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THE SUBCELLULAR BASIS FOR THE MECHANISM OF INOTROPIC ACTION OF CARDIAC GLYCOSIDES

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I. INTRODUCTION

In the search for the mechanism of the positive inotropic action of cardiac glycosides (CG), almost all major areas of cellular activity in the myocardium have, at one time or another, been the subject of investigation. This has resulted in a bewildering array of data on the action of CG, but compounding the complexity of the problem is the fact that many authors claim to have discovered the "true" mechanism of CG action in the limited scope of their experimental area. In addition, interest in CG has been aroused among biologists in general, because of the specific influence of this class of compounds on transport mechanisms in tissues other than heart. Yet, to date, the enormous information about the effects of CG has not provided a clear mechanism of positive inotropism on a reasonably firm experimental basis.

This review is concerned with the subcellular basis of the inotropic action of CG. Studies on clinical and other aspects which are not relevant to this immediate purpose will not be discussed. An attempt will be made to present, with the emphasis on the latest findings, those data which are deemed pertinent for the elucidation of the inotropic action of CG. An attempt has been made to minimize the prejudices of the authors by presenting, impartially, the views of various workers as they have stated them. However, some prejudice will be unavoidable. No effort has been made to include all of the literature existing in this field, and some studies of significance may not have found a place in this review because of limitations of space. The present authors do not pretend that they have the answer to the problem, but they have made a serious attempt to correlate many known findings and to narrow the area as much as possible where the true mechanism of CG may reside.

II. ACTIONS OF CG ON NONFAILING HEART

It is proper to begin this review with this problem because of its many implications. If the CG increase the force of contraction only in failing hearts, then this implies that whatever the defect in the failing heart, it is the site of digitalis action. On the other hand, if CG increase the contractility of normal as well as failing hearts, then the area of action would not be subject to this limitation.

Until recently, many investigators considered that the positive inotropic effect of the CG is dependent on the functional state of the myocardium. This concept was based on the belief that a hypodynamic state of myocardium was essential for manifestation of the positive inotropic effect of CG in isolated heart muscle preparations (457, 546), and that these drugs failed to increase the cardiac output of normal hearts *in situ* (164, 195, 373, 423, 461, 463, 494).

It is well established that in isolated heart muscles the *relative* increase in contractile force produced by CG is greater when the degree of hypodynamia is more pronounced (401, 438, 457). However, the definitive positive inotropic effect has been shown to occur in presumably nondepressed isolated heart muscle preparations (278, 438). Recently, it has become increasingly evident that the positive inotropic action of CG occurs in the nonfailing heart *in situ*. This has been shown in studies in which the isometric wall tension, the intraventricular pressure, and the maximal rate of rise of intraventricular tension were measured under well controlled conditions (40, 81, 342, 442, 450, 484, 528, 543, 549). The failure in some previous studies to demonstrate the positive inotropic effect of CG in the normal heart may be due to concomitant changes of some hemodynamic parameters (*e.g.*, effect on venous return) which interfered with an actual increase in cardiac output (81, 119, 462, 484). Thus, it can be certain that CG do produce positive inotropic effects, regardless of the functional state of the myocardium. Therefore, the mechanism by which CG increase the force of contraction is probably not simply a reversal of the processes which induced the hypodynamic state of the heart.

III. THE INFLUENCE OF CG ON CONTRACTILE MECHANICS OF THE CARDIAC MUSCLE

Recent experiments on isolated cardiac muscle preparations (mainly papillary muscles) have shown that the interacting behavior of three different components, one contractile and two elastic, which are well known to be involved in the contractile mechanics of skeletal muscle (215) must also be considered in the analysis of the contractile mechanics of heart muscle. None of the inotropic interventions studied so far has been shown to have an appreciable influence on the properties of the passive elastic components (110, 238); therefore, inotropic alterations of the myocardium have to be achieved through an influence on the behavior of the contractile component only. The action of CG on the contractile dynamics of the heart muscle may be described in terms of the following features of the isometric twitch: 1) the rise of tension in the early part of contraction, which is roughly proportional to the onset and the rise of the active state (AS), but not necessarily related to the maximal rate of shortening of the contractile elements; 2) the peak tension, which may give a clue to the maximal intensity of the AS if due allowance is made for the time course of the isometric twitch; and 3) the time to peak tension and the total duration of contraction, which indicate alterations in the time course of activation. The principal effects of CG on these parameters of myocardial contraction, as observed in isolated heart muscle preparations, are an increase in the rate of rise of tension, an increase in the maximal tension developed, and (especially in ventricular preparations) reduction of the time to peak tension and of the total duration of contraction (52, 110, 112, 276, 278, 403, 405, 465, 481). Quite similar observations have been made on intact hearts under controlled hemodynamic conditions, in which it was found that CG increased the rate of rise in the intraventricular pressure, augmented the maximal ventricular pressure developed, and shortened the duration of systole (40, 41, 81, 361, 442, 465, 484, 493, 524, 525, 539, 543, 549, 577).

IV. VARIOUS PARAMETERS MODIFYING THE ACTION OF CG ON CARDIAC FUNCTION

A. Frequency-dependence of CG action: Onset of action

It has been shown that the rate of onset of CG action is dependent on myocardial activity (283, 321, 326, 352, 437, 520, 552). The rate of onset in guinea pig ventricle preparations was strictly proportional to the beat frequency and followed first order kinetics, and extrapolation to the zero frequency resulted in nearly zero action (437). A similar observation was made by Vasalle *et al.* (520) in dog ventricle strips in their study on the appearance of toxic signs of digitalis at different stimulation frequencies. A detailed analysis of the relationship between the myocardial activity and the digitalis action on rabbit atria by Moran (352, 353) showed that the time required for development of a certain effect was inversely proportional to the frequency of stimulation, and that the cumulative number of contractions for half-maximal effect was almost the same at all frequencies tested (15–120/min). On the other hand, exposure of resting preparations to ouabain for up to 30 min did not result in a positive inotropic effect when the preparations were stimulated after this period (the ouabain-containing incubation medium was removed prior to the stimulation). From these findings, Moran (352, 353) concluded that the rate of onset of digitalis action is related to the total number of beats, rather than to the frequency of stimulation or to the time of exposure to the drug. These results were confirmed by Holland (224) and Lock (328). The dependence of the development of CG actions on myocardial activity was explained by Moran (352, 353) on the assumption that CG probably are not bound to specific receptors in resting state or that such receptors are not present in the resting muscle.

On the other hand, numerous investigations do not confirm the above frequency-dependence of CG action. Garb and Penna (144) did not detect any influence of driving rate on the development of the inotropic effect of ouabain in the papillary muscle. In atrial and ventricular preparations of different species, the positive inotropic effect could be demonstrated to develop and persist even at very low stimulation rates with intervals up to 5 min ("resting state contraction") (55, 139, 278, 279, 523). In some studies, the development of positive inotropic effect was almost independent of stimulation. Toxic signs, such as contracture and electrophysiological changes, developed clearly even in completely quiescent preparations (520). Indeed, Schatzman and Witt (446) found an increased loss of K^+ from quiescent skeletal muscle preparations after exposure to strophanthidin, and Page *et al.* (380) obtained similar results (K^+ loss, Na^+ uptake) on quiescent cat papillary muscles by high doses of ouabain. A careful analysis of this interrelationship in guinea pig atria (523) showed that the rate of onset of ouabain action was almost strictly time-dependent at low frequencies of stimulation, whereas it was dependent on the number of beats at higher frequencies (intervals less than 3 sec). Atria incubated in the presence of ouabain without stimulation for 50 min showed significantly increased contractile force following the first stimulus. This means that CG may alter the cellular charac-

teristics (to bring about conditions which produce the positive inotropic effect) even in the nonactive cardiac muscle. Since the onset of digitalis action is speeded up above certain frequencies, it appears that onset of CG action proceeds at a certain basal rate in cardiac muscles at rest, or near rest, and this rate is increased when the beat interval becomes less than a certain critical duration.

The discrepancies in the results concerning the dependence of CG action on stimulation frequency might be explained on the basis of certain differences in the experimental conditions (523). The strict beat-dependence was found at low temperatures (30°C or less) (352, 544, 552), whereas at high temperatures (35°C or more) time-dependence on the onset of CG action was found to be predominant (144, 277, 278, 279, 523). It is assumed that the basal rate of onset of CG action (in the absence of myocardial activity) is rather slow and, in addition, is highly temperature-dependent, which makes it detectable at high temperatures but not at low temperatures. At lower temperatures this basal process might be overshadowed by the frequency-dependent component. Moran (353), who initially thought that the species difference might be the cause of the divergent results, also agrees that there is a component in the inotropic CG action which is independent of contraction (488). The weight of available data clearly indicates that digitalis actions may develop even in the absence of any myocardial activity. On the other hand, there is no doubt that the rate of onset of CG action is greatly enhanced by increased myocardial activity.

B. Frequency-dependence of CG action: Magnitude of CG effect

The influence of stimulation frequency on the magnitude of the positive inotropic action of CG on isolated heart muscle preparations is comprehensively described by Koch-Weser and Blinks (279).

It has long been known that the degree of the positive inotropic effect of CG is dependent on the frequency of contraction. In earlier days it was thought that the higher the stimulation frequency, the more effective was the action of CG (544). However, more recent studies by Tuttle and Farah (515) showed that, in both atrial and ventricular preparations, CG increased the absolute contractile force more at lower frequencies than at higher frequencies. Also, a detailed study on cat heart muscle strips showed that the absolute changes in contractile strength, induced by CG in lower concentrations, were nearly the same at all frequencies, which resulted in a parallel shift of force-frequency curve to the higher level (278). When rather high concentrations of CG were employed, the positive inotropic effect was nearly independent of the frequency, bringing the contractile strength to the same maximal value over the whole range of frequencies (139, 187, 189, 278, 283, 515). These results indicate that high concentrations of CG abolish frequency-dependent changes in contractile strength, such as staircase phenomena, rest potentiation, poststimulation potentiation, and post-extrasystolic potentiation (139, 209, 211, 278, 283), whereas low concentrations of CG have only minor influences on these phenomena (144, 278, 279).

This divergent effect of frequency on the CG action was explained by Kruta *et al.* (283) on the basis that the inotropic actions of digitalis and those induced by

frequency changes are additive at a given condition, and that the maximal contractile force is determined by the intrinsic limit of contractility ("ceiling of contractility").

There are, indeed, surprising similarities between the positive inotropic effect produced by CG and that brought about by changes in the frequency of contraction in experimental conditions, where an increase in frequency of stimulation is accompanied by an increase in contractility. As with CG, the positive inotropic effect of an increase in frequency is characterized by an increase in the rate of tension development, a decrease in the time to peak tension, and a reduction of the total duration of contraction (1, 29, 275, 404, 408). The underlying process seems to be an increase in the intensity and rate of onset of active state, combined with a reduction of the duration of the active state (111, 114), which is quite similar to the CG action on these parameters.

Therefore, the synergistic effect produced by CG and increasing the frequency of stimulation is in keeping with the hypothesis of a common mechanism of action present in these two inotropic interventions. The ceiling of contractility, quoted above, just would mean the highest degree of activation of the contractile system that can be achieved under the prevailing conditions (110). A further analysis of the nature of the cellular mechanism by which digitalis and a frequency change may alter the contractile strength was made by Koch-Weser *et al.* (276) and Koch-Weser and Blinks (278, 279). They postulated that the maximal tension of an isometric twitch is determined both by the strength of the "rested state" contraction (contractile response uninfluenced by previous beats, an equivalent of the basal contractile ability of the myocardium) and by a positive and a negative inotropic influence produced by preceding beats. This merely descriptive procedure allowed them to characterize the positive inotropic effect of CG further, and to come to the conclusion that CG act only through their effect on the rested state contraction. The time course of production and decay of the positive and negative inotropic factors is thought to be largely uninfluenced by CG. It is not yet possible to interpret this effect of CG in terms of influence on a particular cellular process or processes, but it may be concluded that the basic action of CG is not at all dependent on the frequency of contraction or on the myocardial activity. It seems established that the altered condition responsible for the positive inotropic effect of CG can be fully induced in the completely quiescent muscle. Therefore, these drugs appear to improve the contractile strength by a favorable influence on the conditions determining the basal contractile ability of the myocardium.

C. Potassium-dependence of CG action

The tolerance to digitalis toxicity was found to be increased by elevated serum potassium levels (333, 444), and the minimal lethal dose of CG is increased by high potassium (203). It was demonstrated in cat papillary muscles (145) and rabbit ventricular strips (57) that high $[K^+]_0$ delayed or even prevented the appearance of the toxic action of ouabain.

With regard to the mechanism of K^+-digitalis antagonism, the following

observations are noteworthy. Ebert *et al.* (104) demonstrated in digitalized dogs that raising K^+ serum level by potassium infusion for 150 min reduced the digoxin content of heart muscle. Cohn *et al.* (74) observed an increased accumulation of digoxin in hearts of mice with potassium deficiency as compared to animals with a normal serum potassium level. These observations indicate that the level of potassium determines the amount of digitalis bound to the heart; *i.e.*, high potassium decreases digitalis binding to heart.

In reviewing the relationship between the toxic effect of CG and potassium, it appears best to explain the relationship on the basis of CG inhibition of Na^+-K^+ ATPase. It is known (as will be seen in section X) that CG inhibit Na^+-K^+ ATPase, and high K^+ antagonizes the inhibitory effect. The inhibition of this enzyme by CG appears to result in toxic effects such as arrhythmias and conduction blockade (see section XI). Since high potassium antagonizes the inhibitory effect of Na^+-K^+ ATPase by CG, high potassium would be expected to antagonize or prevent many of the toxic effects of CG.

In contrast to the marked influence of potassium on the toxic effects of CG, several investigators have reported no significant influence of altering $[K^+]_0$ on the inotropic effect of CG (145, 318, 320). However, it should be noted that the relationship between the effects of potassium and those of CG on the contractility of heart is difficult to interpret, since myocardial contractility depends on the algebraic sum of all positive and negative inotropic influences at the same time. Under control conditions, changes in $[K^+]_0$ appreciably modify the myocardial contractile force: lowered $[K^+]_0$ may increase the contractile strength (75, 188, 320); moderate changes of normal $[K^+]_0$ do not appreciably alter the cardiac tension developed (75, 145, 165, 172, 320); high levels of $[K^+]_0$ usually produce negative inotropic effects (143, 433, 487). Thus, the change in $[K^+]_0$ itself may markedly modify the basic contractility of the heart, and this may overshadow the possible specific interference of K^+ with the positive inotropic effect of CG. Cohn *et al.* (75) reinvestigated the interaction of potassium and the positive inotropic effect of ouabain on guinea pig heart preparations, taking the variation in baseline tension caused by potassium into consideration. He observed that in higher concentrations (around 10 mM) potassium significantly diminished the positive inotropic effect of ouabain. However, alteration of $[K^+]_0$ in a lower range (2–5 mM), which influenced toxic effects, did not influence the inotropic effect of CG. Similar differential actions of potassium on the therapeutic and toxic effect of ouabain were reported (145). In dogs, it was found that the application of potassium suppressed the ouabain-induced arrhythmias but did not alter the positive inotropic effect (556). These data indicate that the toxic effect of CG is always antagonized by K^+ but the inotropic effect is not necessarily influenced by K^+. This dissociation of the influence of $[K^+]_0$ on the toxic effect and that on the inotropic effect has an important implication. It may suggest that the toxic effect is caused by the K^+ loss from heart muscle but the inotropic effect is not.

D. Sodium-dependence of CG action

The maximal extent of the positive inotropic effect of CG on atrial and ventricular muscle preparations was reduced, and the onset of the inotropic action

in frog hearts was delayed in low Na^+ medium (57, 128, 403, 504). In most of these experiments the ratio $[Ca^{++}]/[Na^+]^2$ was kept constant so as to maintain the same contractile force during the experiments (403, 504). Thus, both $[Ca^{++}]$ and $[Na^+]$ were lowered during investigation of the inotropic effect of CG. But lowering $[Ca^{++}]$ at a constant $[Na^+]$ did not have as pronounced influences on CG action as lowering $[Na^+]$ at a constant $[Ca^{++}]$ (403, 504). Furthermore, it was shown in studies on spontaneously beating rabbit atria that a reduction of $[Na^+]$ was able to prevent the occurrence of ouabain-induced arrhythmias (509); but once toxicity developed, lowering of $[Na^+]_0$ could not reverse this toxicity.

This sodium-dependence of the positive inotropic action on heart muscle resembles the Na^+-dependence of the inhibitory action on the active sodium transport system at the cell membrane and on isolated Na^+-K^+ ATPase. It has been repeatedly asserted that the inhibitory action of CG on the membrane ATPase is facilitated by sodium and reduced by lowering the sodium concentration (411, 412, 414). In addition, the ouabain-induced inhibition of sodium efflux in squid axons develops approximately 10 times faster in sodium-containing medium than in sodium-free medium (14), and the rate of K^+ turnover in quiescent cat papillary muscles is not affected by ouabain (10^{-5} M) in the absence of sodium, whereas it is inhibited in the presence of sodium (380). These parallelisms make it tempting to assume (*e.g.*, 57, 504, 509) that the Na^+-dependence of CG action on cardiac function may be related to the inhibition of Na^+-K^+ ATPase of the cell membrane. But this relationship may also reflect differences in the uptake (or distribution) of CG by (or in) heart muscle in different concentrations of extracellular $[Na^+]$ (see section VI).

