
Control (300 min)	6		5.3 ± 0.3	9.7 ± 0.7
Inotropic dose (120 min)	4	3.8 ± 0.3	4.9 ± 0.4	9.4 ± 1.1
Inotropic dose (300 min)	6	5.3 ± 0.7	4.6 ± 1.2	8.8 ± 0.9
Arrhythmic dose (300 min)	5	13.6 ± 1.0	4.7 ± 0.3	6.9 ± 0.16†

* Reprinted by permission from Rhee *et al.* (104).

† Significantly different compared to the inotropic group (300 min) based on unpaired *t*-test ($P < .005$).

ever, that a reduction in cardiac Na^+,K^+-ATPase activity was observed with nontoxic doses of ouabain in their studies, although the difference from control value was not statistically significant. They should not conclude that digitalis *does not* alter Na^+,K^+-ATPase activity, since such a decision should be based on a different set of statistical analyses. It should be emphasized, too, that in the group of animals receiving the arrhythmic dose of the glycoside, a reduction of Na^+,K^+-ATPase of only 29% was observed. It is certainly reasonable to expect that the magnitude of the drug effect would be much less when a lower concentration of the drug is used. The variability of the data and the number of animals used will determine whether or not a relatively small change observed with a low drug concentration is statistically significant. It should also be pointed out that the control values reported in this study (104) are markedly lower than those reported earlier by the same group using the same technique (42), or by other groups using similar techniques (12, 24, 58).

In similar experiments described from our laboratory using a low dose of ouabain, enzyme activity in the treated group was inhibited as much as 28% (fig. 6). This value was not statistically significant from control when the data were analyzed by group comparison. Thus, the failure of Rhee *et al.* (104) to demonstrate a significant change does not warrant the conclusion that there is no difference in the data (or that the two groups of data are derived from the same population). Whether the small change in Na^+,K^+-ATPase activity observed would still not be significant after a larger number of experiments, or with a less variable method for the estimation of enzyme activity, is unknown. Recently, Hougen and Smith (67a) have reported that, using nontoxic concentrations of ouabain under experimental conditions similar to Rhee *et al.*, they could find a substantial decrease in sodium pump activity at a time when there was a marked increase in cardiac force of contraction (Section IV B).

In 1974, Murthy *et al.* (92) reported that ouabain potentiates acetylcholine-induced contractions of the rabbit myometrium. This effect could be observed within 10 min. When pieces of myometrial tissue are incubated in a sodium-rich media, however, neither ouabain nor digitoxin inhibits ion movement after the 10-min incubation, although ion recovery is significantly blocked after 30 min. While it is tempting to suggest that there is a ouabain-induced increase in the force of contraction in the absence of

Na^+,K^+-ATPase inhibition, it should be emphasized that, again, the force of contraction and enzyme activity were not studied in the same tissue preparation and that many factors influence the binding. It is indeed to be anticipated that the cardiac glycosides should have a later onset of action in noncontracting tissue (91). In the absence of estimates of actual drug binding studies, these data could be used to *support* a positive correlation between ouabain-induced Na^+,K^+-ATPase inhibition and the potentiation of acetylcholine-induced contraction.

Two reports have been sometimes cited in refutation of a correlation between Na^+,K^+-ATPase inhibition and the digitalis-induced increase in force of contraction. Dal Pra *et al.* (37) suggest that the increased force of contraction is related to the stimulation of transport ATPase activity in the frog heart. Because of inadequate statistical analysis, it is not possible to determine the validity of their findings. The other study is that of Thomas *et al.* (125) who examined a series of cardenolide analogs and found differences in the ability of several of these compounds to inhibit Na^+,K^+-ATPase and increase force of contraction of guinea pig atrial muscle. Again, these authors failed to measure the above parameters in the same heart. In fact, the physiological measurements were compared to enzyme studies performed a year earlier.

Thus, definitive evidence to show a dissociation between Na^+,K^+-ATPase inhibition and the positive inotropic action of digitalis is lacking. Rather, studies on the relationship between these two events overwhelmingly support the hypothesis that the interaction of digitalis with the enzyme is related to the inotropic action of this agent.

D. Enzyme Inhibition in Other Tissues

Na^+,K^+-ATPase is an enzyme which plays an important role in almost all animal tissues and has a wide distribution (27). Thus, it would be reasonable to expect that if Na^+,K^+-ATPase is the receptor for digitalis, tissues other than the heart might also be affected. Of particular interest is skeletal muscle, which shares many common features with cardiac muscle. Digitalis fails to produce a clear positive inotropic effect in skeletal muscle under normal circumstances. The difference in the source of Ca^{++} needed for contractile activation (105) or the difference in the permeability of the sarcolemma for digitalis (52) has been proposed as the reason for the glycoside insensitivity of skeletal muscle. Na^+,K^+-ATPase of skeletal muscle, however, is 10 to 1000 times less sensitive to cardiac glycosides than the corresponding cardiac enzyme in guinea pig, rabbit and rat, although the magnitude of the difference is variable, dependent upon the animal species (39). Our earlier data (12) also demonstrate that at a time when a glycoside-induced positive inotropic action and the concomitant inhibition of dog cardiac Na^+,K^+-ATPase activity are observed, the skeletal muscle enzyme is not inhibited. In fact, among those tissues studied (dog brain, kidney and skeletal muscle), only cardiac Na^+,K^+-ATPase is affected when amounts of digitalis which produced inotropic effects were infused intravenously. Tissue sensitivity can thus explain the selective action of digitalis on cardiac muscle.

