CORRELATION OF THE SUBCELLULAR DISTRIBUTION OF DIGOXIN WITH THE POSITIVE INOTROPIC EFFECT IN THE GUINEA PIG HEART

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ABSTRACT

We have investigated the effect of insulin, chlorpromazine and aldosterone on the positive inotropic effect and the subcellular distribution of ^{3}H-digoxin in isolated, electrically driven guinea pig hearts. Insulin increased the positive inotropic effect of ^{3}H-digoxin and the uptake of the drug by the microsomal fraction, but chlorpromazine and aldosterone decreased the positive inotropic effect of ^{3}H-digoxin and uptake of the drug by the microsomal fraction. However, the total tissue uptake and the tissue:medium ratios of the radioactivity did not differ significantly from those in untreated hearts. Since all measurements were made in the same experiments, we were able to show a significant correlation between the positive inotropic effect and the content of ^{3}H-digoxin in the microsomal fraction, but not in the mitochondrial or nuclear fractions. Approximately one-half of the bound drug was released into the supernatant when the microsomal pellet was shaken in Krebs-Henseleit solution and centrifuged. There was a greater correlation of the inotropic effect with the loosely bound drug than with the total microsomal binding, and no correlation between contractility and the drug remaining in the microsomal pellet. Approximately 25% of the total microsomal digoxin was released when the microsomal pellet was resuspended in sucrose solution containing EDTA. Addition of 2.5 mM calcium to the sucrose

resuspension medium released an additional 25%, so that the total was comparable to the digoxin released by Krebs-Henseleit solution. Resuspension of the microsomal fraction in 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, which we have called the calcium-labile fraction, was thus the same as that caused by the addition of calcium to the sucrose solution containing EDTA. In a dose-response study with the positive inotropic range of digoxin concentrations, the amount of digoxin in the calcium-labile fraction was correlated well with the positive inotropic effect; however, digoxin uptake by the total tissue and the digoxin content in the total microsomal fraction were correlated equally well. The ratio of calcium-labile to urea releasable digoxin was not changed significantly. The effect of insulin and aldosterone on the digoxin binding on the calcium-labile site was also investigated. Aldosterone significantly decreased the ratio of calcium-labile to urea releasable digoxin, but insulin did not affect this ratio significantly. There was a significant correlation of the inotropic effect with the calcium-labile digoxin, but not with the urea releasable digoxin. Gel filtration of Krebs-releasable fraction showed that the calcium-labile digoxin was not bound to a macromolecule. These results suggest that the digoxin released by calcium may be bound to the receptor site responsible for the positive inotropic effect of the drug.

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I INTRODUCTION

A. Historical Background

The cardiac glycosides were known as a medicine to the ancient Egyptians and were mentioned 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 the name Digitalis purpurea because the flower resembles a finger and is purple. Primitive people have used various crude extracts of 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 (1925) 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.

1. Binding of digitalis glycosides to cardiac tissue

a. Total tissue uptake and the positive inotropic effect

Tissue distribution studies indicates that the organs concerned with metabolism and excretion, such as the gstrointestinal tract, liver and kidney, contain the highest concentration of digitoxin and its

metabolites (Okita, et al., 1955; Doherty, 1968). Various studies have confirmed that little digoxin is metabolized by mammals, either in vitro or in vivo (Lage and Spratt, 1965; Marcus, et al., 1964; Kuschinsky, et al., 1967). 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 (Lage and Spratt, 1965). 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 (Gonzales and Layne, 1960). In an attempt to determine the locus of action of digitalis, several investigators have reported on the intracellular distribution of cardiac glycosides in cardiac muscle using differential centrifugation (St. George et al., 1953; Spratt and Okita, 1958). 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 (1955), 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 may be related to the amount of the glycoside taken up by the myocardium (Conrad and Baxter, 1964; Kuschinsky et al., 1967).

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.(1971) 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., (1963) 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 may be affected by a smaller amount of ouabain than is required to affect conduction in the ventricle. Dutta and Marks (1969a) have recently shown that the accumulation of ouabain by isolated guinea pig heart is inhibited by pharmacologically active cardiac glycosides. They have also reported that insulin increased ouabain accumulation (Dutta and Marks, unpublished observation), and phloretin or phlorizin reduced ouabain uptake by the heart (1969b). This is consistent with the suggestion that the effect of phloretin and insulin on the inotropic responses to ouabain or K-strophanthidin (Bailey and Dresel, 1969; 1970) may be related to alterations of accumulation of the glycoside or aglycone by heart tissue. Their study (Dutta and Marks, unpublished observation) 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 (Dutta et al., 1969b).