E. Dependence of CG action on $[Ca^{++}]_0$

From the early work of Ringer (419) on the action of ions in the bathing fluid on the function of the frog's heart, it is known that Ca^{++} ions play a dominant role in the regulation of the contractile force of heart muscle. The contractile force is gradually increased with increasing Ca^{++} concentrations up to a maximal value; then if the Ca^{++} concentration is further increased, arrhythmia, contracture, and finally systolic cardiac standstill develop. On the other hand, the reduction of calcium concentration $[Ca^{++}]_0$ reduced the contractility gradually and resulted finally in uncoupling excitation-contraction (420). This dependency of the myocardial contractile force on the Ca^{++} concentration of the incubation medium has been confirmed repeatedly on different heart preparations of various species (216, 354, 355, 551).

This effect of calcium on the myocardial contractility is very similar to that of the CG, except that the Ca^{++} effect is much faster (319, 354, 551). Furthermore, increasing calcium concentrations abolish the staircase phenomenon, as do CG (355). On account of these similarities in their cardiotonic action, the dependency of the CG effect on the calcium concentration of the medium has been studied extensively, and in most of these experiments a synergistic or additive action could be demonstrated. This kind of interaction was investigated as early as 1910 by Werschimin (545), who observed that an increase in the calcium concentra-

tion of the incubation medium hastened the onset of ouabain-induced contracture in isolated hearts of frogs. More detailed studies with this preparation by Clark (71), Konschegg (280), and Loewi (329, 330) revealed that the incidence of toxic signs of CG was increased by a simultaneous increase in $[Ca^{++}]_0$ and was reduced by removal of Ca^{++}. This interdependence was repeatedly confirmed and extended to other heart muscle preparations (*e.g.*, 12, 15, 63, 132, 137, 433, 434, 435, 439, 540, 554) and could be demonstrated *in vivo* (477). When calcium is applied with CG, the toxic effects of CG are enhanced, arrhythmias and other changes in the ECG are developed earlier or at lower doses of CG than without additional calcium, and the lethal doses of CG are reduced (158, 159, 160, 324, 340, 347, 477). This synergistic effect of Ca^{++} and CG has been observed repeatedly in the clinic (21, 24, 25, 32, 36, 106). In addition, it is known that intravenous injections of calcium, alone, may produce changes in the electrocardiogram which are very similar to the effects of CG (217, 218, 325, 327, 470). On the other hand, CG-induced arrhythmias can be prevented or abolished by producing a hypocalcemic state by the administration of either EDTA or citrate (22, 47, 73, 79, 116, 182, 239, 241, 357, 379, 425, 439, 478, 480, 498, 499, 501). Furthermore, the protective effect of a low $[Ca^{++}]_0$ level against the toxic CG action is well known (12, 182, 501). Some of these reports claim that lowering $[Ca^{++}]_0$ specifically antagonizes the CG-induced arrhythmias (*e. g.*, 47, 116, 239). However, similar antagonistic effects of low $[Ca^{++}]_0$ on other kinds of arrhythmias were observed (*e.g.*, 73, 480, 498, 499) which suggest that some basic properties of the myocardial cell membrane are influenced by low $[Ca^{++}]_0$.

All of these observations indicate a close relationship between the cardiotoxic actions of CG and of Ca^{++} ions. But observations in patients (*e.g.*, 24, 25) and in isolated heart muscle preparations (57, 128, 204, 216, 433, 434, 435, 551, 554) also demonstrated a synergism of the therapeutic actions of CG and calcium. Important observations on the quantitative nature of this synergism were made on frog hearts by Salter *et al.* (433, 434, 435). The contractile force of this preparation was reduced stepwise by lowering $[Ca^{++}]_0$, and the amount of CG needed to restore the contractile force to normal was determined. They found a linear relationship in a certain concentration range of CG. Calculations showed that the effect of 1 mol of ouabain was equivalent to the effect of 6400 mol of Ca^{++} under these conditions.

With regard to the nature of the Ca^{++}-CG synergism, Loewi (329, 330) and Burridge (54) suggested a sensitization of the heart to the action of calcium ions. According to this hypothesis, the CG have no direct influence on the contractile force, are ineffective in the absence of calcium, and only potentiate the response of the heart to calcium ions. Such a hypothesis, however, is only a description of certain aspects of the calcium-CG interaction and does not explain the underlying mechanism.

Another possible mechanism of the calcium-CG synergism was postulated on the assumption that both calcium and CG bring about similar changes in the electrolyte milieu of the myocardial cell (451). The suggested common denominator was a disturbance of the myocardial potassium turnover. This kind of inter-

action appears to be rather doubtful because it has been demonstrated that, unlike high CG concentrations, moderately increased Ca^{++} concentrations do not cause a loss of potassium from either heart muscle (402, 497), skeletal muscle (167, 560), or erythrocytes (338). However, in some reports, an interference of increased $[Ca^{++}]_o$ with the sodium and potassium turnover in cardiac muscle (244, 274), in skeletal muscle (468), and in erythrocytes (242) has been stated. Another explanation of the Ca^{++}-CG synergism which has been favored more recently (271, 551) is that the CG primarily influence the myocardial calcium metabolism, which results in a higher intracellular concentration of calcium and leads to an increase in calcium availability. This possible interrelationship has been studied in detail during recent years and will be discussed in section X.

V. SOME FACTORS THROUGH WHICH DIGITALIS MAY CAUSE THE INOTROPIC EFFECT INDIRECTLY

In the past, some investigators have suggested that the inotropic action of CG may be mediated indirectly through 1) a release of catecholamines or 2) prevention of the loss of a cardiotonic component.

A. Possible release of catecholamines by CG

Tanz (505) found that pretreatment of cats with reserpine resulted in an alteration of response of isolated papillary muscles to ouabain, and this was confirmed by others in subsequent studies (133, 485). Thus, papillary muscles isolated from reserpine-treated cat hearts were found to be less sensitive or even insensitive to the inotropic effect of ouabain. Combined treatment with dichloroisoproterenol and reserpine had a greater inhibitory effect on the ouabain inotropism but did not influence the response of muscles to calcium inotropism. From these data it was proposed that ouabain either increases the force of contraction by release of catecholamine or requires the presence of catecholamine for its inotropic effect.

Both reserpine and guanethidine, which release catecholamine from the heart muscle, also reduced the ouabain-induced augmentation of cat Langendorff preparations. In the isolated auricle preparation from rabbits pretreated with reserpine or guanethidine, a positive correlation was found between the effect of ouabain and the catecholamine content of heart (88, 322). In the intact anesthetized dog, the prior administration of propranolol markedly reduced ouabain-induced augmentation, as well as toxicity (332). Also, pronethalol, an adrenergic blocking agent, antagonized the inotropic effect of ouabain in frog hearts (89). From these data a suggestion was made that at least part of the positive inotropic effect of ouabain is mediated through activation of beta-adrenergic receptors or the release of catecholamines (89, 507).

However, a host of workers could not observe either a correlation between the catecholamine content of heart and the response to digitalis, or the influence of adrenergic blocking agents on digitalis action (37, 38, 105, 559, 578, 580). Tanz *et al.* (506) attempted to explain the failure to observe an effect of catecholamine depletion on the glycoside-induced augmentaion by suggesting that the degree of depletion was not sufficient. These authors found that partial repletion of cate-

cholamines to 8.5% or more appeared to be sufficient to restore the response of the heart to ouabain. However, denervation of the heart decreased the catecholamine content to less than 5%, yet the denervated heart responded to digitalis just as strongly as the nondepleted hearts (486). Also, the ventricular catecholamine content at the time of peak CG effect was not different from the control value before the addition of CG (506).

In view of numerous results which show no correlation between the catecholamine content of the heart and the response to digitalis in the denervated heart, as well as in other well controlled experiments, it is difficult to see how the action of CG is mediated through release of catecholamines. Also, effects of catecholamines on the contractile mechanics and electrophysiological parameters of heart muscle are different from those of CG. Thus, Tanz and Marcus (507) concluded that the presence of a certain amount of catecholamines may be needed for manifestation of the full effect of CG, but release of catecholamine itself is not likely to be the mechanism of CG action.

B. Prevention of loss of a cardiotonic factor

Another suggestion for the mechanism of action of CG has been put forward on the basis of results obtained from gas-perfused hearts. Small cat hearts were perfused with a warm, moist mixture of 95% O_2–5% CO_2, and hearts were found to beat more strongly and fail much more slowly than did hearts perfused with a substrate-free Krebs solution (141). In later work with the gas-perfused heart, Gabel *et al.* (142) found that intermittent administration of small volumes of Krebs solution caused progressive failure of the heart, accompanied by loss of a protein from the hearts. This protein material was found to have a cardiotonic action. Preperfusion with small amounts of digitalis glycosides prevented loss of the protein material during periods of subsequent washing procedure and at the same time protected hearts against failure. The implication is that CG may exert their therapeutic effect by slowing or preventing the loss of an "intrinsic inotropic substance" from failing hearts (142). In view of lack of further information on the subject, it is difficult to evaluate such a mechanism for CG at the present time. Another interesting suggestion linking the CG action to the protein has been made by Hamrick and Fritz (190), who postulated that CG increase myocardial contractility by promoting the synthesis of specific proteins. The evaluation of this finding is difficult at present, in view of the lack of further information.

VI. LOCALIZATION OF CG IN HEART MUSCLE

The effectiveness of drug action depends not only on the drug reaching the target organ, but also on its interaction with appropriate receptors. Since so-called nonspecific binding of drug to tissues is not uncommon, the localization of a drug at certain sites does not necessarily mean that the mode of action of the drug is mediated at that site. However, the elucidation of the binding sites of drugs woud be meaningful in the understanding of the drug action if other pharmacological parameters could be correlated with the localization of drugs. For this reason,

attempts have been made in the past to localize the site of CG binding in heart muscle. In discussing this problem, the specific binding of CG to Na^+-K^+ ATPase, which is rather well defined, will be deferred to a later section, and the present discussion will be concerned mainly with uptake and distribution of CG.

A. Uptake and subcellular distribution of CG in heart muscle cell

The intracellular sites of CG binding in cardiac muscle have not yet been clearly elucidated. For a long time it has even been questionable whether CG are able to enter the cell, or if they are reacting only at the outer surface of the cell membrane. In earlier experiments, it could only be shown that a certain portion of CG was extracted from the perfusion medium by the heart, and this portion was presumably bound to the heart (42, 156, 198, 341, 471, 489, 490). More recently, however, the uptake of CG by cardiac muscle cell has become increasingly evident; but uncertainty still prevails as to the exact subcellular site or localization of the drug.

Three different experimental approaches to this problem have been utilized: 1) measurement of the exchange kinetics of CG in intact heart muscle preparations; 2) autoradiography combined with light or electron microscopy; 3) differential centrifugation of heart muscle homogenates. These techniques, however, have their limitations, and while they are listed above in the order of increasing conclusiveness, there is also an increasing risk of inducing artifacts.

Kinetic studies on the uptake and release of ^3H-labeled CG (ouabain, digoxin, digitoxin) on isolated atria of guinea pigs have shown a difference in the rate as well as the extent of uptake of these agents by heart muscle (286, 287, 288). Only a small amount of ouabain was found to be bound to the tissue, and it was suggested that most of this bound fraction was reacting with "specific" receptors directly involved in producing its positive inotropic action (286, 287, 288). On the other hand, the atria accumulated a far greater amount of digitoxin (and digoxin). Since the amount of digitoxin used produced a relatively small inotropic effect, most of the binding was considered to be pharmacologically unimportant. From this type of study, however, one cannot expect to derive exact information on the localization or nature of the subcellular binding sites, because it is difficult to assign a kinetically distinct fraction to a particular cellular compartment.

In a similar way, the results obtained on the distribution of radioactive labeled CG (digoxin-^3H, digitoxin-^3H) in hearts of various species (frog, cat, dog, cattle, rat), by the use of autoradiography combined with light microscopy (534) or electron microscopy (77, 134, 476, 482, 513) are not conclusive with regard to the exact subcellular binding sites of these agents. An unavoidable disadvantage of this method would be the possible or even probable redistribution and loss of bound CG during histological preparation (fixation, embedding, etc.) (see 77, 134, 513), which may obscure the true original pattern of subcellular distribution. In addition, interpretation of results is limited by the relatively poor discrimination of closely related structures. In most of the early experiments the CG were thought to be localized to the greatest extent in the myofibrils, especially at the A-band area (77, 134, 476, 513, 534), and to a lesser extent in the cell membrane

(77). It has to be considered that in most of these studies the hearts were used relatively early after application of the drugs to the animals, or soon after perfusion of the isolated heart with ^{3}H-digitalis; thus, a good deal of nonspecifically localized CG (such as localization in the extracellular space) might have been present. Furthermore, the close association of the radioactivity to the sarcomeres does not prove that the contractile elements themselves are the binding sites. Because of resolution limits, investigators were unable to distinguish between structures as closely related as myofibrils and the tubules of the sarcoplasmic reticulum (see 134). Indeed, in later work which employed tritiated digoxin and autoradiography, the radioactivity was almost exclusively localized in the sarcotubular reticulum system (96, 134, 482).

Any attempt to localize the subcellular binding sites by means of differential centrifugation has to consider the unavoidable loss and redistribution of the CG present in different compartments of the heart muscle cell in the course of homogenization and separation procedures. But some of the artifacts can be corrected if dual control experiments are performed (96, 97, 135, 147). The earliest studies of this kind (198, 489, 490), however, were performed under unfavorable conditions and did not allow for such corrections. Early studies on isolated hearts of guinea pigs revealed a distinct binding of radioactive digitoxin to the particulate fraction of the heart muscle homogenate (198), but no clear preference for any one of the subcellular fractions could be observed. In rat hearts, most of the CG was found "unbound" in the supernatant (489, 490), and it was also found that, on the whole, CG show a very low binding affinity to rat hearts (96). In subsequent studies, it became known that the microsomal fraction had the highest affinity for CG (96, 97, 135, 147). The ultracentrifugal distribution pattern of ^{3}H-digoxin in bullfrog hearts indicated a close association of this agent with the microsomal fraction (131). This was further supported by the extensive investigation of Dutta *et al.* (96, 97) in guinea pig hearts. Following perfusion of isolated hearts with the same concentration (10^{-7} M) of several CG, a different amount of each drug was taken up by hearts, resulting in total myocardial contents which varied by a factor of 100. In all hearts, by far the highest concentration of the drug was associated with the microsomal fraction (97).

In an attempt to elucidate further the relationship between the site of localization and the site of action of CG, the time course of uptake (and release) of labeled ouabain and digitoxin by various subcellular components of guinea pig hearts was followed in relation to development and disappearance of the positive inotropic effect. Simultaneously, concentrations of a drug in subcellular fractions were compared with pharmacological effects (135, 147) of that drug at corresponding moments. Ouabain and digitoxin were markedly different in the rate and extent of uptake, or release by or from the heart; but, with both drugs, the highest concenteration was found in microsomal fractions, and the best correlation between the drug concentration and the positive inotropic effect was found in this fraction. The second best correlation between the concentration and the effect was obtained in the debris fraction, the first sediment, which is supposed to contain mainly nuclei, membranes, and myofibrils. The mitochondrial fraction showed a small

uptake of CG and a very poor correlation between binding and the effect. These findings make it difficult to determine whether both the microsomal and membrane fractions contain "specific" binding sites, or if the aforementioned best correlation with the microsomal fraction is due to contamination by membrane fragments in this fraction, or if the debris fraction owes its good correlation to contamination by the sarcoplasmic reticulum fragments. It should be mentioned in this connection that Schwartz *et al.* (453) found in dog and calf hearts that ^{3}H-CG was bound to membrane ATPase specifically, and very little to the sarcoplasmic reticulum fragments. This finding may suggest, then, that the distribution of ^{3}H-CG in the microsomal and nuclei fraction, as observed above, may be an expression of the ^{3}H-CG binding to membrane fragments. On the other hand, it is possible, as reported by Marks and his coworkers (96, 97, 98), that CG may be actively transported into the cell and bound specifically to the sarcoplasmic reticulum (see next section).

B. Factors influencing myocardial uptake of CG

The mechanism of uptake and binding of CG is still obscure, but recently some information has become available with regard to the environmental factors influencing these processes, and the evidence suggests the existence of "specific" transport mechanisms for CG.

Ebert *et al.* (104) reported a reduced uptake of digoxin-^{3}H by dog hearts in hyperkalemic animals. On the other hand, Cohn *et al.* (74) observed an increased accumulation of digoxin in hearts of mice with K^+ deficiency. Similarly, Harrison and Brown (193) found a retention of digoxin-^{3}H in hearts of K^+-depleted rats 24 hr after injection. In addition, Harrison and Wakim (194) described an inhibition of digoxin-^{3}H binding to dog myocardium of about 50% when animals were subjected to hemodialysis against a low sodium medium (resulting in a reduction of the serum sodium from 146 to 69 mEq/liter, and the myocardial sodium from 34 to 20 mEq/kg).