IV. Action of Digitalis on Sodium Pump Activity

A. General Considerations

After it had been shown that an ATPase existed within the membranes of most tissues which could be activated by the simultaneous presence of Na^+ and K^+ (117), it became apparent that the enzyme was identical with, or part of, the membrane ion pump (56, 118, 119). The action of digitalis on transmembrane ion movement, however, was recognized before the discovery of the enzyme system. Inhibition of ion transport by digitalis had been shown in various animal tissues such as kidney, red blood cells, blood vessels, stomach, large intestine and thyroid. It was not long before an association was suggested between the therapeutic effects of digitalis and its ability

to affect the transport of Na^+ and K^+ across the cell membrane. In their review in 1959, Hajdu and Leonard (62) concluded: "Cardiac glycosides, in therapeutic as well as toxic concentrations, cause a loss of potassium from cardiac muscle.... It does not appear that the glycosides have any direct effect on cellular calcium." Thus, when Na^+,K^+-ATPase activity was first described, the action of digitalis was examined soon after, and inhibition of enzyme activity was offered as evidence to indicate that this enzyme system is related to ion transport (118, 119).

Nevertheless, a number of investigators failed to recognize that the inhibition of ion transport was related to the therapeutic action of this agent. In 1964, Glynn (56) reviewed the action of the cardiac glycosides on ion movement and concluded that while the sensitivity of the Na^+ and K^+ transport mechanism to the cardiac glycosides was adequate for that transport system to be altered by therapeutic concentration of the drug, it was doubtful whether such concentrations of the glycoside caused an appreciable lowering of $[K^+]_i$. He suggested that the positive inotropic action of glycosides was probably associated with an increased uptake of Ca^{++}.

B. Sodium Pump Inhibition and Force of Contraction

The question that had to be answered before any causal relationship between the inotropic action of digitalis and pump inhibition could be established was whether the Na^+-K^+ pump in cardiac muscle was inhibited during the inotropic action of digitalis. Further, one had to determine whether such an inhibition was of sufficient magnitude to evoke significant biochemical or physiological changes in the myocardium.

The relationship between Na^+,K^+-ATPase and the sodium pump has been firmly established (56, 119). Because it had also been shown that during the inotropic action of digitalis Na^+,K^+-ATPase activity is inhibited by approximately 40% (12, 24, 58), it was not unreasonable to expect that sodium pump activity might also be substantially reduced at this time. Early attempts to show such an inhibition by estimating $[Na^+]_i$ during the inotropic action had not yielded definitive data (82). This might be anticipated, however, if one considers that in the intact cell, the sodium pump activity is controlled by $[Na^+]_i$. Let us assume that the glycosides moderately inhibit sodium pump activity and as a result of this inhibition there is a slight increase in Na^+ concentration at a site from which Na^+ is transported out of the cell. This would then increase the residual (or uninhibited) sodium pump activity and any significant Na^+ accumulation would not be detected. Thus, it is mandatory to use a more direct method of assessing sodium pump activity.

One approach to measuring pump function is to determine the ouabain-sensitive ^{86}Rb uptake into the cardiac cell after preloading the cell with Na^+ (8, 73). This method eliminates the possible "sodium effect" on the transport system since that ion is no longer rate-limiting. When isolated guinea pig hearts are perfused with digitoxin and ventricular slices are prepared during the inotropic action, the ouabain-sensitive ^{86}Rb uptake by these slices is significantly reduced (8). The degree of inhibition is related to the magnitude of the inotropic effect. When the heart is perfused with drug-free solution after a 20-min exposure to digitoxin, the inotropic effect decrements with a half-time of approximately 40 min. Concurrent with the loss of the inotropic effect is the recovery of sodium pump activity. The amount of uninhibited Na^+,K^+-ATPase can also be estimated in these same hearts by determining the initial velocity of the binding of [^{3}H]ouabain to the enzyme. When this is done under the same conditions as above (during inotropic action and after drug washout), it is found that the changes in enzyme activity parallel the differences seen in sodium pump activity.

A recent study by Hougen and Smith (67a) is of interest. They observed that

active transport of monovalent cations is inhibited in the myocardium when anesthetized dogs with an intact nervous system are treated with therapeutic concentrations of ouabain. With the use of a biopsy technique, ventricular myocardial samples are obtained before ouabain and at two time periods after the administration of the glycoside using a loading dose and infusion schedule which produces stable plasma ouabain concentrations. Left ventricular maximum *dP/dt* is significantly increased at 1 and 2 hr after glycoside treatment. Myocardial monovalent cation transport is inhibited at these times. Thus, inhibition of active monovalent cation transport appears to correlate with the increased force of contraction produced by nontoxic concentrations of ouabain.

1. Pump inhibition and intracellular sodium. Although the degree of sodium pump inhibition observed at the time of the inotropic action of digitalis is substantial, how do we know that this reduction is of physiological significance or if the reserve capacity of the transport system can compensate for this inhibition? At first glance, it would appear that the sodium pump has the capability of functioning normally in the face of such an inhibition. For example, in their review, Lee and Klaus (82) affirm that digitalis fails to alter $[Na^+]_i$ and $[K^+]_i$ at the time of inotropic action. Even where changes are observed, such findings need to be evaluated with caution. Changes in ion concentrations are most frequently found when isolated perfused or superfused hearts are used. In such preparations, generally, the supply of oxygen is marginal. While adequate oxygen levels are probably maintained in control preparations, they may not be adequate when oxygen demand is increased as with a glycoside-enhanced increase in the force of contraction. Thus, changes in $[Na^+]_i$ and $[K^+]_i$ may be the result, rather than the cause, of the digitalis-induced positive inotropic effect.

Recent studies, using improved technology, seem to indicate that there is a small but consistent increase in $[Na^+]_i$ after a therapeutic concentrations of digitalis (81). However, it should be noted that this increase is always much smaller than the magnitude of the sodium pump inhibition. One may conclude from the above that normally the myocardial sodium pump does have a reserve capacity and is able to compensate for a moderate inhibition of the pump. If compensation of the pump can occur, then the physiological significance of any observed inhibition is open to question.

Langer (78) proposed that the increase in $[Na^+]_i$ caused by digitalis-induced pump inhibition produces a significant increase in Ca^{++} influx which is coupled to the Na^+ efflux (Na^+-Ca^{++} exchange reaction). This concept is similar to that put forth by Baker *et al.* (20) for the giant axon of the squid. Langer contends that a small increase in $[Na^+]_i$ would be adequate to cause a substantial increase in calcium influx over the normal level. His suggestion for the mechanism of the inotropic action of digitalis is illustrated in fig. 8.

