Journal of the Pharmaceutical Society of Korea
18, 165-182 (1974)

The Positive Inotropic Effect of Digoxin and Its Subcellular Distribution

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(Received May 15, 1974)

The cardiac glycosides were known as a medicine to the ancient Egyptians and were metioned in the Ebers Papyrus (ca. 1500 B.C.). Digitalis, or foxglove, was mentioned in 1250 in the writing of Welsh physicians. It was described botanically 300 years later by Fuchsius in 1542, who gave it *Digitalis purpurea* because the flower resembles a finger and is purple. Primitive people have used various crude extracts of the plants containing cardiac glycosides for arrow and dart poisons from time immemorial, and during the 16th and 17th century as an expectorant and emetic. It had fallen into relative disuse until Withering revived its use in 1785. The mechanism underlying the therapeutic effect of digitalis was unkown at the time of Withering's original observations, and it was not until the early twentieth century that the primary effect of the drug was shown by Cushny[1] to be an increase in the force of myocardial contraction. Only within the last 50 years has it become firmly established that the main value of digitalis is in the therapy of congestive heart failure. However, the underlying cellular mechanisms remain uncertain to this day, although a great number of studies on the effect of ionic milieu on the inotropic action of digitalis have been conducted and all known cellular and subcellular systems have been studied with respect to possible site of action[2]. This review is mainly concerned with the correlation of the subcellular distirbution of cardiac glycosides with the positive inotropic effect.

Binding of digitalis glycosides to cardiac tissue—Tissue distribution studies indicate that the organs concerned with metabolism and excretion, such as the gastrointestinal

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tract, liver and kidney, contain the highest concentration of digitoxin and its metabolites[3,4]. Various studies have confirmed that little digoxin is metabolized by mammals, either *in vitro* or *in vivo*[5-7]. Degradation of this cardiac glycoside *in vivo* takes place mainly in the liver. Digoxin is metabolized *in vitro* by liver slices but not by heart muscle slices or homogenates[5]. Thus, the digoxin accumulated by the heart is unchanged digoxin.

The heart appears to have a selective affinity for digoxin in comparison to most organs such as skeletal muscle and smooth muscle[8]. In an attempt to determine the locus of action of digitalis, several investigators have reported on the intracellular distribution of cardiac muscle using differential centrifugation[9,10]. In these studies no appreciable binding was observed in the subcellular particles in rats. Perhaps this was due to the well known insensitivity of rats to the cardiac glycosides. However, Harvey and Pieper[11], using isolated guinea pig heart perfused with ^{14}C-digitoxin, showed that there were higher concentrations of radioactivity in the particulate fractions than in the supernatant fractions. Since this work considerable evidence has accumulated to suggest that the positive inotropic effect of digitalis might be related to amount of the glycoside taken up by the myocardium[7,12].

Studies to define the relation between the myocardial uptake of cardiac glycosides and the positive inotropic effect have yielded conflicting results. For example, Luchi *et al.*[13] studied the relationship between myocardial content of ouabain and inotropic or toxic manifestation of the drug in electrically stimulated dog hearts. They showed that the inotropic effect was associated with myocardial content of ouabain greater than 0.2 nM/g dry wt. Toxic manifestations, *i.e.* either sustained ventricular tachycardia or ventricular fibrillation was associated with myocardial contents of ouabain 1.0 nM/g dry wt. or greater. Since digitalis-induced arrhythmias are thought to originate in Purkinje fibers, Dutta *et al.*[14], measured the distribution of ^{3}H-digoxin in heart muscle and reported that sheep Purkinje fibers have a lower concentration of ouabain than ventricular muscle. They concluded that conduction in the Purkinje fibers might be affected by a smaller amount of ouabain than was required to affect conduction in the ventricle. Dutta and Marks[15] have recently shown that the accumulation of ouabain by isolated guinea pig heart was inhibited by pharmacologically active cardiac glycosides. They have also reported that insulin increased ouabain accumulation[16], and phloretin or phlorizin reduced ouabain uptake by the heart[17]. This is consistent with the suggestion that the effect of phloretin and insulin on the inotropic responses to ouabain or K-strophanthidin[18,19] may be related