These observations were confirmed and extended further in the extensive investigations of Marks and his associates (97, 98) on isolated perfused hearts of guinea pigs. It was demonstrated that the cardiac uptake of ouabain-^{3}H from perfusion medium is not based on a simple diffusion process, but is governed by a particular transport mechanism [as previously suggested by Wilbrandt (550) on the basis of the uptake kinetics]. The uptake of ouabain-^{3}H was inhibited by other CG and dependent on the ionic environment. The presence of sodium was required, and the extent of uptake was directly related to the sodium concentration of the perfusion medium. Furthermore, the uptake of ouabain-^{3}H was found to be inversely proportional to the potassium concentration of the perfusion medium. On the basis of these relationships, it was suggested that transport of (polar) CG into the intracellular compartment may be intimately linked to the N^+-K^+ pump (97). Lowering the calcium concentration had only a minor influence (reduction) on the rate of CG uptake (98, 286). The inhibitory effect of potassium on ouabain uptake might be explained by the assumption that at the external surface of the cardiac cell membrane, potassium and CG may compete

for the transport, as indicated by the competition between digoxin and potassium for binding in the isolated purified Na^+-K^+ ATPase (343). However, the interference of potassium with the binding of CG on intracellular binding sites cannot be excluded, because Marks and his associates observed that potassium antagonized the binding of CG to sarcoplasmic reticulum isolated from beef hearts (97, 98).

The Na^+-dependence of the ouabain-^3H uptake (98) meets a fundamental requirement of a carrier-mediated transport system (83, 86) and gives further support to the postulate that the transport mechanism operative in the heart muscle membrane may be similar to that proposed in the intestine, where the uptake of CG requires sodium and is an active process (295, 296, 297). The participation of both sodium and potassium in CG transport suggests strongly the involvement of Na^+-K^+ ATPase in the myocardial transport mechanism of CG proposed by these workers (97).

The observation (97) that the intact heart of guinea pig distinguished between ouabain and dihydroouabain in the uptake process, whereas sarcoplasmic reticulum fragments isolated from beef heart do not, led to the proposal (97, 147) that the differential uptake mechanism in the surface membrane of the heart muscle cell for different CG determines the cardiotonic potency of a given CG; namely, the easier the penetration of a drug into the cells, the higher the cardiotonic potency. This observation is in good agreement with the assumption of a specific transport mechanism for CG in the heart muscle, as previously suggested by Wilbrandt (550).

The existence of specific transport mechanisms for CG has quite recently been demonstrated for 1) convallatoxin on small intestine (295, 296, 297), on the basis of the saturability of this process, the inhibition by other CG, and Na^+-dependence; and 2) ouabain on liver slices (284), on the basis of saturability and inhibition by anaerobic conditions, metabolic inhibitors, or other CG. This transport mechanism seems to be predominant in the uptake of the more polar CG, whereas nonpolar CG are taken up mainly by diffusion processes, as indicated by studies in small intestine (173) and in guinea pig hearts (see 98). The presence of an active transport mechanism for CG in the cardiac cell membrane, if it is proved to exist, implicates the possibility that CG may have an intracellularly localized site of action.

VII. ACTION OF CG ON CONTRACTILE PROTEINS

One of the mechanisms by which CG may increase the contractile force of the heart muscle is through their direct effect on the contractile proteins.

A. Influence of CG on polymerization of g-actin

The influence of CG on the physicochemical behavior of isolated actin has been investigated by several workers. The most prominent property of this protein studied under *in vitro* conditions is the polymerization process, namely, the process of transformation of (globular) g-actin into (fibrillar) f-actin occurring in the presence of ATP at a certain ionic strength of incubation medium.

The rate of polymerization of the cardiac actin was found to be accelerated by ouabain or digitoxin (2 to 4 $\times$ 10^{-6} g/ml), whereas skeletal muscle actin was completely uninfluenced by even higher concentrations of CG (10^{-4} g/ml) (228). This stimulatory effect of CG on actin polymerization was confirmed by others (479, 569, 579). But this action of CG seems to be rather unspecific because it is absent in fresh actin preparations (285, 569) and it has no correlation with the cardiotonic potency of different glycosides (569). In aged actin preparations, the same degree of acceleration of polymerization could be induced by the same concentration (20 μg/ml) of cardioactive (*e.g.*, scillaren A, emicymarin) or inactive CG (*e.g.*, hexahydroscillaren, alloemicymarin). In addition, it has been shown more recently that polymerization of highly purified g-actin was uninfluenced by digitalis (236). According to these observations, the causal relationship between the CG-induced changes in physicochemical properties of actin and the cardiotonic action of these drugs can be excluded.

B. Influence of CG on enzymatic and physicochemical properties of myosin

Both inhibition and stimulation of the myosin ATPase activity by ouabain in different concentrations have been reported (334); also, changes in the physicochemical properties of myosin prepared from animals treated with digitoxin have been observed (372). However, several investigations employing myosins of different degrees of purity, and actomyosins isolated from normal as well as failing hearts, indicate that various CG in a wide concentration range do not influence ATPase activities of these preparations, whether calcium in different amounts is present or not (235, 251, 335, 407, 495). The ATPase activity of actomyosin isolated from normal or failing hearts was also not influenced by CG (235, 251, 335, 407, 495). Thus, no convincing evidence of a direct action of CG on myosin has been established.

C. Influence of CG on viscosity of actomyosin solution

The simultaneous extraction of both actin and myosin or the combination of these protein moieties after extraction results in formation of a protein complex, actomyosin, due to the high affinity between the proteins. Actomyosin solutions show an abnormally high viscosity. The addition of ATP results in the initial dissociation of actin from myosin, and this is manifested by the initial fall of viscosity. This initial fall is followed by gradual rise of viscosity due to ATP splitting by free myosin ATPase. The influence of various CG on these viscosity changes of skeletal muscle actomyosin was studied in detail by Waser (531, 532, 533, 535). He observed slight dose-dependent decreases in the viscosity of actomyosin solutions in the presence of CG, but this effect was unrelated to the cardiotonic potency of the drug tested. It went roughly parallel to the degree of protein binding of these substances (530, 531, 532, 533). Other authors (19, 210, 254), however, have failed to detect any influence of CG on the viscosity of actomyosin solutions prepared from cardiac muscle, and no conformational change occurred in the presence of CG as measured by the optical rotatory dispersion method (254).

Some years ago, Waser (531, 532, 533) measured the influence of CG on the viscosity fall caused by rotation movements, so-called thixotropy, in solutions of skeletal muscle actomyosin. In these experiments, cardioinactive CG increased the thixotropy of actomyosin solutions, whereas cardioactive CG reduced it, the degree of reduction depending on their concentrations and on their different cardiotonic potencies. The cardiotonic CG increased the binding of potassium and calcium ions to myosin simultaneously. However, a recent careful study from the same laboratory reported that CG did not influence the viscosity and the thixotropy of purified natural actomyosin or reconstituted actomyosin from calf hearts (237).

D. Influence of CG on actomyosin ATPase activity

In some earlier works the ATPase activity of myosin A was found to be stimulated by CG (107, 183). Recently, superprecipitation and ATPase activity of cardiac myosin B were found to be accelerated by ouabain or digoxin (233, 495). However, this stimulating effect of CG on myosin B ATPase was observed only under certain experimental conditions (233). The ATPase activity of actomyosin obtained from rat hearts after treatment of the animals with digoxin (0.5–2.0 mg/kg) was found to be greater than control (190), although a rather crude actomyosin preparation was used. Previously, it was reported that the actomyosin ATPase activity of failing hearts was found to be less than normal (349), and Kako and Bing (247) suggested that CG, together with Ca^{++}, may remedy this defect in failing hearts.

However, many investigations on normal and on failing hearts showed that the ATPase activity of cardiac actomyosin, either natural or reconstituted (251, 252, 254, 349, 396, 495, 535), and that of myofibrils (166), are not influenced by CG in the presence of different calcium concentrations in medium. CG, even in high concentrations, had no effect on ATPase activities of myofibrils obtained from normal and failing hearts (166). Thus, no consistent effect of CG on the actomyosin ATPase was observed.

E. Influence of CG on "contractile" behavior of actomyosin preparations

The "contractility" of isolated actomyosin can be studied in media of appropriate ionic strength and composition by measuring the shortening of threads or bands of actomyosin, or by recording the turbidity changes of actomyosin solutions, or by determining the volume change of actomyosin after addition of ATP. These reactions are supposed to be at least qualitatively equivalent to the contraction of the actomyosin system in intact muscle preparations.

The "contraction" of actomyosin gels induced by ATP was not influenced by digitoxin in a concentration as high as 10^{-4} M (285). More detailed studies on actomyosin threads, however, revealed an accelerated rate and an increased degree of shortening in the presence of ouabain (421). This effect was more pronounced in cardiac than in skeletal muscle actomyosin (35). However, Kako and Bing (247) observed no effect of CG on shortening of actomyosin bands prepared from normal and failed human hearts, although increased shortening by CG was

noted when CG was given in the presence of Ca^{++} (1 mM). The superprecipitation of natural actomyosin was found to be accelerated by CG in low concentrations in the range of 10^{-7} to 10^{-9} M (349, 495). But recent experiments on more highly purified or reconstituted actomyosin failed to demonstrate any effect of CG over a wide range of concentrations (10^{10}–10^{-4} M) on both the rate and the extent of superprecipitation (237, 251).

F. Influence of CG on glycerol-extracted muscle preparations

In studies of the enzymatic and physicochemical properties of isolated contractile proteins, the highly ordered structural organization of these proteins in the intact muscle cannot be reproduced. Glycerol-extracted fibers, on the other hand, were found to preserve the original structural organization as the living muscle (199, 537). The results obtained in these preparations with CG, however, are conflicting. In cardiac muscle preparations (166, 302, 496) no direct effect of CG could be demonstrated. On the other hand, ouabain was reported to increase the extent of ATP-induced shortening of cardiac or skeletal glycerol-extracted fibers if calcium (1 mM) or creatine phosphate was present in the medium (108, 109, 302). However, more recent experiments on glycerol-extracted fibers could not confirm this stimulating effect of CG on the tension development in the presence of creatine phosphate (120).

G. Summary

According to these observations on isolated contractile proteins, no conclusive evidence for a direct interference of CG with the physicochemical or enzymatic properties of the actomyosin is available. Most of the results obtained more recently on better purified (or even reconstituted) contractile protein preparations do not support the hypothesis of a direct influence of CG on the contractile protein system. Most of the previously found influences of CG on the actiomyosin system proved later to be due to the effect of CG on some moieties contaminating the contractile protein. The positive influence of CG on contractile proteins is often observed in experiments employing less prurified preparations (natural actomyosin, partially extracted fibers, etc.) which might be contaminated by mitochondria (127), microsomal particles (536), or some unknown cellular components. These observations tend to support the view that CG do not exert a direct influence on the contractile proteins but, rather, modify the properties of this system secondarily through the primary influence of CG on other subcellular components accompanying the contractile protein.

VIII. INFLUENCE OF CG ON MYOCARDIAL ENERGY METABOLISM

Numerous studies in the past indicated that CG have a variety of effects on many metabolic aspects of the heart. The metabolic effect of CG in relation to the inotropic action has been previously discussed in detail by many authors (265, 299, 315, 371, 430, 563). However, no definitive importance has been assigned to a known metabolic effect of CG with regard to the causal relationship to the positive inotropic effect. Recent findings indicate that many of the metabolic

actions of CG which were formerly thought to be the primary effect of CG appear to be indirect phenomena secondary to alteration of intracellular electrolytes caused by CG.

The most convenient method to study drug actions on the myocardial energy metabolism is to study their influences on the oxygen consumption. Most studies on quiescent heart muscle preparations, such as ventricle slices from different species, showed that the oxygen consumption of these muscles was increased by CG in the low concentration range and was decreased in the high concentration range (53, 85, 90, 130, 146, 212, 213, 298, 300, 367, 429, 432, 503, 562, 563). This biphasic effect of CG on the oxygen consumption of heart muscle preparations was not observed in heart muscle homogenates or in isolated cardiac subcellular components (90, 146, 292, 301, 428, 432, 562, 563). The metabolic effect of CG is not observed in muscle preparations which lost the structural integrity, and this suggests that the cellular structure is required for the metabolic effect of CG (407, 492, 562, 563, 570). The magnitude of CG action on the myocardial oxygen consumption is dependent on the age of the animals (320, 568) and on the oxygen consumption levels prior to CG addition (130, 292). Furthermore, the increase in oxygen consumption caused by CG is most pronounced in media containing glucose (85, 130, 212, 292, 562); is less when lactate or oxaloacetic acid (212, 213) is used as the substrate; and is not observed when pyruvate, acetate, malate or succinate is used as the substrate (212, 213). An important aspect is the dependence of the metabolic effect of CG on the ionic composition of the incubation medium. Thus, the more calcium or the less phosphate in the medium, the greater is the CG-induced increase in oxygen consumption (85, 90, 130, 212, 292). This suggests the possibility that these ions are involved in the CG action.

The influence of CG on the oxygen consumption of beating preparations has been extensively studied, with the use of isolated hearts, heart-lung preparations, and hearts *in situ*. It has been shown that CG increase the work output of failing hearts to a greater extent than the oxygen consumption, and sometimes the oxygen consumption is even reduced after CG administration (26, 28, 157, 162, 175, 176, 184, 233, 381, 386, 431, 442). It is clear, then, that CG increase the mechanical efficiency of failing hearts. On the other hand, some studies in nonfailing hearts reported that CG increased the oxygen consumption without altering the cardiac output (26, 115, 348), indicating that the mechanical efficiency was rather decreased by CG in these nonfailing hearts. These opposite effects of CG on the mechanical efficiency observed in failing and nonfailing hearts were probably due to the difference in the hemodynamic states in those hearts.

In failing hearts CG have dual effects. The direct effect of CG is to increase the oxygen consumption of heart through its direct effect on the muscle cell as described above. The indirect effect of CG is to decrease the oxygen consumption secondarily through their primary effect of reducing the end-diastolic pressure and the heart rate of failing hearts. It is known that the action of any drug which decreases heart size is to increase the mechanical efficiency. In nonfailing hearts, no indirect influence brought by reduction of the rate and the end-diastolic pressure is present, and only the direct effect of increasing the oxygen consump-

tion is observed after the administration of CG. On the other hand, in failing hearts the indirect effect of CG interferes with the direct effect. This explanation is supported by the finding that the oxygen consumption of the heart was always increased by CG when the end-diastolic pressure was kept constant (82, 265, 350, 386, 424).

The analysis of results from studies on isolated heart muscle preparations such as papillary muscles is easier, since the complication resulting from hemodynamic changes is eliminated. It was found in cat papillary muscles that CG increase the contractile tension without increasing the oxygen consumption in the early inotropic stage of the drug action (298, 301). However, more recent investigations employing improved polarographic techniques showed that the oxygen consumption of heart muscle strips was increased simultaneously with an increase in the contractile tension following the administration of CG in the low concentration range, and the oxygen consumption was decreased with the toxic concentration of CG (70, 76, 168, 264, 362, 401, 467, 474, 475).

From these observations it may be concluded that CG stimulate both the myocardial oxygen consumption and the contractile force simultaneously at the inotropic stage. It appears that the increase in oxygen consumption is the result of the increase in cardiac work induced by the inotropic effect of CG.

The numerous investigations on the effect of CG on cellular levels of various intermediary metabolites proved to be unfruitful. For example, measurements on the glycogen and lactate content of heart muscle as influenced by CG did not reveal consistent results (62, 118, 397). Furthermore, measurements of the steady state concentrations of these metabolites do not permit the analysis of CG action on the metabolism, since the turnover rate of a metabolite may change without alteration of the steady level of the metabolite. More precise information has been obtained by measuring the oxidation rate of substrates by use of radioactively labeled substances. Wollenberger (564, 565, 567) showed that oxidation of glucose and lactate in heart muscle slices was increased by ouabain in large doses, and simultaneously lactate production and glucose extraction were reduced. Kien *et al.* (257, 258) observed on dog hearts perfused *in situ* that digoxin induced a preferential oxidation and utilization of glucose over other substrates. Crevasse and Schipp (84) and Kreisberg and Williamson (281) reported that CG increased the rate of uptake and oxidation of glucose and the rate of lactate formation, and these CG effects were found to be dependent upon the calcium concentration in the medium. Kien *et al.* (257), using nonutilizable galactose in dog myocardium, showed an increase in membrane permeability to sugars caused by CG. This effect is similar to the insulin action. Also, CG were reported to have a glucagon- or epinephrine-like action on glucose oxidation and glycolysis (281). However, ouabain was found to have no effect on the phosphorylase activity (345). The effect of CG on isolated metabolic enzyme systems was studied, and results have been uniformly disappointing. Many isolated enzyme systems participating in anaerobic glycolysis or in transfer of electrons through the respiratory chain have been investigated, and none of these enzymes tested was found to be definitely influenced by CG (90, 209, 407, 443, 458, 562, 563). It is clear that effects of CG

on the oxidative and other metabolic pathways of heart muscle preparations, such as slices, are not observed when the drug is applied to heart muscle preparations which do not have the structural integrity. These findings strongly suggest that the stimulatory effect of CG on metabolism may be mediated by some other primary effect of CG which requires the integrity of cellular structure.