2. Inotropic effect without an increase in intracellular sodium. Several investigators have proposed theories for the inotropic action of digitalis which do not require that the myocardial $[Na^+]$ necessarily be increased. Among these is one which suggests that the function of Na^+,K^+-ATPase is to transport digitalis to its site of inotropic action (which is not Na^+,K^+-ATPase). This concept has been put forth by Dutta *et al.* (41) and Park and Vincenzi (97). Evidence which correlates the binding of digitalis to Na^+,K^+-ATPase with the development of the inotropic effect obviously supports this hypothesis, as well as that which proposes that the enzyme is indeed the receptor for the inotropic action of digitalis. The "carrier mechanism" theory, however, is much less credible because of the slow release of such glycosides as ouabain, digoxin and digitoxin from the enzyme in most mammalian species. The enzyme would be an inefficient mechanism for the transport of the potent cardiac glycosides because an effective system requires binding on one side of the membrane and rapid

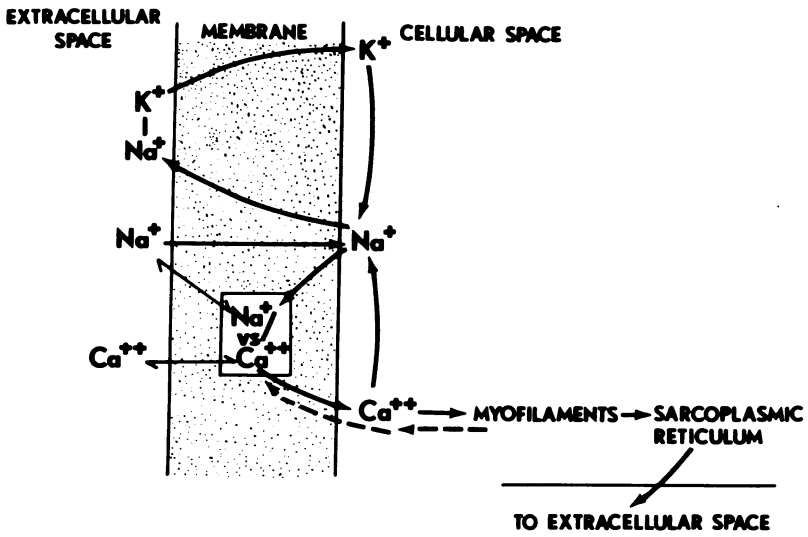


FIG. 8. Langer's proposal for interrelation among Na^+,K^+ and Ca^{++} movements at and through the myocardial membrane. Upper portion of the figure represents the Na^+,K^+-ATPase-mediated Na^+-K^+ exchange reaction. Lower portion represents the Na^+-Ca^{++} exchange reaction. Inhibition of Na^+,K^+-ATPase causes an increase in $[Na^+]_i$, which in turn enhances calcium influx via the Na^+-Ca^{++} exchange reaction. Enhanced calcium influx then increases the force of contraction. (Redrawn by permission from Langer (78).)

release of the material on the other side. An exception would be the rat, in which cardiac Na^+,K^+-ATPase is capable of releasing digitalis very rapidly (130). The rat, however, is notoriously insensitive to the cardiac glycosides.

The most serious problem with the "carrier mechanism" theory is that there is no evidence which indicates that the glycosides act on the inside of the cell to produce their inotropic action. Although an intracellular site of action for digitalis has been proposed for the increased force of contraction seen in crab skeletal muscle (52), in the dog heart, the specific binding of the glycosides is exclusive for Na^+,K^+-ATPase (15). No other organelle or "enriched" system has a high affinity for digitalis. In fact, the "low affinity" site of the enzyme would not appear to be low enough. The $K_{0.5}$ value of the "intracellular low affinity" ouabain binding sites of the Na^+,K^+-ATPase obtained from guinea pig heart is about 0.63 μM (48). If Na^+,K^+-ATPase is the "carrier system" for the glycosides, then the drug binding to the "inotropic site" should be of a higher affinity than that which occurs between digitalis and the enzyme. If this were not so, the drug would be unable to move from the "carrier" to the "inotropic site." Such an alternative site has not been found.

Another theory that relates the specific binding of digitalis to Na^+,K^+-ATPase with the inotropic action of digitalis, but does not require sodium pump inhibition, has been proposed by Besch and Schwartz (25) and has most recently refined by Schwartz (111). According to this concept, digitalis binds to the enzyme and induces a specific conformational change in the protein. This conformational transition alters the affinity of Ca^{++} for lipids associated with Na^+,K^+-ATPase and, thus, increases the amount of Ca^{++} released into the cell. It is also possible that sodium pump inhibition with its resultant increase in $[Na^+]$ at some intracellular site may alter the affinity of a phospholipid component for Ca^{++}. Thus, they propose that pump inhibition may or may not be necessary for the inotropic action of digitalis (111). Evidence to support the thesis that the interaction of ouabain and Na^+,K^+-ATPase alters the affinity of the enzyme preparation for Ca^{++} has been presented (54). It is not known, however, whether such an alteration in Ca^{++} affinity enhances the release of membrane bound

Ca^{++} after membrane excitation. If the above concept is correct, then the amount of digitalis-enzyme complex formed, rather than the degree of sodium pump inhibition, determines the extent of the increase in force of contraction, and hence the magnitude of the positive inotropic action would be greater in cardiac tissues in which the sodium pump sites per unit of membrane surface were more numerous. Thus, certain pharmacological interventions which increase Na^+,K^+-ATPase and sodium pump sites in cardiac tissue (36) might increase the efficacy of the inotropic action of digitalis. In contrast, the positive inotropic effect would be smaller in such tissues if the residual sodium pump activity determines the force of contraction, or unaffected by the concentration of sodium pump sites if the force of contraction is related to the percent inhibition of sodium pump activity. Data are presently not available to decide between these alternatives.