to alterations of accumulation of the glycoside or aglycone by heart tissue. Their study[16] showed that cellular energy is required for the accumulation both of ouabain and digitoxin by the isolated guinea pig heart. The same conclusion was reached in experiments which showed that dinitrophenol inhibited cardiac glycoside accumulation by the heart[10]. The increase in contractile force for a variety of cardiac glycosides occurs within a small range of concentrations in guinea pig atria[20]. However, the tissue uptake of the glycosides were independent of their potency and concentration in the bathing medium, that is, the tissue: medium ratio varied by tenfold for a single concentration of glycosides in the medium. For example, the tissue: medium ratio was 0.6 when 1×10^{-7} g/ml ouabain was contained in the bathing medium. In contrast, the same concentration of digitoxin produced a tissue: medium ratio of 9. The disparity occurred despite the fact that digitoxin is at least two times as potent as ouabain in this particular preparation[21]. The tissue: medium ratio was 3 when 1×10^{-7} g/ml digoxin was added to the bathing medium[7]. The tissue: medium ratios vary directly with changes in the lipid solubility of the drugs. In the order of decreasing polarity the sequence is: ouabain>digoxin> digitoxin[22] and this is accompanied by an increased accumulation. These findings suggest that the cardiac glycosides are probably bound to lipid- and protein-containing structure of the cells. The binding of the cardiac glycosides to serum protein also increases in the same sequence: no measurable protein binding could be demonstrated for ouabain, whereas about 80% of the ^{3}H-digitoxin or its genin are firmly bound to serum protein[23,24]. Moreover, previous removal of lipid material from atrial tissue by extraction with aqueous glycerol reduced the tissue: medium ratio for ^{3}H-digitoxin, but without change for that of ouabain[25]. If we assume that a certain degree of receptor occupation is related to a given positive inotropic effect and that this degree will be about the same for all the glycosides, the large differences in tissue: medium ratios suggest that total tissue uptake is not related with the increase in contractility. Morgan and Binnion[26] in their investigation on the distribution of ^{3}H-digoxin in dog heart suggested that a large part of the ^{3}H-digoxin accumulated by the canine myocardial cell is attached to non-specific cell binding sites, while only a small percentage would be attached to sites responsible for the positve inotropic effect. Kuschinsky *et al.*[27], hypothesized that the cellular uptake at different concentrations of digoxin may be the sum of two different components. A linear component which was not saturable and possibly represents the passive movements of digoxin across the cell membrane. The other component, which was saturable, probably represents the

accumulation of digoxin at the active site. On the contrary, Lüllman *et al.*[20], suggested that the different durations of action of cardiac glycosides *in vivo* is caused by differences in elimination and/or metabolism and not by differences in fixation to the heart. This suggestion was based on the observation that the loss of the positive inotopic effect was not parallel with the loss of total tissue concentrations. Their suggestion is in agreement with the conclusion of Okita[28] and other investigators[6,29] that the long duration of action of nonpolar cardiac glycosides is due to their lipid solubility, which permits retention in the enterohepatic circulation as a result of reabsorption of the drug across the lipid membrane of the intestinal mucosa and the kidney tubules.

Moran[30,31] has demonstrated a direct correlation between the positive inotropic effect of ouabain on isolated rabbit atria and the number of contractions from the time of exposure to the glycoside. He also showed that exposure of a quiescent atrium to a low concentration of ouabain gave little detectable effect if the drug was removed from the bath before initiating contractions. He confirmed his hypothesis in a later study that showed the positive inotropic effect of the cardiac glycosides to be largely contraction dependent, and suggested that the binding of cardiac glycosides to the myocadial receptor and the positive inotropic action are determined by the number of contractions. In contrast, Vincenzi[32] concluded that the rate of onset of the positive inotropic effect is nearly independent of myocardial activity, except at beat intervals less than 3 sec. Byrne and Dresel[33] have found that exposure of quiescent atria to ouabain may lead to an inotropic effect; however, the ability of ouabain to act on quiescent muscle is dependent upon the experimental conditions, notably temperature and calcium concentration.

Two reports have dealt with the relationship between contractile activity of heart muscle and the uptake of ^{3}H-digoxin. Contrary to Moran's suggestion is the finding of Roth-Schechter *et al.*[37] that there was no significant difference in the level of digoxin concentration between quiescent and contracting atria in spite of difference in the magnitude of the positive inotropic effect. They also reported that there was no difference in drug retention or binding affinity between contracting and quiescent atria as evidenced by similar drug half-lives of 48 min. This was further confirmed by the presence of only a single exponential component over a three-hour washout period in drug-free buffer. Kuschinsky *et al.*[7] found that contracting guinea pig atria took up ^{3}H-digoxin more rapidly than did quiescent atria, but after 3 hrs maximum uptake by both types of atria did not differ.