A considerable amount of investigation was carried out on the effect of CG on the energy-rich phosphate content of heart muscle. The myocardial content of high energy phosphates was found to be uninfluenced (31, 140, 563, 566), reduced (397), or increased (174, 491, 541) under the influence of CG. Thus, Wollenberger (563) came to the conclusion that the myocardial content of these substances is most probably not changed by therapeutic concentrations of CG. This conclusion was mainly based upon results obtained in his studies on dog heart-lung preparations (566), which have been confirmed by more recent experiments on guinea pig atria and cat papillary muscles (138, 315, 316). On the other hand, toxic concentrations of CG were found to decrease the myocardial content of creatine phosphate and ATP and to increase the content of ADP, AMP, and inorganic phosphate (31, 138, 174, 563, 566). These findings suggested that CG in toxic concentrations might uncouple oxidative phosphorylation. The uncoupling of oxidative phosphorylation by dinitrophenol was potentiated by high concentrations of CG (177, 178), and Lee *et al.* (313) observed an increase in oxygen consumption of mitochondria in the absence of phosphate acceptors in mitochondria (which is considered to be a sign of uncoupling of oxidative phosphorylation) isolated from CG-intoxicated hearts. However, in the same work (313) it was shown that CG added *in vitro* into the incubation medium containing isolated cardiac mitochondria did not influence oxidative phosphorylation. Many subsequent investigations seem to have established that CG, even in high concentrations, do not influence oxidative phosphorylation in isolated mitochondria (177, 178, 292, 309, 313, 388, 429, 563, 566). Only Lamprecht (289) and Goldschmidt and Lamprecht (161) observed a stimulation of the oxygen consumption of isolated mitochondria by CG, but the oxidative phosphorylation was not influenced by high concentrations of ouabain. On the other hand, it was reported that oxidative phosphorylation is somewhat uncoupled in mitochondria isolated from failing hearts, and this coupling was restored in the presence of ouabain in media (289, 290, 291). However, other investigators observed only very slight changes (if any) in the phosphorylation rate of mitochondria isolated from failing hearts (387, 454, 502), and, in addition, the above "phosphorylation-restoring" effect was also produced by dihydroouabain, which has very weak inotropic action (291). Thus, it must be concluded that CG do not influence the energy-rich phosphate metabolism of the myocardium.

From these negative results of CG on the energy production and energy conservation phases, most workers have come to the conclusion that the effect of CG on the energy metabolism of heart must reside in the energy utilization phase (140, 563). However, very little is known about the effect of CG on this phase of energy metabolism.

It is clear from the above discussion, then, that CG do show definitive in-

fluences on many metabolic functions of intact heart muscle, and yet none of these metabolic effects could be clearly demonstrated in isolated enzyme systems or in heart muscle preparations in which the integrity of cellular structure is lost. This suggests strongly that the metabolic effects of CG are mediated through some other primary effects of CG which require the presence of cell membrane for manifestation. Since CG are known to have a profound effect on movement of electrolytes through the cell membrane (see section X), it is reasonable to suspect that the metabolic effects of CG are secondary manifestations of the primary CG effect on the electrolyte movement. There are accumulating data supporting the view that many metabolic effects of CG are indeed mediated by alteration of the cellular electrolyte movement brought about by CG.

The stimulation of the oxygen consumption and the glucose oxidation in cardiac muscle tissue by CG requires the presence of extracellular calcium in the medium (130, 281). This is not limited to the cardiac tissue. In a calcium-free Ringer's solution, exposure of brain slices to ouabain in concentrations inhibitory to potassium uptake caused a decrease in the rate of oxygen uptake (547). However, the same treatment of brain slices in a medium containing calcium resulted in as great a loss of tissue potassium as that occurring in the absence of calcium, but the rate of oxygen uptake was not decreased (571) and was even increased (452). Thus, the stimulating effect of CG on the oxygen consumption of heart and brain slices is highly dependent on the calcium concentration of incubation medium (34, 452, 500, 548, 563, 570). In studies on brain cortex slices, Tower (510) found that ouabain increased Ca^{++} intake in brain cortex slices only in the presence of sufficient calcium in the incubation medium, and most of calcium gained by the tissue in the presence of ouabain was found in mitochondria. Similar results were obtained in experiments on the intact heart tissue (385). These findings suggest that an increase in the cellular calcium content or an intracellular mobilization of bound calcium induced by CG (see section XII) results in an accumulation of calcium by mitochondria, and this causes the increase in oxygen consumption observed with CG. It is known that presence of Ca^{++} stimulates an active calcium transport mechanism of mitochondria which is coupled to oxidative processes (43, 66, 87, 91, 519). Thus, the increase in tissue oxygen consumption brought about by CG may be mediated through the influence of CG on calcium movement, resulting in an increase in the free intracellular calcium concentration.

Another piece of evidence suggesting the role of calcium in modulating cellular metabolism was provided by Ozawa *et al.* (377, 378). These authors have shown that a preparation of phosphorylase b kinase, prepared by a modification of Krebs method, is activated at very low concentrations of calcium ion. Even at 5×10^{-7} M calcium, the kinase was found to be activated markedly. These findings indicate strongly that calcium ion has an additional important role as the trigger of glycolysis. If CG increase the intracellular free calcium concentration, then, according to the above finding, glycogenolysis should be stimulated by CG. Thus, some of the effects of CG on the glucose metabolism mentioned previously may be mediated by CG-induced alteration of calcium movement. It is also possible that the disturbance in sodium and potassium movement caused by CG

through their inhibitory effect of Na^+-K^+ ATPase may cause secondarily many metabolic alterations (547).

In conclusion, then, no definitive metabolic effect of CG has been demonstrated in isolated and well defined enzyme systems. Most of the metabolic effects of CG observed in intact tissue preparations appear to be the secondary sequence of primary effects of CG on cellular electrolyte movement.

IX. EFFECT OF DIGITALIS ON MOVEMENT OF MYOCARDIAL POTASSIUM AND SODIUM

A. Effect of CG on total myocardial potassium and sodium content

Calhoun and Harrison (56) found, in dogs, a marked decrease in myocardial potassium content after administration of CG in toxic and lethal doses but did not observe changes after treatment with lower "therapeutic" doses. Numerous subsequent investigations have confirmed the effect of toxic doses of CG, namely, loss of potassium (and sometimes gain of sodium) after application of rather high doses of CG. The potassium loss following large doses of CG was confirmed in patients (208, 398) and on isolated heart muscle preparations and hearts *in vivo* of dogs (8, 9, 10, 16, 27, 30, 49, 51, 132, 191, 207, 400, 464, 573, 574); cats (303, 316, 561); rabbits (3, 58, 185, 245, 222, 395, 516, 561); guinea pigs (171, 196, 226, 267, 268, 269, 497, 521, 522); rats (374, 448); frogs (249); turtles (538); and pigeons (249).

In contrast to this well established toxic effect of CG on the myocardial potassium and sodium content, the effect of low, "therapeutic" concentrations of CG is still a matter of some controversy. In some studies where it was reported that exposure to "low" concentrations of CG resulted consistently in reduction of the cellular potassium content of heart muscle (*e.g.*, 374, 395, 438, 574), the concentrations of CG actually used were rather high.

A recent work employing radioactive potassium for the purpose of determining the total cellular potassium content (nonflux) is noteworthy. Müller (356), using sheep trabecular muscle and ^{42}K, followed changes in the intracellular content of potassium. The muscle preparations were equilibrated with ^{42}K-containing solutions, and the radioactivity in the muscle after 5-min washout periods was considered to be equivalent to the intracellular potassium content. Ouabain reduced the radioactivity in a dose-dependent manner, which was taken as evidence that CG in low concentrations induce a loss of cellular potassium. These measurements, however, reflect only the total amount of ^{42}K present in the muscle preparations, which included the activity in the intra- and extracellular space. From the data presented it was obvious that the control muscles were beating very poorly, whereas the ouabain-treated preparations showed a markedly increased contractility (up to 300% and more). Thus, the removal of the extracellular radioactivity during the 5-min washout period (low flow rate) could have been different under these conditions. This fact was not taken into consideration in interpretation of data by Müller.

Numerous studies indicate that if concentrations of CG used are clearly within the low therapeutic range, neither a significant potassium loss nor sodium gain

occurs. This was shown *in vivo* in dogs (56, 464), cats (39, 538), rabbits (151, 185), and pigeons (480). It was also shown *in vitro* in isolated heart muscle preparations from rabbits (516, 561), guinea pigs (227, 267, 269), cats (315, 316), and turtles (538). In some experiments even an increase in myocardial potassium or a decrease in myocardial sodium content or both were found with CG concentrations which ordinarily produced only the positive inotropic action (39, 58, 185, 267, 269, 351, 516). The reduced cellular potassium content of failing hearts was found to be increased both in man (72) and in dogs (30) following digitalis therapy. However, the last effect may have been due to changes in the extracellular space (caused by edema), since no appropriate correction was made for this complicating factor in those studies.

One may claim that CG cause very small changes (less than 3% of the intracellular potassium concentration) which are of physiological importance but cannot be detected because of the insensitivity of measurement (188). This claim, however, is only relevant to those reports where no significant changes were observed, but they do not hold in all other cases where significant *increases* in the myocardial potassium content and *decreases* in the sodium content were observed. Hagen (185) found a slight increase in myocardial potassium content in rabbit hearts treated with low concentrations of digilanid, and Boyer and Poindexter (39) observed a significant increase in potassium content of cat hearts treated with CG (up to the moment when ECG changes occurred). More recently, Tuttle and his coworkers (516, 561) have reported a reduction of the spontaneous potassium loss from isolated rabbit atria under the influence of positive inotropic (50–60% increase in contractile force) concentrations of ouabain, resulting in higher potassium contents of CG-treated preparations than of control preparations after a certain period of exposure to the drug. Quite similar results were obtained by Holland *et al.* (227) on isolated perfused guinea pig hearts, and by Carslake *et al.* (58) on isolated rabbit atria.

These data indicate that positive inotropic actions may be produced by low doses of CG without concomitant loss of intracellular potassium or gain in intracellular sodium or both. At least, the repeated observations of opposite changes cast doubt as to the causal relationship between changes in the intracellular potassium and sodium concentrations and the mechanism of the positive inotropic action of CG.

Another aspect to be considered is the magnitude of changes of intracellular sodium and potassium necessary to bring about the alteration of contractility. Even if we assume that a slight loss of myocardial potassium or a gain of intracellular sodium or both might occur at the inotropic stage of CG action, still the question remains whether such net changes in undetectable amounts are sufficient to cause the positive inotropic effect. For example, an increase in contractile force obtained by exposing cat papillary muscles to potassium-free medium is accompanied by a considerably greater change in intracellular ion concentrations (loss of potassium, gain of sodium) than that which is associated with the toxic stage of ouabain action, and yet the extent of the maximal inotropic effect induced in potassium-free medium is still below that seen with ouabain at its maxi-

mal inotropic stage (316). This quantitative aspect strongly supports the thesis that the primary mechanism of the inotropic effect of CG is not related to an alteration of the myocardial sodium and potassium content.

B. Influence of CG on potassium efflux from heart muscle

The subject of potassium loss from the heart following the administration of digitalis has been studied extensively by means of determination of the A-V difference, since a change in potassium content is much more easily measured than a change in sodium content in the perfusion medium.

A recent reinvestigation of the relationship between the potassium balance and the contractility of the heart revealed that an increase in contractility following injection of acetylstrophanthidin directly into the coronary inflow of dog hearts is accompanied by concomitant potassium loss from the hearts, and this potassium loss was dose-dependent (440). A similar degree of potassium loss was observed during the rate- or pressure-induced homeostatic autoregulation (69, 440). From these findings it was concluded that a loss of myocardial potassium and an increase in myocardial contractility are not entirely independent variables (440). However, caution should be exercised with regard to postulating an intimate causal relationship between potassium loss and contractility changes. The measurement of the A-V potassium difference revealed that in some experiments hearts were continuously losing or gaining potassium without any evidence of concomitant change in contractility (49, 51, 351, 441). Also, some agents causing a negative inotropic effect, such as quinidine (50), induce potassium loss. On the other hand, the negative inotropic effect of pentobarbital is not accompanied by any change in potassium balance (50).

There are numerous investigations reporting potassium loss from the heart caused by digitalis. Wood and Moe (574) observed an increase in venous potassium concentration in heart-lung preparations of dogs treated with different doses of lanatoside, the potassium loss being roughly related to the increase in contractility. Quite similar results were obtained in patients (208) and intact dogs (27, 191, 400) following injection of CG, the loss being transient with low doses and severe with toxic doses (10, 16, 131, 422). Experiments by Areskog (8, 9) on dog heart-lung preparations with fast acting CG in doses causing arrhythmia within 10 to 30 min showed a correlation between the magnitude of potassium loss and the decrease in contractility, which was an early sign of toxicity. The slight loss of potassium before development of toxicity did not appear to be related to the positive inotropic action. Vick and Kahn (522) did not observe appreciable effects of ouabain on the myocardial potassium balance of guinea pig hearts in concentrations producing positive effects up to 80% of the maximal effect. Only larger doses producing more than 80% of the maximal therapeutic effect or toxic effects caused a progressive potassium loss which was quantitatively related to the development of toxic signs. Similar observations, namely, no potassium loss with inotropic doses of CG but potassium loss with toxic doses, were reported on guinea pig hearts (246, 497). Thus, it appears that toxic doses of

digitalis invariably cause K^+ loss. However, the positive inotropic effect of CG is not always accompanied by potassium loss.

In contrast to reports on potassium loss, there are some reports on gain of myocardial potassium following administration of digitalis. Brown *et al.* (51) investigated the potassium balance of dog hearts injected with the therapeutic dose of dihydroouabain and found a slight and transient potassium loss immediately after injection, which was followed sometimes by a potassium uptake. Smaller doses (2 to 3 times positive inotropic threshold doses) induced an uptake of potassium into the myocardium, and only higher "pretoxic" doses causing occasional cardiac irregularities produced consistently a loss of myocardial potassium (49). Quite similar biphasic observations were made with veratridine (a CG-like acting compound) under the same experimental conditions (351). This substance proved to be capable of producing either a gain or a loss of myocardial potassium. The potassium loss was always associated with ventricular irregularities and occasionally occurred shortly before development of toxicity. When cardiac arrhythmia did not occur, the positive inotropic effect was associated with a gain of potassium by the heart, and then it could be observed that successive small doses were followed each time by an increase in both contractility and potassium uptake by the heart. In patients treated with digitalis, Gonlubol *et al.* (163) were unable to detect changes in myocardial potassium balance, and Nawata (361) also did not obtain consistent effects of digitalis under similar conditions. Also, effects of digitalis on myocardial potassium balance were found to be inconsistent in guinea pigs (246, 521, 522).

A survey of the above results indicates that CG cause potassium loss consistently in toxic doses. Digitalis in doses which cause the positive inotropic effect does not cause potassium loss or causes occasional potassium loss, which is inconsistent. Sometimes the inotropic effect of digitalis is accompanied by even a significant gain of myocardial potassium. These results obtained by the measurement of A-V difference of potassium content do not support the view that a negative potassium balance of heart determines the strength of contraction in digitalis action.

Before coming to a conclusion on the relationship between the potassium balance and the inotropic effect of digitalis, a critical evaluation of data which show a loss of potassium with no sign of toxicity should be made. It is true that potassium loss may occur without toxic manifestations following digitalis, or the negative change in myocardial potassium balance may precede any sign of toxicity in some experiments. However, the interpretation of data should be made after due consideration is given to the possible bias built in the experimental conditions. In experiments on isolated perfused guinea pig heart, Greeff *et al.* (171) observed that even very small doses of digitalis were able to cause loss of myocardial potassium. Moreover, the magnitude of this effect was correlated with the positive inotropic effect of the drug, and it increased markedly with doses inducing toxicities. In their experiments drugs were injected into the perfusate. This may be the source of error in the interpretation. In most experiments employing the injection technique, drugs were injected in a small volume of concen-

trated solutions to avoid the artifact caused by volume dilution. For example, Greeff *et al.* (171) injected, within 10 sec, 1 to 40 μg of ouabain into the perfusion fluid at 1 cm distance away from the heart. This procedure would cause a rather high concentration of ouabain entering coronary arteries. Calculated on the basis of the reported perfusion rate of 3 to 4 ml per min, the concentration of the drug in the perfusate would be at least more than 10^{-6} M, and the transient local toxic effect consequent to the high concentration may be manifested as a transient loss of potassium from the affected tissue. The subsequent distribution of the injected dose over the entire heart would result in a low overall effective concentration inducing only a positive inotropic effect, without any sign of toxicity. In this sequence of events, the transient potassium loss cannot be related to the inotropic effect of the injected drug. This behavior pattern of distribution of injected drug may explain the only transient potassium loss observed in their experiments and the biphasic time course of potassium loss or gain observed by many other investigators immediately after injection of digitalis in low doses (8, 27, 51, 171, 207, 208, 398, 400, 521, 522).

Some attempts to dissociate experimentally the positive inotropic effect of CG from the disturbance in K^+ balance have been made. Insulin was reported to produce complete dissociation of these CG effects on dog hearts (27, 399); however, more recent work did not confirm this (440). On the other hand, administration of CG in combination with diphenylhydantoin, which is known to possess an antiarrhythmic action in CG toxicity (447), resulted in a dissociation of these two effects. In dog hearts *in situ*, continuous intravenous infusion of CG caused a steady loss of potassium, both in the presence and in the absence of diphenylhydantoin; however, the rate of potassium loss was more rapid with digitalis alone than with the combination of both drugs, whereas the time courses of increase in the maximal rate of rise of pressure development induced by CG were the same in the presence and in the absence of diphenylhydantoin. Furthermore, during the inotropic stage of CG the application of diphenylhydantoin converted the potassium loss to an uptake of potassium by the heart without affecting the progress of the inotropic effect. From these observations, it has been concluded that the CG-induced loss of myocardial potassium is not directly associated with the inotropic action of these drugs and that the antagonistic effect of diphenylhydantoin on the toxic actions of CG results only in an increase of the therapeutic range of CG action (447).