V. Other Agents that Inhibit Na^+,K^+-ATPase or Sodium Pump Activity

As discussed above, the binding of digitalis to Na^+,K^+-ATPase is related to the inotropic action of this agent. Whether such binding is required for sodium pump inhibition or some other event may be assessed by inhibiting the enzyme (or the pump) and estimating the resultant change in the force of myocardial contraction. If the inotropic action of digitalis is the result of enzyme inhibition, then Na^+,K^+-ATPase inhibitors should produce positive inotropic effects regardless of the mode of enzyme inhibition. Thus, the effect should be independent of the particular enzyme conformation induced by the agent and depend solely on the overall reduction in enzyme activity. Among Na^+,K^+-ATPase inhibitors, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), prednisolone-3,20-bisguanylhydrazone, ethacrynic acid, fluoride, doxorubicin and sanguinarine (38, 40, 55, 96, 115, 120, 132) produce positive inotropic effects in the isolated heart (35, 49–51, 59, 115, 132, 137). Although common correlates for these agents appear to be Na^+,K^+-ATPase inhibition and the positive inotropic effect, some of these compounds are known to possess the ability to alter normal cell biochemistry. It is difficult, therefore, to attribute unequivocally the observed increase in the force of cardiac contraction to inhibition of the transport enzyme.

A. Sulfhydryl Inhibitors

We investigated in some detail the mechanism by which certain of the sulfhydryl inhibitors produce their inotropic effects using field-stimulated guinea pig left atrial preparations (123). The sulfhydryl inhibitors, NEM, PCMB and *p*-chloromercuribenzene sulfonic acid (PCMBS), are capable of producing dose-related positive inotropic effect in isolated heart preparations. The time to maximal response could be correlated with the relative lipid solubility of each of the agents, NEM having the most rapid and PCMBS the least rapid onset of action. This observation is consistent with an earlier suggestion (108) that the essential sulfhydryl groups for sodium pump activity are localized on the inside of the cell membrane. Sodium pump activity can also be estimated in these hearts by determining the activity of glycoside-sensitive ^{86}Rb uptake at the time of the maximal inotropic effect produced by the sulfhydryl inhibitor (or digitoxin which was used as a positive control). The data for each of the sulfhydryl inhibitors, as well as for digitoxin, are shown in Fig. 9. It is apparent that these agents are all capable of inhibiting sodium pump activity during the inotropic action as indicated by the decrease in specific ^{86}Rb uptake.

To test the possibility that the sulfhydryl inhibitors might produce their inotropic action by altering cell membrane permeability, the effects of NEM, PCMB and PCMBS (in concentrations which increase the force of contraction but do not cause arrhythmias) were examined on the resting membrane potential and the several parameters of the normal action potential. A summary of these findings, shown in table 3,

indicates that they fail to alter the resting membrane potential. Further, the small changes observed in some parameters of the action potential are not related to the observed positive inotropic actions of these compounds. Although the data would tend to support the proposition that inhibition of the sodium transport pump by the sulfhydryl inhibitors is causally related to the observed increase in the force of contraction, it is still possible that the inotropic effect is the result of an unknown mechanism.

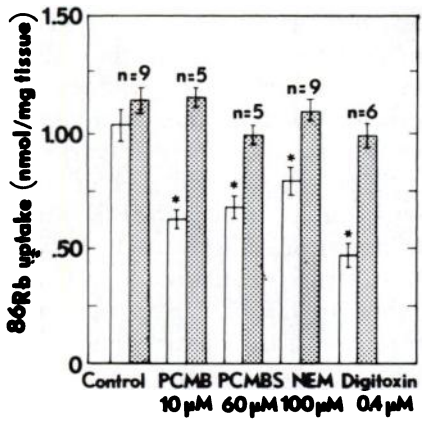


FIG. 9. Effect of perfusion with sulfhydryl inhibitors or digitoxin on sodium pump activity. Langendorff preparations of guinea pig hearts are perfused with one of the agents until the maximal positive inotropic effect is obtained, or with Krebs-Henseleit solution for a comparable period of time (control). At this time, ventricular slices are prepared and ^{86}Rb uptake (an estimate of sodium pump activity) is assayed in the absence or presence of 0.3 mM ouabain. n indicates the number of experiments. Open bars, ouabain-sensitive ^{86}Rb uptake. Dotted bars, ouabain-insensitive (nonspecific) ^{86}Rb uptake. Vertical lines, S.E.M. * Significantly different from control ($P < .05$).

B. Cassaine

Of particular interest was the observation that cassaine, an erythrophleum alkaloid, was capable of inhibiting Na^+,K^+-ATPase (28) by interacting with the cardiotonic steroid binding site of the enzyme (98, 128) prepared from heart, brain, red blood cells and other tissues. Further, cassaine has a positive inotropic effect in the isolated heart (69, 85, 93, 128). Dose-response data indicate that the potency of cassaine to increase the force of contraction is similar to that of ouabain. Cassaine lacks the structural characteristics of the digitalis compounds typified by the cyclopentanophenanthrene nucleus with the A/B *cis,* B/C *trans,* and C/D *cis* fusion of the four-ring structure, the C-14 OH group and an unsaturated lactone ring in a *beta* configuration on C-17. In spite of this, cassaine produces a digitalis-like inotropic effect (128). This is all the more remarkable since the above structural requirements are stringent for digitalis activity and ordinarily only a small change in molecular structure, such as saturation of the lactone ring, results in an almost complete loss in inotropic activity and affinity for Na^+,K^+-ATPase. The chemical structures of ouabain and cassaine are shown in Fig. 10.