Roth-Schechter *et al.*[35] have reported the dissociation between the persistence of the positive inotropic effect and the retention of strophanthidin-3-bromoacetate in isolated rabbit atria. When the positive inotropic effect has completely disappeared, there is still a high concentration of strophanthidin-3-bromoacetate left in the atrium. They used strophanthidin-3-haloacetates since these compounds are believed to alkylate protein receptors and have a strong and persistent binding affinity[36].

Several autoradiographic studies which are attempted to locate the site of action of digitalis by localizing the sites of its binding within the heart are of interest in this connection. However, there is considerable disagreement between various groups of workers. For example, Smith and Fozzard[37] and Tubbs *et al.*[38] found, by a combination of autoradiographic and electron microscopic methods, digoxin mainly in the A-band region of the contractile element. Whereas Sonnenblick *et al.*,[30] and Fozzard and Smith[40] in a later study showed the cardiac glycoside to be concentrated in T-tubule. Conrad and Baxter[12] reported glycoside radioactivity to be concentrated in the cell membrane as well as intracellularly in the A-band. However, Luchi and Conn[41] showed that isolated contractile proteins do not bind the drug. It should be emphasized that such studies may not solve the question, since the area of the greatest concentration of the drug need not necessarily be the site of its primary action[27,42]. Consequently, morphological studies on the localization of digoxin or digitoxin within the cell may not allow any relevant conclusions concerning the binding of these drugs to specific receptors.

The microsomal uptake of cardiac glycosides—Dutta and Marks showed that ^{3}H-digoxin[43] and other glycosides[44] are concentrated in the microsomal fraction to a greater extent than in the other subcellular fractions of guinea pig hearts and have suggested that the microsomal uptake of various glycosides may be involved with the positive inotropic effect. They have shown that the accumulation of ouabain by isolated guinea pig heart is at least partially saturable, is inhibited by other pharmacologically active glycosides[44]. Dutta and Marks[44,15] have reported that ^{3}H-digoxin uptake by the microsomal fraction was related to the concentration of sodium and potassium ions in the perfusion medium in a manner consistent with the known effect of these ions on the positive inotropic effect of the drug. However, they did not measure the positive inotropic effect in the same heart. They studied the uptake of various glycosides by freshly prepared beef heart sarcoplasmic reticulum fragments and showed that ATP was required and 10 mM KCl reduced the binding of cardiac glyosides by sarcoplasmic reticulum

fragments. Dutta *et al.*[44] postulated that the potassium-dependent site for cardiac glycosides in the sarcoplasmic reticulum may be the receptor for the positive inotropic effect in the heart. In addition, Kuschinsky *et al.*[21] on the basis of kinetic studies with ^{3}H-ouabain, suggested that the digitalis receptor site may be located on the membrane. Their results suggest that that the receptor is readily accessible to the extracellular space and that the combination of drug with the receptor probably reaches the equilibrium quite rapidly. The reason for the long latency for digitalis response is not known, but it has been suggested that the digitalis transport system is located on the cardiac cell membrane and the digitalis must be transported into the intracellular space before it can bind to the specific digitalis receptors[44].

Their studies and those of Kuschinsky *et al.* and Kuschinsky and Van Zwieten[45] suffered from the fact that tissue uptake or distribution of the glycoside was not measured in the same preparations in which the pharmacological effect was determined. Taking this additional precaution, we[46,47] found that the action of insulin to increase the positive inotropic effect of ^{3}H-digoxin in perfused guinea-pig hearts was directly related to the increase in the uptake of the glycoside by the microsomal fraction, but was not related to the total content of digoxin of the hearts, to the tissue/medium ratio, or to the binding to the crude nuclear and mitochondrial fractions. Our results were in agreement with those of Kuschinsky *et al.*[21,27] and Kuschinsky and Van Zwieten[45] and showed that the total uptake of a given compound by the heart under constant conditions was not necessarily an index of drug binding to a spiecfic receptor. We suggested that the total uptake by the microsomal fraction might also not be the most appropriate index of the portion of bound digoxin which might be responsible for the pharmacological effect.

The objective of our work was to augment or interfere with the positive inotropic effect of digoxin or with its binding to various subcellular fractions by agents which unlike the changes in ionic concentrations examined by Dutta and Marks would not cause major changes in total tissue uptake of digoxin but which might affect the cellular distribution of the glycoside.

The effect of insulin—Insulin is known to increase the uptake and pharmacological effects of a wide variety of drugs *in vivo*. Bailey and Dresel[18,48] have shown that highly purified insulin increases the rate of development of the positive inotropic effect of ouabain and K-strophanthidin in rabbit atria. The increased positive inotropic response to ^{3}H-digoxin in the presence of purified insulin was directly related to the uptake of

^{3}H-digoxin by the microsomal fraction when the hearts were exposed to digoxin for 30 min at a constant rate, but was not related to the uptake by any other subcellular structure.