The weight of data presented leads one to believe that the positive inotropic action of digitalis can be induced without any concomitant negative change in potassium balance, or even with a slight gain in myocardial potassium. At most, it seems certain that a loss of myocardial potassium is not an absolute prerequisite for development of the positive inotropic action.

C. Influence on transmembrane potassium and sodium turnover

A more precise analysis of the CG influence on the myocardial electrolyte metabolism consists of the measurement of fluxes by use of radioactive tracers. This technique, combined with determination of the cellular ion content, gives

rather accurate values for the extent and the rate of transmembrane electrolyte turnover and allows calculation of the electrolyte balance.

Systematic investigations on the influence of two distinctly different categories of drug concentration, namely, inotropic and toxic, have shown that two different effects on the transmembrane potassium turnover and on the intracellular ion content in isolated auricles of guinea pigs and rabbits are produced by these two concentrations (267, 268, 269, 517). Tuttle *et al.* (517) found that small doses of ouabain resulted in an increase of potassium influx and a decrease of potassium efflux in isolated beating atria from rabbits. With higher doses, opposite effects were observed. Klaus *et al.* (267, 268, 269) demonstrated a temporary increase of potassium influx in isolated, electrically stimulated auricles of guinea pigs with a low concentration of digitoxigenin, which increased the contractile tension up to 160 % over control. Potassium efflux and total cellular potassium content were found to be unaffected (probably because of the insensitivity of the method used for potassium determination) under this condition, but the intracellular sodium concentration was significantly reduced. Toxic concentrations had similar effects during their initial transient positive inotropic action, potassium influx being stimulated even more than at low digitoxigenin concentrations, but only for a short period. The potassium influx was not reduced until toxic signs developed. During the toxic stage of CG action potassium influx was decreased, potassium efflux was increased, and the combination of both effects resulted in a marked loss of cellular potassium with a concomitant increase in sodium content. An increase in potassium efflux has been demonstrated on auricles of guinea pigs by the use of ^{42}K and high concentrations of CG (181, 267, 268, 517).

Although the mechanism of the initial increase of potassium influx at low inotropic concentrations is not clear, it is almost certain that the later inhibition of K^+ influx brought about by the toxic range of CG is due to its well known inhibitory effect on Na^+-K^+ ATPase. The initial increase of this inhibition of potassium influx at high concentrations of CG has been almost universally observed and clearly established. In contrast to this established effect of high concentrations of CG, the influence of the drug in the low inotropic range has been found by most investigators to be variable and inconsistent. However, it seems to be clear that CG in the low dose range does not inhibit potassium influx significantly. In numerous investigations where CG has been employed in low inotropic concentrations, there has been no influence on potassium fluxes in rabbit auricles (395), in chick embryo hearts (273), and in amphibian ventricles (232) and auricles (331). However, in most of the above experiments toxic concentrations of digitalis were found to cause inhibition of potassium influx.

D. Summary

On the basis of the aforementioned numerous data obtained on the content, balance, and turnover of cellular sodium and potassium, it can be agreed unanimously that in the heart muscle cell CG, in high concentrations producing toxicity, increase and decrease the intracellular concentrations of sodium and potassium, respectively. This effect is the result of interference with the active

sodium transport mechanism by the CG inhibition of Na^+-K^+ ATPase. This interpretation is based on the well established effect of CG on the Na^+-K^+ ATPase enzyme and the concept of passive distribution of potassium secondary to the active sodium transport. There are many experimental data supporting this interpretation, for example, that potassium influx in cat papillary muscles was markedly decreased by high concentrations of ouabain in the presence of $[Na^+]_0$ but not in the absence of $[Na^+]_0$ (380). On the other hand, the influence of CG in the inotropic range is not definitively established, except that no significant inhibition of potassium influx occurs. There is some evidence that positive inotropic effects may be connected, at least temporarily, with a stimulation of the active ion transport processes (resulting in a low $[Na]_i$ and high $[K]_i$), and it might be tempting to assume that this action is based on the repeatedly observed stimulation of the Na^+-K^+ ATPase activity by CG in low concentrations or during the initial period of action of CG in high concentrations (see section X). But direct proof for this sort of relationship is still lacking, and this interpretation of the data is an unorthodox interpretation (which cannot be excluded, however, on the basis of the data available).

It is apparent, then, that no consistent change in intracellular potassium content can be related to the inotropic action of CG. Similarly, the relationship between the inotropic action of CG and the intracellular sodium content is unknown at present, mainly because of methodological limitations in measuring the sodium content in the cell. Some reports indicate that in the low concentration range of CG even an increase in potassium content or decrease in intracellular sodium content or both may occur during the inotropic stage of CG (39, 58, 185, 267, 269, 351, 516). Thus, it must be concluded that the inotropic effect of CG is caused neither by an increase in intracellular sodium content nor by a decrease in potassium content.

X. EFFECT OF CG ON NA^+-K^+ ATPASE

A. Inhibition of Na^+-K^+ ATPase by CG

This subject was reviewed in this Journal by Glynn in 1964 (155). The present review will consider principally literature since that time but will occasionally refer to pre-1964 material.

The discovery of Na^+-K^+ ATPase by Skou in 1957 (472) laid the foundation for an enzymatic explanation of the sequence of events involved in the supply of energy for active sodium transport. It is now established that Na^+-K^+ ATPase is intimately identified with the active transport system of sodium from the heart muscle cells (among others, see Glynn, 155). It seems certain that the sarcolemma contains this Na^+-K^+ ATPase, but the location of this enzyme in other membrane structures of heart cells is not clear. Wollenberger (570), on the basis of a histochemical study of heart muscle, reported that the sarcotubular system does not have Na^+-K^+ ATPase. Since the sarcotubular system is considered to be an extension of the plasma membrane and is important for the inward conduction of membrane excitation, the lack of Na^+-K^+ ATPase, if it is confirmed, has important implications in the interpretation of excitation-contraction coupling.

Since the microsomal fraction from homogenates of cardiac muscle also contains the Na^+-K^+ ATPase activity, the presence of this enzyme in the subcellular structure giving rise to microsomal particles, namely, the sarcoplasmic reticulum (187, 466), was and still is suspected. However, the elegant work by Wallach and his associates (248, 526, 527) on Ehrlich ascites carcinoma cells showed that, on further density gradient separation of the microsomal fraction, this enzymatic activity was found to coincide with the peak of distribution of surface cell antigens, as determined by an immunological technique with intact cells. This indicates that small fragments of plasma membrane sediment collected with "microsomes" provide the major Na^+-K^+ ATPase activity of microsomal fractions of cell homogenates. The exclusive localization of this enzyme on the cellular membrane is interesting from the mechanistic viewpoint of CG action, as will be discussed in section XII. It is now well known that the Na^+-K^+ ATPase activity is specifically inhibited by the CG (5, 6, 23, 155, 272, 411, 412, 453, 456). In fact, the inhibition of Na^+-K^+ ATPase by CG is so specific that the intimate correlation between the inhibition of the active sodium transport and that of the enzyme is one of the main reasons to believe the identity of these two functions. By virtue of this specific inhibitory effect, the CG have become the most important ally of biologists exploring the frontiers of membrane transport in search of the mechanism of utilization of energy for uphill transport work.

The Na^+-K^+ ATPase isolated from heart muscle of digitalis-sensitive species (*e.g.*, guinea pig) is detectably inhibited by low concentrations of CG (in a range of 10^{-9} M), with an appropriate half-maximal inhibition occurring at a concentration of 5×10^{-7} M (391). The reported sensitivity of Na^+-K^+ ATPase to CG ranges rather widely, from more than 10^{-4} M to 10^{-8} M. The complicating factors are conditions of experiment, such as the time of exposure to the drug, species difference, and temperature. As discussed by Glynn (155), most of the experiments on inhibition of ATPase activity by drugs have not taken into consideration the fact that the longer the exposure to the drug, the more inhibitory a low concentration may become. However, in general, it appears that the concentration necessary for 50% inhibition of Na^+-K^+ ATPase is of the order of 10^{-6} to 10^{-7} M, which is on the borderline between so-called therapeutic and toxic concentrations of inotropic action seen in the heart muscle from digitalis-sensitive species.

The species variations in the inhibitory effect of cardiotonic glycosides have parallel relationships to species variations in the inotropic effect (413). In the same species, among different CG preparations the potency of inhibitory effect on Na^+-K^+ ATPase parallels that of inotropic effect (312, 411, 414). Both the inhibitory effect on the ATPase and the inotropic effect of CG are antagonized by potassium and are reduced by reduction of extracellular sodium concentration or the temperature of incubation (412).

These many parallelisms appear to suggest a causal relationship between the inhibition of Na^+-K^+ ATPase activity and the inotropic effect of CG. However, caution is required for the interpretation of these parallelisms. For example, with regard to the potassium antagonism, it is found that the presence of potassium decreases binding of the CG to the membrane (392, 456) and decreases the

penetration of CG into the cell (96, 97). Therefore, if digitalis causes its inotropic effect through an effect on any function of the cellular membrane or intracellular membranes, then the effect of potassium, which decreases binding of the drug to the membranes, would decrease the efficacy of the drug. Because of the multitude of membrane functions, the site of the drug effect would not necessarily be limited to Na^+-K^+ ATPase. Also, the similar effects of a reduced sodium concentration on both the CG effect on the ATPase and that on the contractility should not necessarily be considered to favor a causal relationship between these two effects. Niedergerke (363) and others (*e.g.*, 365, 366) showed clearly that a reduction of sodium in the medium favors calcium influx into the heart muscle and increases the contractility. If digitalis causes its inotropic effect by increasing the intracellular free calcium concentrations, then the cardiotonic effect of digitalis would be relatively decreased when there is already an increase in calcium influx. The reduction in the inhibitory effect of CG on Na^+-K^+ ATPase due to the reduction of sodium in the medium is entirely due to the fact that less sodium in the medium decreases the degree of sodium activation of the ATPase, resulting in a reduced ATPase activity on which the CG could act. It is obvious, then, that a decrease in the inotropic efficacy of digitalis at low sodium concentrations in the medium does not necessarily support the belief that the inhibition of sodium transport is related to the cardiotonic effect.

A similar situation also applies in the case of the temperature effect. Lowering temperature is known to produce the prolongation of the plateau phase of action potential, which favors an increase in calcium influx (418, 572), and also markedly decreases the outward movement of calcium (311). Such effects would be expected to result in an increase in contractility at lower temperatures, and under this condition the inotropic effect of digitalis would be less. Therefore, the fact that the low temperature decreases both the inhibitory effect of CG on Na^+-K^+ ATPase and the inotropic effect does not necessarily indicate a causal relationship between these effects of digitalis.

It also has been reported that a certain percentage of the Na^+-K^+ ATPase activity is inhibited in cardiac tissue biopsied at the time when the inotropic effect of CG is manifested in dog experiments *in vivo* (4, 23), and this finding has been taken as a proof for the causal relationship. However, any quantitative comparison between an effect *in vitro* and an effect *in vivo* requires caution. A considerable safety factor is built in *in vivo*. It is known that the diuretic effect of carbonic anhydrase inhibitors does not become evident until well over 90% of this enzymatic activity is inhibited. Most enzymes operate with a fraction of their maximal capacity *in vivo*. On the other hand, the enzymatic activity *in vitro* is usually at its maximum, because of the experimentally constructed environment favorable for its activity. Thus, it is completely unknown how much inhibition of Na^+-K^+ ATPase activity *in vitro* is required in order to conclude that interference with the sodium-potassium exchange has actually occurred *in vivo*. In the case of Na^+-K^+ ATPase inhibition, there is no known basis to justify a comparison between data *in vitro* and *in vivo*. In addition, it is reported that there is no such quantitative correlation between the inhibition of the Na^+-K^+ enzyme *in vitro* and the inotropic effect *in vivo* (370). Thus, the above parallelisms

between the inhibitory effect of Na^+-K^+ ATPase and the inotropic effect might well occur even if the CG caused the inotropic effect by mechanisms other than inhibition of Na^+-K^+ ATPase. However, the above discussion should not be viewed as denying the possibility of a causal relationship between the ATPase inhibition and the inotropic effect. The purpose is merely to point out that the analysis of the mechanism should be based on tangible evidence, and not on apparently favorable data which may be interpreted in other ways.

B. Binding of CG on Na^+-K^+ ATPase

Recent studies have contributed much to the elucidation of the mechanism of inhibition by the CG. Albers *et al.* (6) showed that, in Na^+-K^+ ATPase of the electric organ, dissociation of the drug-enzyme complex does not occur to a significant extent. Schwartz and others (6, 453) showed that there is a parallel relationship between the degree of binding and the extent of inhibition of Na^+-K^+ ATPase activity by CG in enzymes obtained from electric organ (6) and from dog heart (453). Conditions which favor the formation of the phosphorylated intermediary, such as the presence of magnesium, ATP, and sodium, also favor binding of CG; and the presence of potassium, which dephosphorylates the enzyme, also reduces CG binding (344, 392, 453, 456). However, the binding of CG does not necessarily involve the phosphorylated form, since many conditions, such as the presence of magnesium alone, without ATP, also increase glycoside binding (456). It is now thought that CG bind nonphosphorylated enzymes also, but only slowly (392). That the binding of CG to Na^+-K^+ ATPase is allosteric in nature (*i.e.*, involved conformational changes of the enzyme) is indicated by the influence of various ions on binding, and also by phosphorylation of digitalis-bound enzyme by inorganic phosphate (6, 456). Recent studies on fluorescent probe and circular dichroism spectra also support the view that digitalis causes the conformational change of Na^+-K^+ ATPase (326, 455).

Schwartz and his coworkers found that a drug concentration of approximately 3×10^{-8} M tritiated ouabain was needed for half-maximal binding to the Na^+-K^+ ATPase isolated from calf and dog hearts (344, 453, 456), and that the inhibition of ATPase paralleled the degree of binding. Only cardioactive glycosides compete with each other for the binding sites of Na^+-K^+ ATPase, indicating the specificity of the binding.

C. Molecular mechanism of interaction between CG and Na^+-K^+ ATPase

The sequence of enzymatic processes involving sodium transport and hydrolysis of ATP has been studied by many workers. From studies with a sodium-dependent nucleotide phosphokinase (123, 124), the following scheme of Na^+-K^+ ATPase was proposed by Albers (5):

$$E_1 + \text{Mg-ATP} \overset{Na^+}{\rightleftarrows} E_1 \sim \text{P} + \text{Mg-ADP} \quad (1)$$

$$E_1 \sim \text{P} \overset{Mg^{++}}{\rightleftarrows} E_2 - \text{P} \quad (2)$$

$$E_2 - \text{P} \overset{K^+}{\rightleftarrows} E_2 + \text{P} \quad (3)$$

$$E_2 \rightleftarrows E_1 \quad (4)$$

E_1 is the enzyme with inwardly oriented cation sites of high sodium affinity, while E_2 is the rearranged enzyme with outwardly oriented cation sites of high potassium affinity. The vectorial force of transport is introduced through reactions 2 and 4, which may represent allosteric conversions. Post *et al.* (392) proposed the following scheme of the enzymatic steps and CG binding sites, on the basis of their results (392) and those of Albers (7, 123, 124):

$$\begin{array}{lcl} \text{ATP} + E_1 & \overset{Na^+,\,Mg^{++}}{\rightleftarrows} & E_1 - \text{P} + \text{ADP} \\ \quad \Updownarrow Mg^{++} & & \downarrow Mg^{++} \leftarrow \text{blocked by NEM} \\ \text{P}_i + E_2 & \overset{K^+}{\leftarrow} & E_2 - \text{P} + \text{H}_2\text{O} \\ \text{Ouabain} \downarrow & & \downarrow \text{Ouabain} \\ \text{P}_i + \text{Ou}.E' & \overset{Mg^{++}}{\rightleftarrows} & \text{Ou}.E' - \text{P} + \text{H}_2\text{O} \end{array}$$

E_1 and E_2 indicate two forms of Na^+-K^+ ATPase in the normal sequence. E' indicates the form of the enzyme when combined with and inhibited by ouabain. E_1-P, E_2-P and E'-P indicate the corresponding phosphorylated forms. The first step indicated on the top, ATP + $E_1 \leftrightharpoons E_1$-P + ADP, namely, phosphorylation of E_1 to E_1-P, requires sodium and low concentrations of Mg^{++} and is a reversible step. The second step, E_1-P $\rightarrow E_2$-P, requires high concentrations of Mg^{++}, and this step is irreversible and is blocked by N-ethylmaleimide (NEM). The third step, E_2-P + $H_2O \rightarrow P_i + E_2$, represents the dephosphorylation of Na^+-K^+ ATPase and requires the presence of K^+. In NEM-treated enzyme, where no reaction proceeds beyond the first step, ouabain did not interfere with phosphorylation of the enzyme. This indicated that ATP + $E_1 \rightarrow E_1$-P + ADP was not influenced by ouabain. Addition of ADP in the NEM-treated enzyme decreased the level of phosphorylated enzyme because of the reaction E_1-P + ADP $\rightarrow$ ATP + E_1, and this ADP effect was not influenced by ouabain. This indicated that E_1-P + ADP $\rightarrow$ ATP + E_1 was not influenced by ouabain and suggested that ouabain did not bind with E_1-P. On the other hand, in enzymes not treated with NEM, ouabain inhibited the dephosphorylation reaction of E_2-P $\rightarrow E_2$ + P_i in the presence of K^+. This indicated that ouabain bound E_2-P. Further experimental results suggested that the structure of E_2 was altered when the enzyme was bound to ouabain. This enzyme with altered structure is represented as E', and the possible binding sequence of ouabain to E_2-P concluded from the experimental results is suggested by arrows in this scheme. As can be seen in this scheme, it is proposed that the pharmacological action of ouabain involves binding to only one form of Na^+-K^+ ATPase, namely, E_2-P.