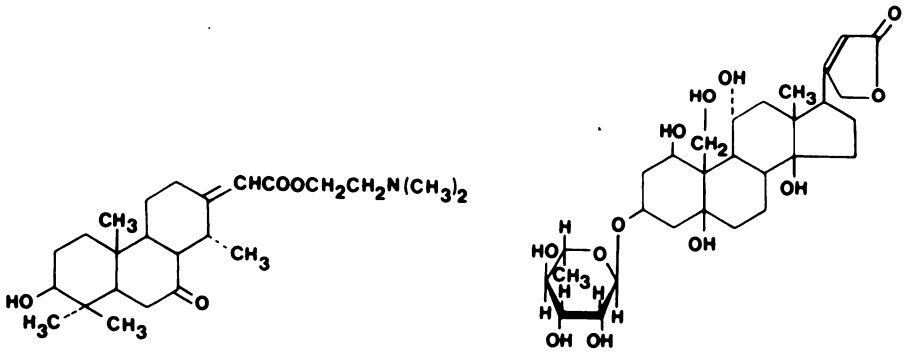
TABLE 3

Effects of sulfhydryl blocking agents on transmembrane potential of reserpine-pretreated guinea pig atria

	Resting Potential	Maximal dV/dt	Action Potential duration (20% Level of Repolarization)
	mV	*V/sec*	*msec*
NEM (100 μM)			
Control	77.1 ± 0.7*	123.0 ± 8.9	24.7 ± 2.1
Maximal Inotropy	76.2 ± 1.0	128.5 ± 3.5	32.9 ± 2.3†
PCMB (20 μM)			
Control	76.7 ± 0.6	120.0 ± 8.2	30.5 ± 1.2
Maximal Inotropy	76.3 ± 0.7	112.0 ± 5.8	20.5 ± 1.4†
PCMBS (20 μM)			
Control	76.4 ± 0.8	120.4 ± 5.0	30.9 ± 1.9
Maximal Inotropy	75.1 ± 0.8	105.1 ± 4.9†	15.9 ± 1.4†

* Each value represents the mean ± S.E.M. of 12 to 18 observations (6–9 preparations).

† Significantly different from control values ($P < .05$).



CASSAINE **OUABAIN**

FIG. 10. Chemical structure of ouabain and cassaine.

C. Monovalent Cations

1. Rubidium and thallium. Several monovalent cations are also capable of inhibiting Na^+,K^+-ATPase activity in the presence of Na^+ and K^+ (99, 129). The advantages of studies with the monovalent cations is that several cations with differential effects on Na^+,K^+-ATPase are available and thus appropriate controls may be utilized. In the presence of Mg^{++} and ATP, Na^+,K^+-ATPase activity may be fully stimulated by the simultaneous presence of Na^+ and K^+. The effect of Na^+ to stimulate phosphorylation of the enzyme cannot be effectively substituted by other cations, whereas the effect of K^+ to promote dephosphorylation may be subserved by monovalent cations such as Rb^+, Cs^+, NH_4^+ and Tl^+ (63). Among these, Rb^+ and Tl^+ have a higher affinity for the enzyme than does K^+. Since the rate-limiting step in the Na^+,K^+-ATPase reaction is the release of K^+ from the dephosphoenzyme, Rb^+ and Tl^+ slow down the turnover of the enzyme when the enzyme is fully activated in the presence of Na^+ and K^+ (99, 129).

Among the monovalent cations, Rb^+ and Tl^+ inhibited cardiac Na^+,K^+-ATPase activity *in vitro* in the presence of Na^+ and K^+ and also produced a sustained, positive inotropic effect in paced, guinea pig atria (74, 75). The inotropic effects observed with both ions were concentration-dependent. The inotropic effect of Tl^+ develops rather slowly in contrast to that of Rb^+, which exhibits a rapid onset of action. It is reasonably well established that the development of the inotropic effect of digitalis is beat-dependent (91, 97, 109). However, with both Rb^+ and Tl^+, the development of the inotropic effect is independent of the number of contractions or depolarizations, a result consistent with the lack of Na^+ dependency of these cations for their interaction with Na^+,K^+-ATPase (75). In contrast to the cardiac glycosides, no species difference in Na^+,K^+-ATPase inhibition or inotropic action is observed with either Rb^+ or Tl^+ (76). Other monovalent cations, such as K^+, Cs^+, NH_4^+ or Na^+ in similar concentrations (0.5–5 mM) have essentially no effect on Na^+,K^+-ATPase activity and produce no sustained positive inotropic effect (75). Furthermore, concentrations of Tl^+ and cardiac glycosides which produce equivalent inotropic effects appear to cause the same degree of pump inhibition. A concentration of Tl^+ (2 mM) sufficient to produce a positive inotropic effect also produced a 40% reduction in glycoside-sensitive ^{86}Rb uptake in guinea pig ventricular slices without altering its nonspecific uptake (table 4). Thus, these ions inhibit Na^+,K^+-ATPase and sodium pump activities and increase the force of cardiac contraction in a quantitatively parallel fashion.

2. Lithium. Li^+ produces a positive inotropic effect in isolated hearts by a mechanism of action somewhat different from

TABLE 4
Effects of Tl^+ and Li^+ on ouabain-sensitive ^{86}Rb uptake

Ventricular slices of guinea pig hearts were incubated with thallous acetate or LiCl for indicated time at 30°C and then preloaded with sodium by incubating at 0°C. Subsequently, ouabain-sensitive ^{86}Rb uptake was estimated at 37°C in the absence of Tl^+ or Li^+. (See Ref. 73 for details of assay method.)

Treatment	Incubation Time	
	15 min	30 min
	nmol/mg tissue	
Control	2.60 ± 0.15 (12)*	2.13 ± 0.10 (12)
Tl^+, 1 mM	1.90 ± 0.15† (7)	1.85 ± 0.20† (5)
Tl^+, 2 mM	1.95 ± 0.10† (7)	1.50 ± 0.15† (5)
Tl^+, 4 mM	1.75 ± 0.15† (7)	1.05 ± 0.15† (7)
Control	2.90 ± 0.15 (17)	2.25 ± 0.15 (17)
Li^+, 29 mM	2.60 ± 0.20 (6)	2.00 ± 0.10 (6)
Li^+, 58 mM	2.35 ± 0.10† (6)	1.35 ± 0.15† (6)
Li^+, 87 mM	1.75 ± 0.15† (6)	1.25 ± 0.10† (6)

* Mean ± S.E.M. Figures in parentheses equal number of separate preparations.