The effect of insulin on the inotropic response to the ^{3}H-digoxin in the guinea pig heart was qualitatively different from that seen in rabbit atria[18,48]. Insulin increased the magnitude of the change in contractile force after treatment with ^{3}H-digoxin for 30 min.

It is well known that insulin increases in concentration of potassium in muscle[49] and in heart[50]. Regan *et al.*[59] have shown that insulin antagonizes the positive inotropic effect of acetylstrophanthidin, probably due to enhanced potassium uptake in the presence of insulin. Insulin caused to increase the uptake of potassium in left ventricles beginning from 5 to 10 min after insulin treatment in intact anesthetized dogs[50]. Dutta *et al.*[44] showed that the uptake of tritiated cardiac glycoside by the fragments of sarcoplasmic reticulum was inhibited by the addition of KCl in the incubatin mixture. Several other investigators[26,51] also showed similar results, *i.e.* a decrease in the myocardial uptake of ^{3}H-digoxin in the hyperkalemic dog. Prindle[52] demonstrated by using cat papillary muscle that the rate of muscle accumulation of tritiated digoxin was inversely related to the bath potassium concentration. These effects of insulin on potassium accumulation are thus opposite to the effects of digitalis. The time relationships of these effects however, may be sufficient to explain the differences in the rate of onset of the effect of digitalis observed in the present work and in that of Bailey and Dresel[18].

It was speculated that the delay in onset of the mechanical response to digitalis was caused by a membrane transport requirement before the drug could be made accessible to its receptor site[44]. It was proposed by them that ATPase is responsible for transporting digitalis from the extra to the intracellular compartment of heart cells, where the interaction with digitalis and inotropic receptor occurs[44]. Drugs such as dihydro-ouabain which have poor affinity for this ATPase of heart membranes, would have reduced activity because they would be unable to be transported into and thus accumulate in the heart. Once in the intracellular space, however, this drug appears to have normal affinity for the particular receptor, as judged by the extent of the binding to isolated sarcoplasmic reticulum fragments.

Dutta and Marks[44] proposed that a digitalis-stimulated ATPase may be a transport enzyme for digitalis and that transport may be the rate-limiting step in determining the onset and intensity of action of digitalis in producing the positive inotropic effect upon

the heart. They have suggested that an important binding site and receptor mechanism for digitalis effects may be present in the sarcoplasmic reticulum. This interpretation is open to question since, however, the muscle fibers of the frog ventricle contain no transverse tubular system[53], but is still sensitive to the cardiac glycosides.

Marcus *et al.*[54] have reported recently that the sensitivity of the dog heart to digitalis toxicity was increased in the presence of hypokalemia and the concentration of digoxin in the myocardium of the hypokalemic dogs was 35% lower than that of the normokalemic dogs at the time of onset of toxicity. They suggested that the myocardial sensitivity to digitalis may be related to altered electrophysiologic properties of the Purkinje cell due to hypokalemia, and not to enhanced cardiac uptake of digoxin[55-57]. In addition, Dutta and Marks[16] observed that the increase of cardiac toxicity by the use of a low potassium medium resulted in a three-fold increase in ouabain accumulation in guinea pig heart. However, they measured only total tissue uptake and used spontaneously beating preparations.

Insulin potentiated the positive inotropic effect of digoxin and its uptake by the microsomal fraction after the hearts exposed to digoxin for 30 min. Then, how can the stimulatory effect of insulin on the digoxin uptake and contractility by 30 min be reconciled with the potassium effect?

Regan *et al.*[50] reported that increased net uptake of potassium after insulin infusion into dogs was decreased by infusion of strophanthidin. This potassium loss occured from 2 to 8 min after injection. Therefore, it seems possible that that the potassium concentration in the heart was decreased by perfusion with digoxin for 30 min even in the presence of insulin. Thus, binding of digoxin by the microsomal fraction was not interfered with by potassium[15].

These results are consistent with the observation by Binnion and Morgan[58], who documented a more than two-fold increase in myocardial concentration of digoxin in the dogs made hypokalemic by infusion of glucose and insulin. They speculated that when glucose and insulin were given, potassium moved from the digitalis receptor site into the cell, enabling digoxin to attach in higher concentrations to the myocardial cell.

Whatever the complex interactions of these two drugs on potassium fluxes may be, it would appear clear that the potentiation which we have observed as a result of additional insulin to the perfusion fluid cannot be due to simple changes in potassium flux or in the potassium content of the cardiac muscle cells.