D. Stimulation of Na^+-K^+ ATPase by CG

Repke (410) was the first to note a stimulatory effect of CG on Na^+-K^+ ATPase in cardiac membrane preparations. Since then, the stimulatory effect of CG on Na^+-K^+ ATPase in a low concentration range (generally below 10^{-8} M) has been well documented by many investigators (314, 382, 383). Lee and Yu (314) observed the stimulation of cardiac microsomal ATPase (Na^+-K^+ ATPase) by

strophanthin at a concentration as low as 10^{-11} M. Klein (272) observed the stimulatory effect of CG on the Na^+-K^+ ATPase from embryonic chicken hearts even with relatively high concentrations of ouabain, and Palmer and Nechay (382) reported the consistently stimulatory effect of ouabain (10^{-8}–10^{-11} M) on Na^+-K^+ ATPase of chicken kidney. However, some authors could not observe the stimulatory effect of CG. The failure to see this stimulatory phase of CG on the Na^+-K^+ ATPase may be explained by the dependency of the stimulating effect on the state of the enzyme (48). Both the magnitude and the direction of CG effect on the Na^+-K^+ ATPase could vary, depending on age, or treatment of the enzyme with chemicals (48). A concentration of ouabain (10^{-4} M) inhibited or stimulated the transport ATPase activity, depending on the experimental conditions. It was concluded that ouabain affects the enzyme conformation in different manners, depending on the prereaction conformation to which the protein is disposed.

Some difficulties in studying this stimulatory effect may result from experimental differences mentioned in the following references: Repke (410) reported that some concentrations of glycosides (0.5 and 5.0 μg/ml) were stimulatory (up to 60%) only briefly, immediately after the start of the reaction (5–10 min), but became inhibitory progressively with increasing incubation time (20–40 min). On the other hand, Palmer *et al.* (383) observed increasing stimulatory effects (16–32%) of ouabain (10^{-8}–10^{-11} M) with increasing incubation times (5–30 min) on rabbit brain and chicken kidney microsomal ATPase. Varying the potassium concentration from 0.01 mM to 25 mM did not influence the stimulatory effect but profoundly influenced the inhibitory effect of CG on the enzyme activity. Aging of the preparation (3–35 days at −20°C) reduced only the stimulatory action of ouabain, whereas the inhibitory effect was unaffected. Similar observations were made by McClane (346) on the sodium transport in the isolated toad bladder as measured by the short circuit method: ouabain at low concentrations (10^{-9}–10^{-6} M) was shown to stimulate the active sodium transport, and this effect was slightly enhanced by increasing the potassium concentration to 14 mM, whereas ouabain at higher concentrations had an inhibitory action which was antagonized by an increase in the potassium level. Essentially similar observations were made previously: Wilbrandt and Weiss (555) showed inhibition of the potential difference across the frog skin by high concentrations of strophanthoside, while lower concentrations caused a stimulation, followed by a later inhibition. This later biphasic effect was confirmed by MacRobbie and Ussing (339), employing high concentrations of ouabain on the same preparation. From these observations, two receptor sites for ouabain were postulated (346, 383), one mediating stimulation and another mediating inhibition. At low concentrations of ouabain, interaction with the stimulatory receptor predominates; at higher concentrations, interaction with the inhibitory receptor predominates (346, 383). Increased concentrations of potassium have a negligible effect on the interaction of ouabain with the stimulatory receptor. This concept postulates the existence of two receptors having different affinities.

E. Influence of other agents on Na^+-K^+ ATPase

The erythrophleum alkaloids, which resemble CG pharmacologically but differ from CG in chemical structure, have both the inhibitory effect on Na^+-K^+ ATPase and the positive inotropic effect (33, 155, 243). However, there are many agents which inhibit Na^+-K^+ ATPase but do not have a positive inotropic effect on heart.

The Na^+-K^+ ATPase enzyme isolated from toad cardiac muscle was depressed by quinidine (10–50 μg/ml) (256). Oligomycin was reported to depress the Na^+-K^+ ATPase activity of erythrocytes and brain microsomal fractions (240, 518). The SH-blocking agents were shown to inhibit specifically Na^+-K^+ ATPase (65, 409). Mersalyl (20 μM) almost completely inhibited the Na^+-K^+ ATPase activity without influencing Mg^{++}-activated ATPase (409). Rendi (409) suggested that mersalyl inhibits the Na^+-K^+ ATPase by preventing dephosphorylation in a manner different from that of strophanthidin. Sodium azide also inhibits Na^+-K^+ ATPase (7). It should be mentioned that all of the above inhibitors of Na^+-K^+ ATPase do not have inotropic effects on heart muscle (307).

With regard to the relationship between the inotropic effect on CG and the inhibition of Na^+-K^+ ATPase, results obtained with bromoacetate derivatives of cardiac glycosides are interesting. These compounds were first described by Hokin *et al.* (221). They have both a potent cardiotonic effect and a potent inhibitory effect on Na^+-K^+ ATPase. The comparative studies on bromoacetate derivatives of cardiotonic steroids, on the activity of the isolated membrane ATPase, and on the contractile force of guinea pig and rabbit heart muscle preparations revealed that the positive inotropic effect was reversible and easily washed away, but the inhibitory effect on Na^+-K^+ ATPase was irreversible (370, 426, 427). It was found that the inhibition of the brain Na^+-K^+ ATPase activity by these agents was also irreversible (136). The dissociation of the inhibitory effect on ATPase and the inotropic effect was further demonstrated by the finding that strophanthidin-3-bromoacetate had a weaker positive inotropic effect, but a stronger inhibitory effect, on Na^+-K^+ ATPase than strophanthidin in guinea pig myocardium (136). The above findings, indicating the dissociation of the inhibition of Na^+-K^+ ATPase and the positive inotropic effect, have an important implication in the understanding of the mechanism of CG action.

XI. EFFECT OF CG ON ELECTROPHYSIOLOGICAL PROPERTIES OF THE MYOCARDIUM

It is generally agreed that the resting membrane potential of cardiac muscle cells is based mainly upon the intracellular and extracellular concentration differences of potassium and sodium which, in turn, are intimately linked to the active ion transport processes and the permeability characteristics of the cell membrane. The instability of the resting membrane potential in specialized tissues of the heart, causing diastolic depolarization and "automatic" generation of propagated action potentials in these cells, can be interpreted in the light of certain time-dependent changes in the ionic conductance (presumably potassium

conductance). The rapid depolarization phase of the action potential is initiated by a specific potential-dependent increase of the sodium conductance across the cell membrane in all parts of mammalian (and frog) cardiac tissue studied so far. In frog myocardium, part of this current (during the terminal phase of depolarization) seems to be carried by calcium ions (186, 365, 366, 375). As to the mechanism of the much slower repolarization phase, there is good reason to believe that the inward current of sodium ions is gradually overpowered by the outward current of potassium ions, because of time- and potential-dependent changes in the ionic conductance of the cell membrane, but the exact mode of regulation is still obscure (512). More recently, certain evidence for participation of calcium ions in the transmembrane ion currents in mammalian heart muscle during a certain stage of the action potential has been obtained. The inward Ca^{++} current is thought to be at least partly responsible for the plateau configuration of the action potential and is presumably directly involved in excitation-contraction coupling (17, 18, 417, 418). These multiple components determining the shape of the action potential are quantitatively different in various parts of the heart (atrial, working myocardium, and Purkinje fibers) and in hearts of different species.

The CG not only distinctly influence the configuration of the action potential of heart muscle, but also markedly modify other electrophysiological properties, such as excitability, conduction velocity, and automaticity.

A. Influence of CG on diastolic membrane potential of myocardial cells

1. Impulse-generating cells. The electrophysiological analysis of the chronotropic effects of CG has to consider all parameters which determine the discharge frequency of the impulse-generating system, such as the rate of diastolic depolarization, the level of the maximal diastolic potential, and the threshold potential (see 219). The observed effects of CG on frequency are mainly attributable to changes in the rate of spontaneous diastolic depolarization, as demonstrated in experiments on Purkinje fibers; thus, in the early stage of the CG action this process was found initially to be reduced slightly in the presence of CG (94, 95, 250); however, after exposure to toxic concentrations, the depolarization rate was enhanced; furthermore, the maximal diastolic potential was also reduced, which would result in more rapid firing (520). Calcium (at concentrations less than 10 mM) also increased the rate of diastolic depolarization of Purkinje fibers, resulting in a positive chronotropic effect (459). Because of this similarity in the actions of the calcium ions and CG on these processes, Seifen *et al.* (459) concluded that the CG-induced changes in the time course of the pacemaker potential might be mediated through the drug-induced alteration of calcium ions.

2. Working myocardium. In working myocardial tissue, in contrast to the impulse-generating structures, CG do not induce a progressive diastolic depolarization process leading to spontaneous activity (94, 95, 250). In lower concentrations CG do not influence the diastolic potential, whereas in higher concentrations the drug causes a general fall in the membrane potential (94, 95, 250, 415, 520). This latter effect is explained on the basis of a disturbance of the

intra-extracellular ion gradients caused by the inhibitory effect of the transport ATPase in the myocardial cell membrane by toxic concentrations of CG.

B. Influence of CG on the action potential

CG have been shown to alter certain characteristics of the action potential of Purkinje fibers (94, 95, 250), ventricular (94, 95, 273, 520), and atrial (415, 473) muscle preparations. The observed changes are rather uniform in all these preparations, but the interpretation of these findings is still a matter of controversy.

1. Rate of rise of action potential. During the positive inotropic stage of CG action, the rapid phase of depolarization initiating the action potential was increased in dog ventricular muscle *in vivo* (394). However, this increase was also observed with all other positive inotropic interventions under the same experimental conditions, which indicates a rather nonspecific nature of this effect. In toxic concentrations, CG uniformly decreased the rate of depolarization in all types of cardiac tissue studied (250, 496, 575). This effect is similar to the action of high $[Ca^{++}]_0$ on frog ventricular preparations (61, 529) but unlike the action of high $[Ca^{++}]_0$ on Purkinje fibers from sheep and dog, where an increase in the rate of rise has been reported (220, 542). In the presence of high concentrations of CG, the S-shaped curve relating the maximal upstroke velocity (dV/dt) to the membrane potential was shifted to the right, with no change in the maximal value (95, 250). Kassebaum (250) suggested that the action of strophanthin in toxic concentrations was associated with a decrease in sodium conductance because of reduction in the available sodium carrier. In the manifested toxic stage of CG action, the decrease in the membrane potential additionally contributes to the decrease in the maximal rate of depolarization and to the decrease in the height of the action potential. These changes in the depolarization rate of the action potential do not appear to constitute an essential component for the inotropic action of CG, but they are closely related to the CG-induced alterations of the conduction velocity.

2. Duration of the action potential. The most prominent effect of CG (even in low concentrations) on the myocardial action potential critically related to the inotropic effect is the influence on the rate of repolarization, which is usually manifested by changes in the duration of the action potential at the plateau phase, namely, at phase 2. The alteration of the duration of phase 2 following CG administration was almost uniformly observed in all types of cardiac tissue studied. Thus, CG may prolong or shorten the duration of the action potential at the plateau level, depending on the experimental conditions such as the ionic environment, the stimulation frequency, the CG concentration, etc. In isolated heart muscle of cat, dog, and sheep, it has been found that after the application of CG to the medium the duration of the plateau (phase 2 repolarization) is slightly prolonged, concomitant with the initial development of the CG-induced positive inotropic effect (94, 95, 250, 520). As the positive inotropic effect increases, the duration of phase 2 repolarization becomes progressively shortened below the control level (94, 95, 250, 520). In accordance with these observations

are earlier results indicating a shortening of the extracellularly recorded monophasic action potential (205, 206) and a shortening of the Q-T interval of the ECG in man (20). Reuter (415) has compared the effects of both "therapeutic" and "toxic" concentrations of digitoxigenin on isolated, electrically stimulated left auricles of guinea pigs. With low concentrations ("therapeutic") of the drug, an initial slight increase in the duration of the plateau phase is observed. On the other hand, with high ("toxic") concentrations, a marked decrease in the duration of the plateau phase was observed. Similarly, Sleator *et al.* (473) reported a shortening of the plateau phase in guinea pig atria with moderately high concentrations of strophanthin but noticed that often an increase in contractile tension was observed without any concomitant alteration of the action potential. The most detailed studies have been performed with Purkinje fibers (94, 95, 250), and identical results have been obtained. During the positive inotropic action of ouabain, an initial increase in the duration of the early phase of repolarization developed, followed by progressive shortening of the duration of the action potential in the later course of the experiments. The contractile force increased steadily throughout the experiment during the biphasic response of the action potential. In addition, the early positive inotropic effect occurred before any change of action potential. Corresponding to the biphasic action of CG on the shap of the action potential, the current-voltage relationship in these preparations revealed an initial increase in the membrane resistance, followed by a decrease in the later period.

At variance with the above-mentioned effects of the cardiac glycosides in prolonging (initially or with low concentrations) the duration of the plateau phase of action potential are recent observations which report only reductions of the duration of the action potentials by digitalis in trabecular muscles from sheep, calf, and dog (114, 356). In these experiments, shortening of the duration of the early repolarization phase of the action potential occurs from the time of administration of CG in parallel with the development of the positive inotropic effect. No increase in the duration of the action potential was observed, even at the early stage of the positive inotropic action of the drug. This lack of the initial prolongation of the action potential by digitalis may be partly explained by the careful study of Kassebaum (250) on influence of CG on the membrane properties of sheep trabecular muscle. He observed an increase in the duration of the action potential by ouabain in all muscles contracting at a frequency of 30/min, whereas in muscles contracting at a frequency of 50/min—the rate which was employed in the other experiments (114, 356)—only a progressive decrease in the duration of action potential developed. From these findings, Kassebaum concluded that shortening of the action potential duration at higher frequencies of contraction may have overshadowed the primary small increase in the duration of the action potential brought about by CG. This dependence of the CG-induced changes in the shape of the action potential on the experimental conditions is further demonstrated by the observation (406) that on guinea pig papillary muscles the same concentration of dihydroouabain causes slight prolongation if the extracellular

calcium concentration is low (0.6 mM), but progressive shortening from the very beginning if the extracellular calcium concentration is higher (3.2 mM). Most recently, the influence of several glycosides on the action potentials of ventricular muscle strips was studied under the same experimental conditions during the positive inotropic effect of their action (230, 231). Two glycosides were found to increase the duration of the action potential in all concentrations used. The other CG increased the duration at lower doses and decreased it at higher doses. From these findings CG were classified into two categories, one possessing the initial prolonging effect on the duration of phase 2, and the other shortening the duration from the beginning.

The effect of cardiac glycosides on the action potential may be summarized as follows: At an early stage of the drug effect, or in low concentrations, digitalis may increase the duration of action potential. At a late stage of the drug effect, or in high concentrations, digitalis causes shortening of the action potential. There are some disagreements about the prolonging effect in low concentrations, or at an early stage of the action of CG. However, it is an almost unanimous observation that digitalis shortens the duration of action potential in the later stage of CG action, or in higher, especially toxic, concentrations. This clearly indicates that the change of the action potential cannot be correlated directly with the positive inotropic effect of the drug. However, this statement should not be interpreted as meaning that no intimate relation exists between effects of digitalis on these two important parameters of muscle function. The interpretation of these observations is still a matter of some controversy. In terms of electrophysiological characteristics, the above biphasic changes in the electrical behavior (rate of repolarization, membrane resistance) of myocardial fibers during exposure to the CG may be explained on the basis of an initial decrease, followed by an increase in potassium conductance of the muscle membrane (95, 250).

In biochemical terms, the influence of cardiac glycosides on Na^+-K^+-activated ATPase, resulting in changes of potassium distribution, may explain the above phenomena. If the cardiac glycosides initially stimulated the Na^+-K^+-activated ATPase, this stimulatory effect would be reflected in a prolongation of the early repolarization stage of the action potential, since potassium levels at the outer surface of membrane would fall temporarily, resulting in the delay in the repolarization (220, 511). On the other hand, inhibition of the membrane ATPase would increase the potassium level at the outer surface of membrane, resulting in an earlier repolarization which would be manifested in the shortening of the duration of action potential. There are, however, some objections to this sort of interpretation. The CG-induced changes in the shape of the action potential would be only temporary if they were due to these postulated disturbances in the local potassium distribution, but they persist even under steady state conditions when complete equilibration of the surface membrane with the incubation medium has to be assumed.