† Significantly different compared to control, $P < .05$.

that of Rb^+ and Tl^+ (75). Li^+ has no effect, or at best a stimulatory action, on Na^+,K^+-ATPase activity when studied *in vitro*. Such an effect had been reported earlier (129, 136). The addition of 10 to 20 mM Li^+ produces a significant increase in the force of contraction of paced guinea pig hearts. This response is unaltered by propranolol and is not the result of a decrease in $[Na^+]$ in the medium. If Li^+ does not inhibit Na^+,K^+-ATPase activity, what is the mechanism responsible for the increase in the force of contraction? There is considerable evidence in other tissues that Li^+ is capable of inhibiting the sodium pump as a result of the ion accumulating intracellularly (136). Recent studies from our own laboratory have shown that high concentrations of Li^+ can inhibit myocardial sodium pump activity (table 4). Although Li^+ may actually be inhibiting Na^+,K^+-ATPase at the higher concentrations (61), it is possible that inhibition of cardiac Na^+,K^+-ATPase *per se* is not an absolute requirement for the cardiotonic effect, but rather it is the sodium pump activity which determines the magnitude of the physiologic response.

VI. Intracellular Sodium Transient

A. General Concepts

Observations presented in the two previous sections lead us to two seemingly conflicting conclusions: that digitalis-induced sodium pump inhibition produces the positive inotropic effect and that a moderate sodium pump inhibition induced by digitalis in nontoxic concentrations may be largely without biochemical effect because of the reserve capacity of the sodium pump. The following considerations may explain why sodium pump inhibition increases the force of myocardial contraction despite the ability of the system to apparently neutralize the inhibition.

The rate of sodium influx during membrane excitation is markedly greater than that in the resting state. Thus, during each cycle of myocardial function, the rate of sodium influx is greater earlier in the cycle and less during the later part of the cycle. Such an alternation of sodium influx would result in a fluctuation of $[Na^+]_i$ at the inner surface of sarcolemma. Since the activity of the sodium pump is primarily determined by $[Na^+]_i$, the sodium pump may be markedly activated early in each cycle of myocardial function but functioning well below capacity at a later time. Thus, inhibition of the sodium pump may have a significant effect on $[Na^+]$ at the inner surface of the cell membrane only during an earlier phase of each cycle, when the sodium pump is operating close to its maximum and has no significant reserve capability.

If the above concept is true, then agents which increase the transmembrane sodium influx should enhance the increase of $[Na^+]_i$ at an early phase of each myocardial cycle when the sodium pump activity is functioning maximally. In fact, the positive inotropic action of several agents has been attributed to their ability to increase the transmembrane sodium influx. These agents include sodium ionophores, veratrum alkaloids, angiotensin II, batrachotoxin, germitrine, and the grayanotoxins (10, 22, 34, 45, 66–68, 72, 116). For example,

the grayanotoxins increase sodium influx without affecting Na^+,K^+-ATPase or sodium pump activity directly (10, 72), although in the intact, nonbeating myocardial cell, sodium pump activity may be indirectly activated as a result of increased intracellular sodium concentration. The grayanotoxins produce positive inotropic effects which are readily reversible in isolated guinea pig atrial preparations at concentrations which increase sodium influx (72). The inotropic action is independent of *beta* adrenergic mechanisms and is antagonized by tetrodotoxin, which blocks the grayanotoxin-induced increase in sodium influx. Thus, drugs which enhance the intracellular sodium transient (a transient increase in the $[Na^+]$ at the inner surface of the sarcolemma associated with each membrane excitation) by separate mechanisms, *i.e.,* by enhancing sodium *influx* with agents such as the grayanotoxins or by inhibiting sodium *efflux* with digitalis, increase the force of myocardial contraction.

In further support of the hypothesis that inhibition of the cellular sodium pump by the cardiotonic steroids is causally related to their positive inotropic effect is the recent study of Ebner and Reiter (43) who demonstrated the dependence of the inotropic effectiveness of a moderate concentration of dihydro-ouabain on the frequency of contraction. A stepwise decrease in frequencies of contraction of guinea pig papillary muscle results in a decrementing of the inotropic effect. Since the extent of the increase in $[Na^+]_i$ depends, when the sodium pump is partially inhibited, on the Na^+ load, *i.e.,* on the number of depolarizations per unit time, they concluded that the glycoside produces its positive inotropic effect by increasing $[Na^+]_i$ and stimulating Na^+-Ca^{++} exchange across the sarcolemma.

B. A Computer Simulation

From the above, it would seem to be appropriate to demonstrate that indeed intracellular sodium transients exist which can be enhanced by either digitalis or by an agent known to increase sodium influx. Unfortunately, technology is not presently available to estimate the $[Na^+]$ in a narrow space adjacent to the inner surface of the sarcolemma. The most promising sodium electrode is too large and has a time constant of several seconds, which is inadequate to follow changes in sodium concentration with a cycle time of less than 500 msec. The results of a computer simulation study (5), however, seem to support the contention that this mechanism is an entirely reasonable one. In this study, the model for the computer simulation is the small compartment adjacent to the inner surface of the sarcolemma (fig. 11). The change in $[Na^+]_i$ in this compartment is determined by the rate of sodium influx modified from values reported by Langer (79) and the rate of sodium efflux resulting from sodium pump activity. The relationship between $[Na^+]_i$ and the rate of sodium pump activity is estimated from the activities of partially purified dog heart Na^+,K^+-ATPase preparations assayed in the presence of various concentrations of Na^+ and ouabain. The rate of change in $[Na^+]_i$ with respect to time was calculated at each half-millisecond in the cardiac cycle for a total of 500 msec (corresponding to a heart rate of 120/min). The results indicate that the