Several lines of evidence suggest that the sugar transport system may be involved in the movement of the cardiac glycosides to their site of action. First, it is well known that the positive inotropic response to digitalis is prevented by a high potassium concentration in the bathing medium[59]. This effect is identical to the effect of potassium concentration on the uptake of the cardiac glycosides[14]. Bihler[60] has reported that the transport of sugar is decreased in a high potassium concentration and increased in a potassium-free bathing medium, but has linked this to the binding of the cardiac glycosides to the Na-K adenosine triphospatase of cardiac muscle[61]. Secondly, Bailey and Dresel[62] have shown that the inotropic response to ouabain was blocked by a concentration of phloretin sufficient to block membrane transport, but no metabolic processes, but this concentration blocked neither inotropic interventions by other agents nor oxidative metabolism which suggests that this effect of phloretin is relatively specific for the cardiac glycoside. Dutta *et al.*[17] have shown that phloretin in similar concentrations decreased the total tissue uptake of ^{3}H-digitoxin by guinea pig hearts.

Thirdly, Bailey and Dresel[18] have indicated that increased positive inotropic effect to ouabain is potentiated by highly purified insulin and we have also shown that the positive inotropic effect of digoxin is significantly correlated with the amount of the drug bound to the microsmal fraction in perfused guinea pig hearts and that insulin increases the inotropic effect as well as the microsomal binding in this preparation. These suggest that sugar transport is required for the initiation of the positive inotropic response to the cardiac glycoside[62-65].

An alternative explanation for these observations is that the major effect of glucose and perhaps of insulin is on the distribution of the digitalis substances to pharmacologic site after they have been transported into the intracellular space. This interpretation is in accord with that of Dutta and coworkers[16,17,44] who worked with isolated spontaneously beating guinea pig hearts that the accumulation of ouabain is dependent upon the function of ATP-generating systems, derived either from glycolytic or oxidative metabolism. This, the increase of glucose transport either by elevating the glucose concentration or by adding insulin serves to increase ouabain accumulation.

They[16] also reported the effect of phloretin on inotropic responses to oubain may be related to alterations of ouabain accumulation by heart tissue. Iodoacetic acid affected greatly on digitalis glycoside accumulation as did phloretin. Iodoacetic acid blocks glycolysis, but it also inhibits mitochondrial oxidative metabolism by reducing the supply of

pyruvate. The effectiveness of pyruvate in reversing the iodoacetic acid effect on ouabain accumulation indicated that iodoacetic acid inhibition of ouabain accumulation was not due to non-specific chemical damage of heart cell membranes, but was specifically due to the blockade of glycolysis. The same conclusion was reached in experiments which showed that dinitrophenol inhibited cardiac glycoside accumulation by the heart[17]. It was also reported that by using fragments of sarcoplasmic reticulum of isolated beef heart that the binding of cardiac glycoside required ATP. These uptake studies were performed by measuring the total tissue uptake. It would be more interesting if they had measured the binding of digoxin by microsomal fraction.

Other reports support the possibility that the effect of digitalis depends on the source of energy in the cell. Wenzel and Nichols[66] showed that fluoride inhibited the effect of ouabain in normal rat ventricular strips and in cat papillary muscles and Berman *et al.*[67] showed the same effect in hypodynamic rat ventricle strips. The findings of Majeski and Berman[68], and of Bennett and Chenoweth[69] are also consistent with such a hypothesis.

It seems possible that the binding of digitalis to the microsomal site, or the expression of its pharmacologic effect is determined in some manner by the activity of the Embden-Meyerhoff pathway, either by the concentration of a metabolite in the intracellular fluid or by the amount of adenosine triphoshate available from this pathway[62]. Factors which decrease glucose metabolism, that is, the absence of a utilizable substrate in the medium, the shift in metabolism when alternative substates are used, the block of glucose transport by phloretin or its reduction in tissues from alloxan diabetic animal, all decrease the positive inotropic effect of digitalis. Insulin, on the other hand, speeds the onset of digitalis action or, in the presents, potentiates the total effect.

The effect of aldosterone and of chlorpromazine—Antagonism of the positive inotropic effect of ouabain by aldosterone was first reported by Lefer and Sayers in 1965 and 1966[71]. There is some disagreement concerning this effect[72] but our results seem fully to confirm those of Lefer. We have no knowledge of a previous report of an anti-digitalis action of chlorpromazine. Neither of these two drugs caused significant changes in the total tissue uptake of digoxin.