Another explanation is offered on the basis of the striking similarities of these CG-induced effects, and of the frequency-induced changes in the shape of the

action potential of guinea pig papillary muscles. Reiter and Stickel (408) observed a similar biphasic change in the duration of the action potential by increasing the stimulation frequency at low $[Ca_{+}^{+}]_0$ (>2.4 mM). In higher $[Ca^{++}]_0$ medium, the frequency-induced prolongation was lost, but the shortening effect was more pronounced. There is an abundant literature on this frequency-dependent shortening of the duration of the action potential.

The mechanism most often suggested for this phenomenon is based on the alteration of the ionic environment, namely, a local accumulation of potassium at sites adjacent to the outer membrane surface (511). Reiter and Stickel (408) tested this hypothesis in comparative studies of effects of increasing stimulation frequencies and increased extracellular potassium or calcium concentrations on the resting and action potentials of guinea pig papillary muscles. According to these experimental results, it is most unlikely that the shortening of the action potential is caused by accumulation of potassium ions on the outer surface, because the potassium concentrations necessary to induce the same degree of reduction in the resting membrane potential as that observed with higher stimulation frequencies produced only a very slight shortening of the phase 2 duration in the action potential, in contrast to the marked shortening accompanying an increase in stimulation frequency. On the other hand, a moderate increase in the extracellular calcium concentration decreased the phase 2 duration of the action potential to the same extent as an increase in stimulation frequency, without an appreciably effect on the resting membrane potential. The shortening effect of increased stimulation frequencies was therefore thought to be due to an extracellular accumulation of calcium in the intimate vicinity of the outer cell membrane and not due to the accumulation of potassium, whereas the slight depolarization of the resting membrane was supposed to be caused by extracellular accumulation of potassium ions (408).

Distinct similarities were found between the frequency-dependent changes and CG-induced alterations in the electrophysiological properties of the myocardial cell membrane. Reiter and Stickel (408) considered, on this basis, that the CG-induced alterations of the duration of the action potential originate from local changes of the calcium concentration in the immediate vicinity of the myocardial membrane. The initial prolongation caused by CG in low $[Ca^{++}]_0$ medium may be ascribed to the lack of membranous calcium, and the shortening caused by CG in higher $[Ca^{++}]_0$ medium may be ascribed to the accumulation of calcium in the cell membrane (for further discussion see section XII).

Linking the electrophysiological changes induced by CG to that induced by an increase in stimulation frequency through a common factor, calcium, has some intriguing implications in the search for the mechanism of the inotropic action of CG. According to such a postulate (408), alterations in electrophysiological parameters brought about by CG are not the consequence of an impairment of the sodium transport mechanism by CG so commonly assumed, but they are, instead, the manifestation of the CG influence on calcium movement in the cellular membrane. The final judgment on this interesting hypothesis must wait further investigations.

XII. EFFECT OF CG ON EXCITATION-CONTRACTION COUPLING

Upon the arrival of a propagated excitation wave, the cardiac cell undergoes rapid depolarization and transient reversal of polarity, followed by a gradual restoration of the resting membrane potential. This sequence of changes is designated as the transmembrane action potential. Recognition of the existence of transversely and longitudinally oriented membrane-limited channels in the sarcoplasm suggested the possible participation of these structures in the internally transmitted signal from the surface membrane to the contractile element, actomyosin, throughout the muscle cell (99, 121, 229, 436, 469). For example, electrical stimulation of skinned fibers caused contraction, which indicates that intracellular membranes may conduct electrical signals (99, 358, 359). A concept of intracellular transmission of excitation by means of a sarcoplasmic reticulum is an attractive hypothesis, but supporting evidence is circumstantial. The internally propagated conduction of the membrane excitation signal appears to be applicable, especially in skeletal muscle, where the time lapse between membrane excitation and contractile response is short and the size of muscle fiber is large. However, in view of the slow time course of contraction, the smaller size of heart muscle fibers and the paucity of the reticulum system in the heart make internal spread of membrane excitation through the specialized system less certain in the myocardium. Whatever the detailed mechanism for the spread of excitation from the surface membrane into muscle fibers may be, it is generally accepted that the depolarization of the surface membrane releases or increases an activator in muscle cells which, in turn, triggers the contraction of actomyosin. The indications provided by several independent lines of inquiry show this activator to be calcium (99, 202, 294, 436, 557, 572).

Evidence suggests that in heart muscle calcium ions are involved in the transmembrane current during the action potential (17, 18). Although the mechanism involving the release of Ca^{++} upon excitation in heart muscle is unknown at present, it is generally agreed that binding of the released calcium to the actomyosin system initiates the contractile process. It is known now, through the work of Ebashi and his coworkers (99–103), that the calcium-sensitive actomyosin system consists of at least four protein components: myosin, actin, tropomyosin, and troponin. Troponin and tropomyosin are distributed along the entire thin filament of actin, and troponin appears to be a key protein through which the regulatory action of calcium is mediated to the contractile system. Troponin is bound to actin through tropomyosin, and the "tropomyosin-troponin" system inhibits the interaction of myosin and actin in the absence of calcium. Binding of calcium to the calcium-receptive protein, troponin, releases the inhibitory effect of these proteins on the interaction of actin and myosin, thus leading to activation of the contractile system. The above sequence of events leading to the final coupling of membrane excitation to the mechanical contraction necessitates the last step of the cycle, relaxation, namely, deactivation of the activator. This is the stage at which the voluminous work on sarcoplasmic reticulum fragments lends itself to various interpretations (2, 97, 128, 129, 252, 304, 483, 536, 557).

It is generally agreed that the sarcoplasmic reticulum system is endowed with a powerful calcium transport mechanism which is capable of lowering the intracellular free calcium concentration below the threshold concentration. However, it is unknown at present to what degree the sarcoplasmic reticulum plays a role in the relaxation of cardiac muscle.

Since calcium plays the central role in the current concept of E-C coupling, the exchangeability and movement of calcium in heart muscle should be mentioned. The localization of the endogenous calcium regulating the E-C coupling in the myocardium is still difficult to determine because of methodological limitations. The analysis of the cellular calcium distribution in heart tissue revealed at least three different calcium fractions (80, 153, 192, 213, 282, 558): 1) a rapidly exchangeable fraction; 2) a labile and reversibly bound fraction which exchanges slowly; and 3) a tightly bound, unexchangeable fraction. The quantitative relation of these fractions seems to be different in various muscles and species, and their magnitude (or existence) is dependent on the experimental conditions and methods used for their analysis.

Studies on the calcium exchange by Langer (293, 294), using the arterially perfused isolated papillary muscle of dogs, showed five different calcium fractions, but only one phase of calcium exchange seemed to correlate with the contractile activity. The half-time of exchange of this phase which was about 25% of the total calcium, was 6 min. This phase appeared to correspond to the superficial calcium of Niedergerke (364), and the "intermediary" locus of Winegrad and Shanes (558). Conditions which increased the contractile force (a low $[Na^+]_0$, a high $[Ca^{++}]_0$, an increase in stimulation frequency) increased this calcium fraction. If the capacity of this compartment for Ca^{++} was exceeded, contracture occurred. Grossman and Furchgott (179, 180) found an additional expansion of Ca^{45} space in the presence of contraction in isolated auricles of guinea pigs and referred to it as the "contraction-dependent calcium pool"; this expansion of Ca^{45} space was found to be related to an increase of exchangeability of calcium in muscle, rather than to an increase in total calcium.

There is no doubt that the intracellular compartmentalization or degrees of binding of calcium are affected by the contractile activity. Most reports agree that the total amount of exchangeable calcium is correlated to the functional activity, but only a rapidly exchanging fraction appears to be directly associated with the contraction (293, 294, 508). This compartment may be small, or even absent, in nonbeating preparations (11, 508, 540), and the size of this compartment is related directly to the contractile activity (11, 179, 294, 508). The rate of exchange in this compartment was found to be rapid enough to account for the speed with which induced changes in the contractility developed, and therefore it was concluded that the concentration of calcium in this compartment is the determinant factor of the contractile force.

It seems reasonable to assume that the following factors are involved in regulating the size of this contraction-associated calcium pool: 1) the inward current of calcium during the action potential, which would increase calcium influx during the activity; 2) intracellular release and recapture of this released Ca^{++}; and 3) factors modifying the state of intracellular calcium binding or redistribution

of intracellular calcium. The intracellular calcium is probably in a state of dynamic equilibrium, and levels of the intracellular calcium releasable upon excitation, which will be called excitation-releasable calcium, are determined by the preceding history of the contractile activity and Ca^{++} movement during rest intervals (572). Thus, the level of free $[Ca^{++}]_i$ achieved during excitation is determined by calcium influx during the action potential and the precontraction level of intracellular calcium bound to excitation-releasable sites; the calcium influx and level of intracellular calcium determine the intensity of active state, which in turn determines the contractile tension developed. The deactivation mechanism, namely, the restoration of calcium to its origin, heralds the relaxation and probably consists mainly of the binding of the active calcium to intracellular membranes (78, 99, 234) and the active outward transmembrane extrusion (311, 445). However, no conclusive evidence is available for the deactivation mechanism of calcium in heart muscle.

In heart muscle, contractile processes may be described in the following sequence of events: 1) propagation of the action potential and inward spread of the excitation through the T-system; 2) flow of the transmembrane inward calcium current and release of calcium from intracellular binding sites; 3) diffusion of calcium to the contractile element and binding of calcium to troponin; 4) interaction of ATP and actomyosin; 5) recapture of the released calcium by intracellular binding elements and transmembrane outward calcium transport; 6) possible redistribution of calcium to the releasing sites. The influence of CG on stages 3 and 6 are totally unknown, and stage 4 was discussed in section VII. Thus, possible effects of CG on stages 1, 2, and 5 will be discussed along with the CG influence on calcium movement.

A. Influence of CG on mechanical threshold

An interesting approach in elucidating the effect of CG on E-C coupling has been made on frog hearts (376). In these experiments the threshold of the mechanical response to depolarization by potassium was found to be substantially reduced by ouabain in positive inotropic concentrations. There was no increase in the maximal contracture tension developed, but ouabain shifted the depolarization-tension curve by about 30 mv toward the resting potential. If applied to the normal excitation process during the action potential, the finding could mean that the action potential may have become more effective as an activator of the contractile system in the presence of CG because of lowering of the mechanical threshold or longer duration of effective period when the inward calcium current takes place. This effect of ouabain in shifting the depolarization-tension curve to lower potential levels was comparable to the effect of increased $[Ca^{++}]_0$ in this preparation (363). This may suggest that the mechanical threshold is lowered by CG through an increase in the amount of intracellular free calcium.

B. Influence of CG on inward calcium current

The effect of CG on the configuration of action potential, especially on the plateau phase, was discussed in section XI. The influence of CG on the transmembrane inward calcium current during the action potential has not been

studied in detail. However, a recent study (449) indicated that under experimental conditions in which catecholamines increased the inward calcium current markedly in Purkinje fibers (416), digitoxigenin in concentrations which manifested a marked positive inotropic effect did not have any effect on the inward calcium current. Since the plateau phase of the action potential is associated with the calcium current (418) and this phase usually is not lengthened during the inotropic effect of CG (see section XI), it appears that CG do not exert their positive inotropic action by increasing the transmembrane calcium inflow during the action potential.

C. Effect on sarcoplasmic reticulum

Some agents which influence the contractility of muscle appear to owe their pharmacological effect to interference with the calcium uptake mechanism of the sarcoplasmic reticulum (SR), which is intimately related to the E-C coupling (234, 305, 306). Lee (302) found that in the presence of ouabain some glycerol-extracted cardiac fibers developed greater contractile tensions than did control fibers without ouabain, upon the addition of ATP. This effect of ouabain was observed in glycerol-extracted fibers which had not been extracted too extensively. Since glycerol-extracted fibers which are not extracted for too long a period have a functionally intact sarcoplasmic reticulum (117, 126), this effect on only short time extracted fibers may mean that ouabain inhibits calcium uptake of the sarcoplasmic reticulum. Further studies in Lee's laboratory indicated that inhibition of the syneresis of cardiac myofibrils by the "relaxing factor" was reversed by passage of electrical impulses, and this reversal was potentiated by CG (317). However, CG did not influence the inhibition of the "relaxing factor" on the syneresis without electrical stimulation (317). Klaus found that the inhibitory activity of cardiac SR on superprecipitation of actomyosin (skeletal muscle) was reduced in a dose-dependent manner by digitoxigenin (260, 261). A further detailed study by Lee *et al.* (312) showed that the superprecipitation of cardiac actomyosin was also inhibited by cardiac SR, and this inhibitory effect of SR was reversed by several CG tested. The degree of reversal by these CG had a positive correlation with the inotropic potency of these CG. This reversal effect of the SR was presumably because of an increase in the free calcium concentration brought about by CG in the presence of SR, and the above studies indicated that CG might influence the active calcium transport in the sarcoplasmic reticulum (59, 306). Later work from the same laboratory showed that the CG did not influence the active calcium transport mechanism of the isolated cardiac SR, but these drugs did alter the calcium-binding property of the cardiac SR (270, 307, 308, 310).

In experiments on the skeletal muscle SR, Fairhurst and Jenden (125) found no effect of ouabain on the active calcium transport, and Portius and Repke (390) found about 10% inhibition with high concentrations of ouabain. On the other hand, Luckenbach and Lüllmann (336) investigated the effect of ouabain on the calcium content of SR isolated from guinea pigs treated *in vitro* and *in vivo* by CG. In all cases, the CG-treated cardiac SR showed a diminished calcium

content, indicating that the CG in some way reduced the calcium binding or the calcium transport of SR, which resulted in release of calcium from SR. Carsten (59) found that CG inhibited the Ca^{++} uptake of cardiac SR. It should be mentioned that Carsten used "aged vesicles," and this may have had some bearing on the inhibitory effect of CG.

Interestingly, the opposite effect of ouabain (namely, stimulation) on the calcium uptake of the cardiac SR was reported under different experimental conditions. Briggs and his associates (46, 152) reported a depression of calcium transport in SR associated with both barbiturate-induced heart failure and spontaneous failure in heart-lung preparations, and this depression of calcium transport, like the contractile failure, was reversed by ouabain. Recently, Entman *et al.* (122) observed that ouabain and *alpha* angelica lactone, which also causes an inotropic effect, increased calcium binding of cardiac microsomal vesicles.

On the other hand, several investigations on the effect of CG on Ca^{++} uptake by cardiac SR produced neither inhibition nor stimulation. In the earlier work of Abe *et al.* (2), ouabain had no effect on the activity of the cardiac relaxing factor system on the myofibrillar ATPase of heart muscle. This inactivity of CG has been confirmed by recent studies. Thus, Chimoskey and Gergley (68) found neither the CG effect nor the antagonism between CG and barbiturates reported previously by Briggs and his associates (46, 214) on Ca^{++} uptake of cardiac SR. Similarly, Pretorious *et al.* (293) found that ouabain, in wide concentration ranges, had no effect on cardiac SR Ca^{++} uptake, although propranolol and caffeine inhibited the uptake. Besch *et al.* (23) isolated cardiac SR from dog hearts which developed the full inotropic effect of ouabain *in vivo* and compared their activity to that of control cardiac SR isolated from normal nontreated hearts. Calcium binding and uptake (in the presence of ATP) by the ouabain-treated cardiac SR were unaltered compared to control values. Furthermore, the addition of ouabain (10^{-7}–10^{-3} M) *in vitro* was without effect on cardiac SR isolated from both control and ouabain-perfused hearts. Similarly, the ATP-dependent Ca^{++} binding of cardiac SR purified on sucrose density gradients was found to be uninfluenced by ouabain (253). In skeletal SR, Worsfold and Peter (576) observed an inhibitory effect of quinidine and chlorpromazine, but no effect of ouabain, on Ca^{++} uptake.

It is apparent, then, that the effects of CG on Ca^{++} uptake of SR range from stimulation to no effect to inhibition, depending on the investigators and experimental conditions. These divergent results may arise mainly from the poorly understood nature of the Ca^{++} uptake process of cardiac SR, as discussed below. First, according to the present concept of active membrane transport, the process of Ca^{++} uptake by SR membrane in the presence of ATP would involve binding of Ca^{++} with the membrane carrier, transport through the membrane, and storage at intramembrane sites. Any one or all of these processes may be ATP-dependent. The nature of involvement of these processes in the Ca^{++} "uptake" of SR is not clear. Thus, Ohnishi and Ebashi (368, 369) showed spectrophotometrically that the ATP-dependent Ca^{++} binding of skeletal SR was completed within milliseconds. Carvalho and Leo (60) found that the Ca^{++} uptake of skele-

tal SR involves the exchange of Ca^{++} with other cations in SR. On the other hand, in the presence of oxalate and ATP, Ca^{++} apparently is actually transported into the SR vesicular lumen (200, 201). The terms "ATP-dependent binding," "Ca^{++} uptake," and "Ca^{++} transport of SR" are used by many authors interchangeably without discrimination because the nature of the "uptake" process is not well enough understood at present to define these terms clearly. This confusion is not limited only to terms, since different experimental conditions, which may favor one process over the other, are employed and their results are presented in any of these terms. Second, so far no cardiac SR has been prepared in pure form. It is contaminated by various other cellular components. In some cases effort has been made to separate SR from other components by density gradients and by other methods; however, the degree of contamination by other cellular elements is unknown in most preparations employed by investigators. Third, cardiac SR is known to be far less stable than its skeletal counterpart. For unknown reasons, after isolation, it rapidly loses the ability to take up Ca^{++} in the presence of ATP. To prevent this deterioration, various measures such as the addition of creatine phosphate, sucrose, and other agents were employed. These measures were found to be effective or ineffective depending on the investigator. It is generally agreed that the presence of oxalate or inorganic phosphate in the incubation medium increases Ca^{++} "uptake" by SR. However, many investigators employ different amounts of these potentiators. This resulted in an undesirable situation in which different experimental conditions are employed by investigators to the point that it is hard to find a given set of experimental conditions duplicated by any other investigator. The experimental conditions, the biochemical state, and the purity of SR preparation are expected to influence the CG effect on the interaction of Ca^{++}-SR, and the above mentioned difficulties in studying the effect of CG on SR would be expected to result in divergent results.