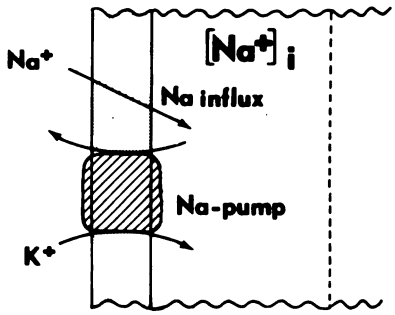


FIG. 11. A model for computer simulation of $[Na^+]_i$. The model describes a small compartment adjacent to the inner surface of the sarcolemma. The change in $[Na^+]$ in this space is determined by the rate of sodium influx (79) and the rate of sodium efflux due to active sodium transport (sodium pump activity). The relationship between $[Na^+]_i$ and the sodium pump activity is estimated from the activities of partially purified dog heart Na^+,K^+-ATPase preparations assayed with various concentrations of Na^+ and ouabain.

initial rapid sodium influx associated with each membrane depolarization results in maximal sodium pump activation. Since the rate of sodium influx exceeds the maximal sodium efflux rate of the sodium pump during the early phase of the action potential plateau, $[Na^+]_i$ increases further and reaches a peak at 75 msec and the $[Na^+]_i$ returns to pre-excitation levels at approximately 350 msec after membrane excitation. Sodium pump activity lessens as $[Na^+]_i$ decreases. Inhibition of Na^+,K^+-ATPase by ouabain increases the peak $[Na^+]_i$ and also increases the time in each cycle at which the sodium pump operates at a higher rate, *i.e.,* a rate close to the inhibited maximal velocity.

With a 40% inhibition of Na^+,K^+-ATPase, the peak $[Na^+]_i$ is approximately 20% higher than control but $[Na^+]_i$ approaches pre-excitation levels at 500 msec after membrane excitation (fig. 12). Thus, therapeutic concentrations of digitalis will produce an enhanced sodium transient and a minimal myocardial Na^+ accumulation at relatively slow heart rates (below 120 beats/min), whereas toxic concentrations of digitalis will produce a progressive myocardial Na^+ accumulation (fig. 12). The computer simulation predicts that a therapeutic concentration of digitalis will cause a myocardial Na^+ accumulation at a higher heart rate but not at a lower one. This prediction was experimentally confirmed in Langendorff preparations of guinea pig hearts (5). Consequently, it appears that some of the results of the computer simulation are experimentally demonstrable.

If an intracellular sodium transient occurs during each cycle of myocardial function, and the effect of a moderate sodium pump inhibition is to enhance such a transient without causing a progressive sodium accumulation, then one can understand why moderate sodium pump inhibition increases the force of contraction without Na^+ accumulation. Additionally, such a concept can also explain why the Na^+-Ca^{++} exchange reaction (calcium influx coupled with sodium efflux) proposed by Langer operates only during the early phase of each cycle of myocardial function, causing transient increases in $[Ca^{++}]_i$ which ultimately lead to a myocardial twitch. If this is the mechanism by which the membrane excitation causes an increase in $[Ca^{++}]_i$ (calcium transient), then enhanced sodium transients should increase calcium transients and hence the force of contraction. Experimental demonstration of such a sequence of events is, as yet, not available.

VII. Digitalis Cardiotoxicity

Figure 12 indicates that a 60% inhibition of sodium pump activity, which may be observed with toxic doses of digitalis (12) causes a progressive increase in Na^+ within the myocardial cell. Since Na^+ and K^+ movements are coupled, this accumulation may result in K^+ loss with subsequent reduced transmembrane Na^+ and K^+ gradients. Such phenomena have been described in hearts exposed to toxic concentrations of digitalis (82).

Because the heart accumulates Na^+ and loses K^+ during glycoside-induced cardiotoxicity and because such changes can result in a decreased action potential upstroke and in a partial depolarization of myocardial cells, the hypothesis that enzyme inhibition is causally related to the cardiotoxicity is widely accepted. The above ionic shifts can also contribute to the well documented changes in ventricular muscle automaticity.

The relationship between Na^+,K^+-ATPase inhibition and cardiotoxicity (arrhythmias) is probably more complex. When isolated guinea pig hearts are exposed to toxic concentrations of digitalis and then perfused with drug-free solution after the onset of arrhythmias, the time to restoration of a normal rhythm is relatively long. The half-time for the recovery of enzyme activity under these conditions, however, is rapid, *i.e.,* less than 10 min (3, 73). Thus, the events which link Na^+,K^+-ATPase inhibition to cardiac arrhythmias may have a long response time (perhaps the time required to effect a significant potassium

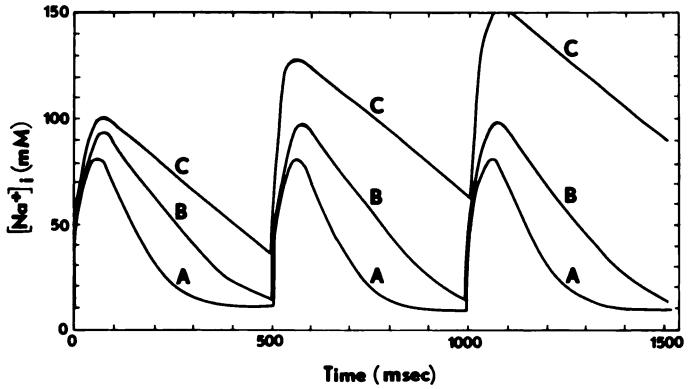


FIG. 12. Computer simulation of intracellular sodium ion concentration. See figure 11. Curve A represents three consecutive myocardial cycles under normal conditions. Curves B and C represent three consecutive cycles when Na^+,K^+-ATPase is inhibited approximately 40(B) and 60%(C) with inotropic and toxic concentrations of ouabain, respectively. With a 40% inhibition of Na^+,K^+-ATPase, the $[Na^+]_i$ transient is enhanced, but Na^+ accumulation does not take place. With a 60% inhibition of the enzyme activity, a progressive Na^+ accumulation occurs. (Redrawn by permission from Akera *et al.* (5).)

loss), whereas the relationship between enzyme inhibition and the inotropic action appears to be a more direct one. It has also been reported that the correlation between Na^+,K^+-ATPase inhibition and the cardiotonic action of several digitalis derivatives is better than that between Na^+,K^+-ATPase inhibition and the arrhythmogenic actions of these compounds (65). Thus, the relationship between enzyme inhibition and cardiotoxicity may be more complicated than generally considered.