Aldosterone decreased the total uptake. This effect on digoxin binding was accompanied by a considerable decrease in the positive inotropic effect. Chlorpromazine, on the other hand, which is known to be bound non-specifically to liver microsomes and concentrated by cardiac tissue[73], decreased the total microsomal uptake of digoxin as much as did aldo-

sterone. However, the positive inotropic effect of digoxin was decreased only slightly.

Despite the apparent dissociation of the positive inotropic effect and the digoxin uptake by microsomal fraction in these hearts, the coefficient of correlation was significant ($r=0.73$, $p<0.05$).

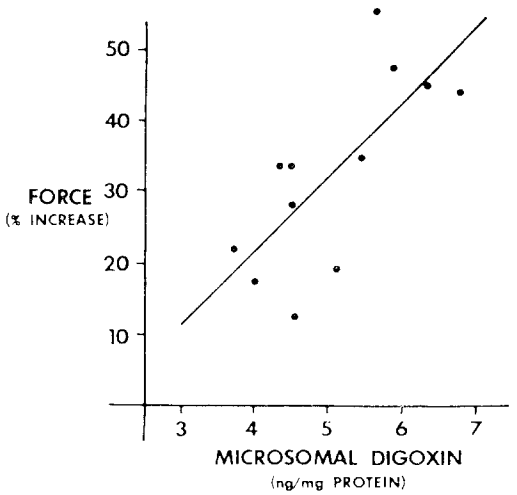

Fig. 1—Correlation between the microsomal uptake of ^{3}H-digoxin and the changes in cardiac contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 minutes with ^{3}H-digoxin alone or in the presence of aldosterone (5.5×10^{-17} M) or chlorpromazine (6.3×10^{-7} M).

Loosely bound digoxin and the positive inotropic effect—Kuschinsky hypothesized that cellular uptake at different concentration of digoxin may be the sum of two different components. The linear component was not saturable and possibly represents the passive movement of digoxin across the cell membrane. The other component, which was saturable, probably represent the accumulation of digoxin at the active site[7]. The availability of the saturable sites was reduced either in the presence of another glycoside or after incubation with metabolic inhibitors[16,74]. It has also been demonstrated that in the guinea pig heart the microsomal uptake of digoxin was inhibited by dinitrophenol[17] and that binding was ATP dependent in fragments of sarcoplasmic reticulum of the dog heart[44]. We have shown a significant correlation between the positive inotropic effect and the uptake of digoxin by the microsomal fraction. We have perfused a few preparations with drug-free medium until contractility returned to control levels. We found that approximately 50% of microsomal digoxin was released after this procedure. Resuspension of the microsomal pellet in a Krebs-

Henseleit solution allowed the total uptake to be divided into two sub-fractions, one which was removed from the microsomes, the other which remained bound to them after this procedure. The correlation between the digoxin released by the resupension procedure and the positive inotropic effect appeared to be better than that of the total microsomal uptake (Fig. 2). The more tightly bound drug was no longer correlated with the positive inotropic effect. These results strongly suggest that it is the digoxin bound loosely to the microsomes which may be causal to the positive inotropic effect whereas that which remained associated with the particulate was not. It should be emphasized that this represents a further fractionation of the bound drug, not of the microsomes, and that no evidence is available at present concerning the constituent of the microsomal fraction which contains the loosely bound drug.

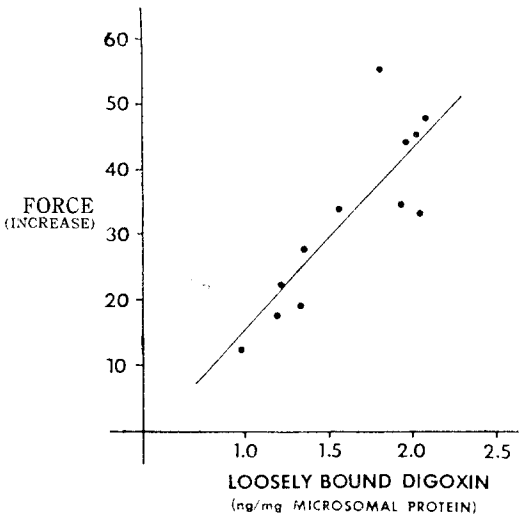

Fig. 2. Correlation between the loosely bound digoxin released from the microsomal fraction during resuspension and the changes in cardiac contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 minutes with ^{3}H-digoxin alone or in the presence of aldosterone(5.5×10^{-7} M) or chlorpromazine (6.3×10^{-7} M.).