It is clear, however, that CG have no consistent effect on SR, and the weight of negative and opposing results appears to indicate that CG has no effect on the Ca^{++} transport mechanism of SR in terms of the Ca^{++} amount "taken" up by SR in the presence of ATP. This conclusion, namely, no effect of CG on the Ca^{++} transport mechanism, is also supported by findings in other membrane preparations. The ATP-dependent Ca^{++} transport mechanism has been conclusively demonstrated in mitochondria (66, 67, 93) and in red cell membrane (64, 311, 445), and CG has been found to have no effect on the Ca^{++} transport mechanism in both mitochondria (93, 308) and red cell membrane (311, 445). It should be mentioned, however, that the lack of effect of CG on Ca^{++} uptake by SR does not necessarily exclude the possibility that CG influence the binding state of Ca^{++}-SR. In fact, it is likely that the CG may alter the Ca^{++}-binding characteristics of SR in such a way as to be manifested only under certain experimental conditions; and this may be the reason why so many divergent, even opposing, effects of CG are observed in many investigations. The fact that the effect of CG on the interaction of Ca^{++}-cardiac SR is found to be varied depending on experimental conditions may be related to the finding that the effect of CG on the Ca^{++} movement is best demonstrated in the contracting heart muscle where SR is presumed to undergo a cyclic change.

D. Influence of CG on myocardial calcium metabolism

The influence of CG on the myocardial calcium metabolism has been studied extensively, employing radioactive calcium, but simultaneous measurements of the total calcium content were made only in a few studies. Since most of the cellular calcium is in a bound form and the exchangeability of bound calcium is variable, depending on the functional state of muscle (see section XII), flux data alone do not permit a comprehensive interpretation of calcium metabolism.

The effect of CG on myocardial calcium turnover was first measured by Harvey and Daniel (197), who determined the influence of several concentrations of digitoxin on the rate of Ca^{45} efflux from perfused guinea pig hearts which were preloaded with Ca^{45}. In these experiments no significant change in Ca^{45} efflux could be detected under all conditions tested, and this result has been confirmed repeatedly by others on different heart muscle preparations (225, 266, 460). On the other hand, Wilbrandt and Caviezel (553) demonstrated a marked reduction of calcium efflux from frog hearts by a high concentration of ouabain (2.5×10^{-6} M). From this observation an increase in the myocardial calcium content was proposed. Since neither calcium influx nor the calcium content was measured, the proposal was made without the necessary experimental evidence. Furthermore, frog hearts were labeled with Ca^{45} in 8 times normal $[Ca^{++}]_0$ solution, whereas washout experiments were performed in normal $[Ca^{++}]_0$ solution (Ringer); thus, the transmembrane calcium exchange was complicated by net calcium movements. More recent experiments on guinea pig atria did not reveal any significant effect of low and high concentrations of CG on calcium efflux (266, 337). Thus, at present no convincing data are available supporting the idea that CG influence calcium efflux from heart muscle.

The opposite direction of calcium exchange, namely, calcium influx, has been investigated extensively on both atrial and ventricular preparations from different species: guinea pig (148, 149, 180, 223, 259, 266, 337); rabbit (44, 45, 170, 225, 226, 255, 460); turtle (169); rat (150). The results obtained were qualitatively quite similar, and in most studies CG increased the uptake of Ca^{45} by the heart muscle, especially when high (toxic) concentrations were used. However, most of these experiments do not allow a detailed analysis of their data because of methodological limitations. The uniformly observed increase in Ca^{45} activity in the heart muscle after exposure to CG-containing solutions may be due to one or more of the following mechanisms: 1) an increase in calcium influx, with efflux unchanged; 2) a reduction of calcium efflux, with influx unchanged; 3) changes of both fluxes, but relatively greater calcium influx; 4) an increase in the rate of calcium exchange in both directions, with measurements made prior to attainment of a steady state; and 5) an increase in the exchangeable fraction of cellular calcium, with measurements made after attainment of a steady state. Among these, mechanisms 1, 2, and 3 would result in a net increase in total cellular calcium content. On the other hand, mechanisms 4 and 5 would not necessarily result in a net increase in the total calcium content.

This complexity of myocardial calcium turnover requires that, to make a comprehensive analysis of the calcium metabolism in heart muscles, the total

calcium content and the degree of attainment of the steady equilibrium of calcium exchange must be known, in addition to information on unidirectional fluxes. Some recent experiments did include measurements of all these parameters. In experiments on isolated, electrically stimulated atria of guinea pigs, Lüllmann and Holland (337) and Klaus and Kuschinsky (266) observed that the steady state equilibration of the cellular calcium with the radioactive extracellular calcium had been attained between 60 and 120 min. The simultaneous determination of the total cellular calcium content revealed that the specific activity of the myocardial calcium after equilibrium was lower than that of the incubation medium. This result indicated that only part of the cellular calcium was exchangeable, and this finding has been confirmed in many other experiments (149, 150, 169, 170, 180, 259). The amount of radioactive calcium present in the tissue after attainment of equilibrium was significantly greater in the presence of CG than in control, whereas the rate of calcium efflux was apparently not influenced by CG (266, 337). From these results, an increase in myocardial calcium content in the presence of CG might be expected. However, the total cellular calcium content did not show a significant change in the presence or absence of CG (266, 337). Other workers (150, 169, 170, 180, 259) also found no significant change in the total Ca^{++} content of heart muscle after CG treatment. It may be argued that the method used for calcium determination was not sensitive enough to detect small increases in the calcium content in this therapeutic range of CG action. But in some of the above experiments (266, 337) with CG concentrations in the therapeutic range there was even a reduction in cellular calcium content; and in many experiments by other workers a significant loss of calcium from heart muscles has been reported in the presence of such concentrations (150, 266, 267, 269). Thus, it is clear that the CG-induced expansion of the exchangeable calcium fraction is not accompanied by a significant increase in the total cellular calcium content. On the other hand, toxic concentrations of CG were found to increase markedly the total cellular calcium content in those experiments (266, 337), and this is in agreement with results obtained by others (149, 169, 170, 259, 267, 269, 316).

Thus, the demonstrated increase in tissue content of radioactive calcium in heart muscles treated with concentrations of CG, in association with unchanged (or even lowered) cellular calcium contents, suggests that CG make more cellular calcium available for exchange, perhaps by increasing the size of the contraction-dependent calcium pool. This CG-induced increase in the exchangeable myocardial calcium fraction, concluded from the experiments mentioned above (266, 337), has been confirmed in other experiments on different heart muscle preparations (148, 149, 150, 169, 170, 259). The magnitude of this increase in exchangeable calcium induced by CG was dependent upon the concentration (259, 266, 337) and the cardiotonic potency of CG (259). With inotropic concentrations the increase in calcium exchangeability was correlated to the inotropic effect, but toxic doses caused a deviation from this relationship because of the concomitant increase in total cellular calcium content (259). Furthermore, the CG-induced increase in calcium exchange was dependent on the stimulation frequency in the

same way as the positive inotropic action. In quiescent preparations no change in exchangeability of cellular calcium could be detected in the presence of CG (148, 169, 180, 246, 460).

According to these observations, the most prominent effect of inotropic concentrations of CG is an increase in the exchangeable myocardial calcium content. This effect can be explained by a shift of the equilibrium between different cellular calcium fractions toward the "functionally available" form of calcium, perhaps by mobilization of otherwise tightly bound calcium by CG.

E. Influence of CG on intracellular calcium distribution

As discussed above, if CG mobilize bound calcium and increase the exchangeability of intracellular calcium, then the distribution of intracellular calcium may be affected by CG. The influence of CG on the metabolism and distribution of myocardial calcium was suggested very early by findings that CG treatment *in vivo* caused a slight increase in the serum level of calcium (24, 25, 106, 154). This observation, which has been confirmed quite recently in experiments on guinea pigs (360), was interpreted as a manifestation of calcium release from heart muscle by CG (24, 25). Net changes in the total calcium content of the heart muscle, however, have never been demonstrated. But on the basis of experiments which showed an increased release of calcium from turtle heart (in low calcium medium) in the presence of strophanthidin, Pohle (389) suggested a shift in the equilibrium along ionic calcium, calcium complexes, and protein-bound calcium in the heart muscle.

The increase in exchangeable calcium fraction in the heart muscle brought about by CG (see the preceding section) suggests that CG mobilize tightly bound intracellular calcium either directly or indirectly by altering calcium distribution or storage. This is supported by an observation that the calcium content of cardiac SR fractions isolated from CG-intoxicated guinea pigs was reduced, whereas the total cellular calcium content was increased, as compared to control preparations (336). Repke *et al.* (411) proposed that changes in myocardial calcium metabolism caused by CG are secondary to inhibition of the sodium transport. A CG-induced increase in $[Na^+]_i$ is supposed to release bound calcium from subcellular structures through competition between sodium and calcium for binding sites. Such a cation competition has been demonstrated in microsomal fractions (384) and in mitochondria (92). However, changes in intracellular sodium concentrations necessary to produce such an interference did not occur in heart muscles in the presence of inotropic concentrations of CG (see section IX). Furthermore, calcium binding to the cardiac SR was not significantly influenced by replacing potassium in medium with sodium (376). Thus, this indirect mechanism of CG action on myocardial calcium movement is still of questionable importance for the inotropic action of CG.

Several studies indicate the direct effect of CG on the intracellular calcium distribution. It was shown that ouabain increases in a dose-dependent manner (range 10^{-7}–10^{-5} M) the "free" calcium fraction (which is releasable in the suspending medium) of the endogenous calcium in isolated preparations of

cardiac SR (261, 262, 270). The active calcium transport system of cardiac SR was not influenced (376). These findings indicate that CG influence the state of physicochemical binding of calcium to SR rather than the specific calcium transport mechanism. This is supported by the observation that ouabain produces qualitatively similar effects on the "free" calcium fractions in mitochondria and in the cellular membrane fragments isolated from both skeletal and cardiac muscles (262, 270). This direct effect of ouabain on the distribution of endogenous calcium in subcellular fractions can best be explained by assuming that CG mobilize bound calcium from binding sites in these subcellular components. This does not necessarily mean that this mobilized calcium fraction is always released into the medium in the presence of CG. It may suggest that tightly bound calcium is transformed by CG into more loosely bound calcium which may be released more easily when proper conditions prevail.

This "calcium-releasing" effect of CG was confirmed in recent experiments on isolated rabbit skeletal muscle SR, but in this study no correlation between this releasing effect and the cardiotonic potency of several CG was found (514). Thus it is possible that this calcium-releasing effect is nonspecific in nature.

In an attempt to localize the site of CG-calcium interaction further, studies were performed on the influence of digitoxin on calcium binding to phospholipids extracted from different subcellular fractions of guinea pig heart homogenates (260). Results indicated that the exchangeability of endogenous lipid-bound calcium with calcium in the suspending medium was increased in the presence of digitoxin (10^{-10}–10^{-6} M).

The effects of CG on the myocardial calcium exchange, on the distribution of endogenous calcium in different subcellular particles, and on the calcium-lipid interaction all lead to the conclusion that CG influence the distribution and the state of binding of calcium in the myocardium.

XIII. CONCLUSION

A drug-induced increase in myocardial contractile force may be brought about by a direct modification of one or more of the steps leading from membrane excitation to mechanical contraction. This contractile process is triggered by the excitation-contraction coupling mechanism, which transmits the membrane excitation to the contractile elements.

A possible direct effect of CG on contractile proteins has been sought in various actin, myosin, and actomyosin preparations of different purities and origins. Several minor alterations in the physicochemical characteristics of some of the contractile proteins studied were found to be induced by CG. However, many of these effects proved to be due to impurities in the preparations tested and are difficult to evaluate because of the unknown nature of the contaminants. Thus, at present no definitive significance can be attached to any known effect of CG on contractile proteins.

With regard to myocardial energy metabolism, CG were found definitely to influence certain aspects of metabolism. Almost all of the metabolic effects of CG were found to occur in heart muscle preparations in which the integrity of

cellular structure was maintained. None of the isolated, well defined enzymes involved in energy liberation systems, such as glycolysis and oxidative processes, has been found to be influenced consistently by CG. Digitalis does not increase the energy-rich phosphate content of the myocardium under various experimental conditions in which the inotropic effect is clearly present. The oxidative phosphorylation of mitochondria appears to be uninfluenced by CG.

Negative results obtained with CG with respect to energy production and the energy storage mechanism lead one to believe that CG increase the efficiency of conversion of energy into mechanical work. It is fairly certain that CG definitely increase the mechanical efficiency in dog heart-lung preparations and in intact hearts in congestive failure. However, more recent investigations on isolated heart preparations *in vitro* indicate that oxygen consumption increases concomitantly with the increase in contractility. Furthermore, the effect of CG on mechanical efficiency may vary under different experimental conditions. From these results it must be concluded that CG do not appear to induce an inotropic effect by influencing energy metabolism.

Beyond the above two areas involved in contractile activity, namely, the contractile elements and energy metabolism, one is led to the excitation-contraction coupling process. Most present evidence suggests that CG increase myocardial contractility by somehow facilitating the E-C coupling process.

The propagation of excitation (action potential) through the muscle membrane mediates the translocation of membranous calcium and the release of intracellular calcium, which in turn triggers the contraction of actomyosin. The releasable calcium related to excitation is presumably located at intracellular membranes such as the inner membrane surface and sarcoplasmic reticulum. The configuration of the action potential of cardiac muscle is prolonged or shortened during the inotropic action of CG, depending on experimental conditions. Thus, an alteration of the action potential by CG is not the cause of the inotropic effect. Also, the inward calcium current during the action potential does not appear to be influenced by CG.

The most consistent effect of inotropic concentrations of CG on calcium movement in heart muscle is an increase in the exchangeable calcium fraction without a detectable increase in total calcium content. This effect of CG is consistently observed in contracting muscle and is often not detectable in noncontracting muscle. This suggests that CG alter the calcium-binding characteristics of cellular and intracellular membranes but do not actually manifest this effect unless the contractile activity is present. The critical problem is the mechanism through which CG bring about these effects.

At present, the singularly established effect of CG on a well defined entity is their specific inhibitory action on Na^+-K^+ ATPase. It is possible that the effect of Na^+-K^+ inhibition by CG may indirectly influence Ca^{++} movement. 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 CG.

According to this view, intracellular sodium would be increased because of the

inhibition of Na^+-K^+ ATPase by CG. This increased sodium would result in better mobilization of intracellular calcium either by competing with calcium for intracellular binding sites or by increasing calcium influx [as shown in the giant axon by Baker *et al.* (13)]. However, the majority of studies on the intracellular distribution of sodium and potassium have indicated that cardiac contents of these electrolytes are not altered by CG at the inotropic stage. In fact, if anything, the intracellular sodium content is often found to be decreased during the inotropic action. Also, results obtained in studies on K^+ flux of cardiac muscle indicate that the inotropic effect of the drug is not necessarily accompanied by loss of K^+ from heart muscle. These findings, among others, indicate that Na^+-K^+ ATPase inhibition, *per se*, is probably not the mode of action of CG. However, it may still be argued that the alteration of intracellular sodium content is compartmentalized in the heart cell and that an increase in sodium concentration at an unknown critical area is responsible for the intracellular mobilization of calcium by CG. In this case, the inhibition of Na^+-K^+ ATPase could be the primary cause of the inotropic action. This kind of argument cannot be proved or disproved with the experimental evidence available at the present time.

On the other hand, it appears that CG bring about a conformational change in the Na^+-K^+ ATPase enzyme, which is an integral part of the cellular membrane. The calcium-binding characteristics of intracellular membranes such as sarcoplasmic reticulum and mitochondria appear to be altered by CG. It is quite possible, then, that CG alter the characteristics of cellular and intracellular membranes in such a way as to release Ca^{++} more easily. According to this view, CG-induced changes in characteristics of cellular and intracellular membranes result in an increased release of bound calcium upon excitation or in an increase in the proportion of free calcium not bound to intracellular membranes or in both. An increase in the amount of Ca^{++} released upon excitation and/or higher resting levels of free Ca^{++} concentration brought about by CG would result in faster onset and development of a more intense active state. This would result in faster attainment of the stronger contractile tension. The inhibition of Na^+-K^+ ATPase activity, which is another manifestation of the change of cellular membrane characteristics caused by CG, is unrelated to the inotropic effect by itself, and it may or may not be present at the time of inotropic effect, depending on the degree of alteration brought about by CG on the membranes.

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