Additionally, it is possible that the arrhythmogenic action of digitalis in the intact animal may stem from an extracardiac action of the drug, although digitalis produces arrhythmias in isolated, denervated heart preparations. The relative importance of this extracardiac effect is unresolved and outside the scope of this review.

VIII. Summary: The Mechanism of Inotropic Action of Digitalis

The binding of digitalis to cardiac Na^+,K^+-ATPase appears to be intimately related to the inotropic action of this agent. Studies counter to the premise suffer either from problems in experimental design or have not been confirmed by other investigators. A hypothesis that digitalis binding to Na^+,K^+-ATPase is required for the transport of the glycoside to its site of action cannot be ruled out at this time, although it is inconsistent with a number of observations (see earlier discussion above). This concept, primarily proposed by Dutta *et al.* (41) is illustrated in fig. 13B. As is shown, digitalis binds to Na^+,K^+-ATPase and is transported across the sarcolemma. This movement of the glycoside is intimately related to the active transport of sodium and potassium ions. Digitalis, by some mechanism not described, interacts with the receptor for inotropic action to produce its cardiotonic effect.

Schwartz (111) has most recently proposed that digitalis binding to Na^+,K^+-ATPase is required to lock the enzyme protein in a particular conformation resulting in an altered affinity of enzyme-associated lipids for Ca^{++}. The hypothesis lacks definitive demonstration that digitalis modifies Ca^{++}-lipid interactions in such a manner as to increase the release of Ca^{++} after membrane excitation. This hypothesis is depicted in Figure 13C. This proposal would obviously necessitate an interaction of the glycoside with the enzyme, but would not require that the sodium pump be inhibited.

The hypothesis put forth in the present article is illustrated in figure 13D. The gly-

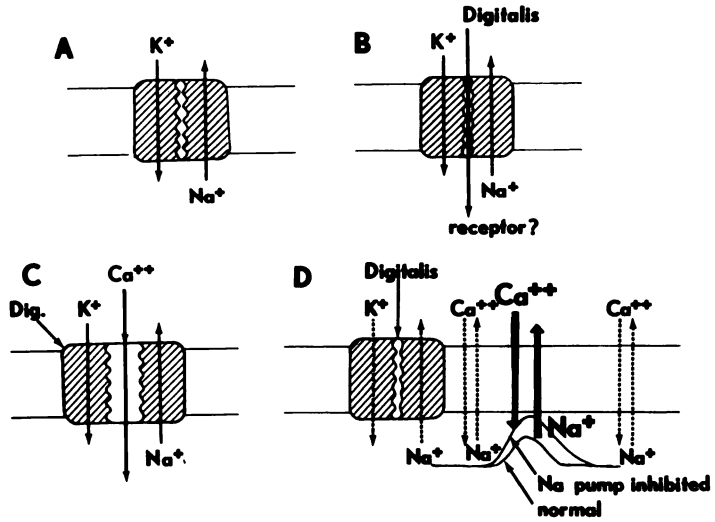


FIG. 13. Schematic diagram representing three theories involving Na^+,K^+-ATPase in the mechanism of inotropic action of digitalis. A. Normal conditions: shaded area represents Na^+,K^+-ATPase which exchanges Na^+_i for K^+_o at the expense of ATP. B. Dutta-Marks hypothesis: Na^+,K^+-ATPase binds digitalis which is transported into the cell and then to a putative digitalis receptor localized inside the cell membrane. C. Schwartz hypothesis: binding of digitalis to Na^+,K^+-ATPase causes the enzyme to assume a specific conformation which promotes Ca^{2+} influx associated with membrane excitation. D. Akera-Brody hypothesis: digitalis binds to Na^+,K^+-ATPase and inhibits the active exchange of Na^+ and K^+. Curves on the right lower part of panel D depict the changes in $[Na^+]_i$ at the inner surface of sarcolemma during a cycle of myocardial function. In the untreated heart, (labeled "normal"), the $[Na^+]_i$ transiently increases with each membrane excitation. During this sodium transient, Ca^{++} enters the cell *via* a coupled Na^+-Ca^{++} exchange mechanism. When the sodium pump is inhibited by digitalis (labeled "Na pump inhibited"), the sodium transient is enhanced, resulting in a greater calcium influx during the early phase of each cycle of myocardial function, and causing a greater contraction. However, there is no intracellular accumulation of Na^+.

coside inhibits the enzyme, which in turn reduces sodium pump activity. As a consequence of the lowered pump function, there is an enhanced transient increase in $[Na^+]_i$, close to the sarcolemma, which occurs *only during the early phase of the cardiac cycle.* The increase in Na^+ results in an enhanced Ca^{++} influx which is reflected in the cardiotonic action of the drug. This hypothesis is a variant of that of Langer (fig. 8) and differs from his original hypothesis in that the increase in Na^+ is cyclic and not cumulative. The present hypothesis is also more consistent with the observations that therapeutic concentrations of digitalis do not cause a marked cellular Na^+ accumulation, and also explains why Na^+-Ca^{++} exchange occurs only during a short time period after each membrane depolarization, resulting in calcium transients. A greater magnitude of enzyme inhibition results in excess Ca^{++} influx, K^+ loss and cardiotoxicity. The mechanism by which the intracellular Ca^{++} transient could evoke a myocardial contraction is well known. Missing is a direct experimental demonstration of the intracellular sodium transient and its enhancement by digitalis, and the mechanism by which the latter evokes the transmembrane exchange of calcium.

The application of new technology should continue to amplify our knowledge of how digitalis acts at the cellular level. This review is an attempt to clarify only one aspect of that knowledge.

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