The correlations which we have shown are based on the use of drugs which decreases the positive inotropic effect of digoxin. This action is recognized for aldosterone[70] but we have no knowledge of a previous report of an anti-digitalis action of chlorpromazine. Neither drug changed the total uptake of digoxin by the tissue, so that differences in their effect on the intracellular distribution may in fact be causal to their differing anti-digitalis actions. It is thus of considerable interest that the two drugs affect differently the ratio of

loosely bound to more tightly bound digoxin. Aldosterone, on the hand, decreased total microsomal uptake, and decreased the quantity of loosely bound digoxin to an even greater extent. This effect on digoxin binding was accompanied by a considerable decrease in the positive inotropic effect. Chlorpromazine, on the other hand, which is known to be bound non-specifically to liver microsomes and to be concentrated by cardiac tissue[73], decreased the total microsomal uptake of digoxin as much as did aldosterone. However, chlorpromazine interfered less than aldosterone with the uptake of digoxin by the labile binding sites. Correspondingly, the positive inotropic effect of digoxin was decreased only slightly, though significantly in the chlorpromazine treated hearts.

Extrapolation of the regression lines (Fig. 1, 2) to zero effect indicates that there may be considerable binding of digoxin before a positive inotropic effect could be observed under the conditions of our experiments. That is, there is probably a threshed concentration of bound digoxin below which no pharmacologic effect occurs. We have perfused a few preparations with drug-free medium until contractility returned to control levels. We found that considerable quantities of digoxin were bound to the microsomes after this procedure.

Calcium-labile fraction and the positive inotropic effect— It was shown that the magnitude of the positive inotropic effect was correlated with the total quantity of digoxin bound to the microsomes in the guinea pig heart, but was more closely related to the fraction of digoxin released by resuspension of the microsomes in a Krebs-Henseleit solution. We have also shown that approximately 50% of total microsomal digoxin was removed during a 20-min washout period and yet a considerable amount of microsomal digoxin remained in microsomal fraction, although the positive inotropic effect of digoxin disappeared after 20 to 30 min washout with a drug-free Krebs-Henseleit solution. These results suggest that the digoxin responsible for the positive inotropic response is the labile fraction of the total digoxin bound to the microsomes. Therefore, we investigated whether any particular component of a Krebs-Henseleit solution might be responsible for the lability of bound digoxin (Fig. 3). Approximately one-fourth of the total microsomal digoxin was released when the microsomal pellet was resuspended in a sucrose solution containing EDTA. Addition of the calcium to the sucrose resuspension medium released comparable amounts of digoxin from the microsomal fraction as did the Krebs-Henseleit solution. An urea solution was used as a resuspension medium. Dutta *et al.*[43] have shown that bound digoxin to the microsomal fraction may be associated with the membrane

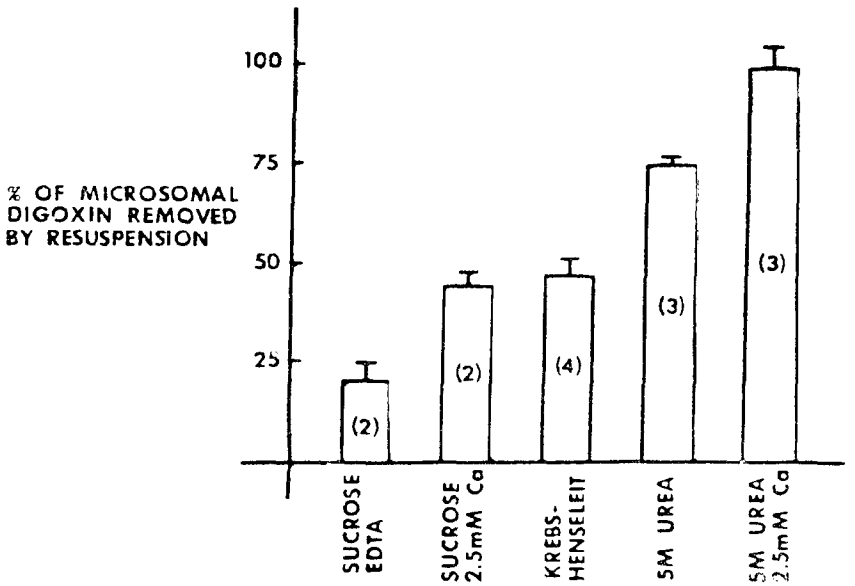

Fig. 3—Comparison of the percent of digoxin released from the total microsomal fraction by various resuspension media. Bars represent standard errors. Guinea pig hearts were perfused with a Krebs-Henseleit solution containing digoxin (2×10^{-7} g/ml) for 30 min. Number in parenthesis represents number of experiments.

macromolecule by hydrogen binding forces. Resuspension of the microsomal fraction in a 5 M urea solution released approximately 75% of the total microsomal digoxin. Addition of 2.5 mM calcium to the urea solution caused the release of the remaining 25% of the bound digoxin. This additional release was thus the same as that caused by the addition of calcium to a sucrose solution containing EDTA. The results suggest that the digoxin bound to microsomes may be categorized into three sub-fractions. A fraction, consisting of about 25% of the bound digoxin, which is released by resuspension in distilled water, sucrose plus EDTA or isotonic NaCl, second fraction, removed by resuspension in 5M urea comprising 50% of the bound digoxin, and a final fraction of approximately 25% of the total mcirosomal bound digoxin which can only be removed when the resuspension medium, either sucrose or urea, contain calcium ion. The result is in harmony with the observation on interaction between digitalis and calcium in the microsomes. Lee and Choi[75] and Carsten[76] found that both ouabain and strophanthidin inhibited the cacium uptake of isolated cardiac sarcoplasmic reticulum fragments. Klaus and Lee[77] showed that cardiac glycosides increased the amount of free releasable calcium fraction of sarco-

plasmic reticulum fragments of dog heart. On the basis of these results, they suggested that calcium releasing effect of cardiac glycosides might be related to the positive inotropic effect. Pfordte and Foerster[78] studied the binding of cardiac glycosides to serum albumin protein *in vitro* and have shown that calcium ions released cardiac glycosides from their protein binding and suggested that calcium ion and cardiac glycoside competed for the same binding site.

In view of these results, we speculated that calcium-labile fraction might be related to the positive inotropic effect and might contain the digitalis receptor. If this hypothesis is correct, then the inotropic effect should be directly related to the absolute amount of digitalis on the calcium-labile site. We have shown previously that aldosterone decreased the positive inotropic effect by decreasing the quantity of digoxin bound loosely to the microsomes, but without affecting the total microsomal content. In addition, insulin has been shown to increase the positive inotropic effect by increasing the microsomal uptake of digoxin. Thus, we tested the effect of insulin and aldosterone on the digoxin binding on the calcium-labile site. The amounts of digoxin on the calcium-labile fraction was obtained from the microsomal digoxin remaining after shaking in the 5 M solution. The correlation between the digoxin on the calcium-labile fraction and the positive inotropic effect appeared to be better than that of the amount of the digoxin on the microsomal fraction and the loosely bound digoxin (Fig. 4). The digoxin released by urea was not correlated with the positive inotropic effect. Aldosterone significanty decreased the ratio of calcium labile to urea releasable digoxin, but insulin did not affect this ratio significantly. This effect on digoxin binding on the calcium-labile fraction was accompanied by a considerable increase or decrease in the positive inotropic effect. These results strongly suggest that the digoxin released by calcium is bound to the receptor site responsible for the positive inotropic effect in the heart.

Calcium labile fraction was also confirmed by the dog microsomes. The pattern of release of digoxin from the dog microsomes was similar to that in guinea pig heart. Therefore, we attempted to isolate the digoxin bound to calcium-labile fraction of the microsomes by the recentrifugation of the microsomal fraction on the discontinuous sucrose density gradient. Bound digoxin was recovered with the highest specific activity between the sample layers and 35% sucrose, in addition, addition of calcium caused to decrease the specific activity of this interface significantly, whereas other interfaces were not affected. These results suggest that the calcium-labile fraction was bound to the light

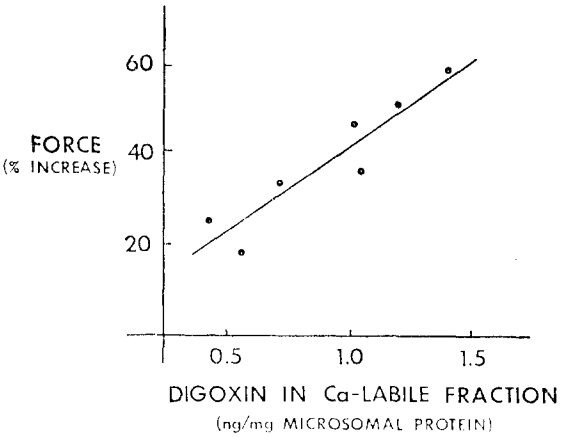

Fig.4—Correlation between the amounts of digoxin on the calcium-labile fraction and the changes in contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 min with digoxin alone or in the presence of aldosterone (2×10^{-7} g/ml) or insulin (2 mU/ml).

microsomal fraction. It was found by electronmicroscopy that this interface did not contain mitochondria fraction and consisted of homogeneous membraneous vesicles.

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