The increase in contractile force for a variety of cardiac glycosides occurs within a small range of concentrations in guinea pig

atria (Lüllman, et al., 1969). 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. This disparity occurred despite the fact that digitoxin is at least two times as potent as ouabain in this particular preparation (Kuschinsky et al., 1968a). The tissue:medium ratio was 3 when 1×10^{-7} g/ml digoxin was added to the bathing medium (Kuschinsky et al., 1967). 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 (Waldi, 1962) and this is accompanied by an increased accumulation. These findings suggests 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 (Scholtan et al., 1966; Kuschinsky et al., 1968). 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 (Kuschinsky et al., 1968c). 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 suggests that total tissue

uptake is not related with the increase in contractility. Morgan and Binnion (1970) 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 positive inotropic effect. Kuschinsky et al., (1969) 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., (1969) 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 inotropic effect was not parallel with the loss of total tissue concentrations. Their suggestion is in agreement with the conclusion of Okita (1969) and other investigators (e.g. Katzung and Meyers, 1965; Marcus et al., 1964) 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 re-absorption of the drug across the lipid membrane of the intestinal mucosa and the kidney tubules.

Moran (1963) 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 (1967) 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 myocardial receptor and the positive inotropic action are determined by the number of contractions. In contrast, Vincenzi (1967) 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 (1969) 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 Okita et al., (1967) and Roth-Schechter et al.,(1970a) 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 (1970a) 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., (1967) 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., (1970b) 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 to protein receptors and have a strong and persistent binding affinity (Hokin et al., 1966).

Several autoradiographic studies which attempt 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 (1963) and Tubbs et al., (1964) 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., (1964) and Fozzard and Smith (1965) in a later study showed the cardiac glycoside to be concentrated in T-tubule. Conrad and Baxter (1964) reported glycoside radioactivity to be concentrated in the cell membrane as well as intracellularly in the A-band. However, Luchi and Conn (1962) 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 greatest concentration of the drug need not necessarily be the site of its primary action (Kuschinsky et al., 1968b; Lüllman and Zwieten, 1969a). 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.

b. The effect of potassium and sodium ions on the cardiac tissue uptake.

Changes in the extracellular concentration of potassium and sodium have long been known to affect myocardial contractility. Since Schatzmann (1953) showed that cardiac glycosides inhibit the transport of sodium and potassium across the membrane of the red blood cell it has become evident that digitalis inhibits active transport in many other tissues, including cardiac muscle (Glynn, 1964; Skou, 1965; Page, 1964; Repke, 1965 and Weatherall, 1966). Hajdu and Leonard (1959) reviewed the literature on myocardial potassium and sodium ion movements during failure and subsequent restitution of contractile force by digitalis. They concluded that loss of intracellular potassium caused by digitalis was causal to the inotropic effect. However, a number of careful studies, particularly those of Klaus and coworkers (1962), clearly demonstrated that this effect of ouabain on ion distribution is not the basis of its inotropic effect. Farah (1969) concluded that the glycoside-induced loss of intracellular potassium is most likely not causally related to the positive inotropic effect but may be related to severe toxic manifestations produced by the glycosides.

There is evidence of an inverse relationship between serum potassium concentration and the myocardial concentration of digitalis necessary to produce the positive inotropic effect. In previous study, Fujino et al., (1964) showed that the presence of 8.4 mM potassium retarded the rate of uptake of digitoxin in the electrically stimulated guinea pig heart in vitro. This study was consistent with the suggestion that the cardiac glycosides compete with potassium for a

binding site possibly on the cell membrane (Page, 1964; Glynn, 1957; Hoffman, 1966). In support of this hypothesis, Ebert and associates (1963), Marcus and coworkers (1969) and Morgan and Binnion (1970) demonstrated a decrease in the myocardial uptake of ^{3}H-digoxin in the hyperkalemic dog. Ebert and colleagues (1963) showed that ^{3}H-digoxin was displaced by potassium although this effect could not be reproduced by other workers (Marcus et al., 1969). Luchi and coworkers (1971) could not demonstrate that hyperkalemia induced prior to the administration of ouabain result in a decreased myocardial content of ouabain. However, they obtained a result similar to Marcus and colleagues when digoxin was used.

The increase in cardiac sensitivity to digitalis glycosides associated with hypokalemia is well documented (Kleiger, et al., 1966; Zeeman et al., 1954) and is clinically important (Lown et al., 1951). In dogs made acutely hypokalemic by dialysis, there was a 60% decrease in the dose of acetylstrophanthidin required to produce toxicity (Lown et al., 1952). One possible explanation for this phenomenon is that low extracellular potassium facilitates the rate of uptake of digitalis by cardiac muscle cells.

Several attempts to define the myocardial uptake of cardiac glycosides as affected by hypokalemia have yielded conflicting results. It was reported by Lefler and coworkers (1968) that potassium-depleted guinea pigs had a higher myocardial and renal concentration of glycosides than their controls. Dutta and Marks (1969a) also observed that there was an inverse relationship between the concentrations of potassium ions in the extracellular medium and the myocardial uptake of tritiated ouabain in isolated guinea pig hearts. Binnion and

Morgan (1970) measured more than a 2-fold increase in the myocardial concentration of digoxin in 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. On the other hand, Harrison and Brown (1968) found a lower concentration of radioactivity in the heart of potassium-depleted rats one hour after injection of tritiated digoxin. Radioactivity in the liver and kidney was higher, as was that in the blood. Cohn et al., (1967) could not detect a higher concentration of myocardial radioactivity in potassium-depleted mice until 24 hours after tritiated digoxin was given. These results are not consistent with the hypothesis that there is competition between potassium and cardiac glycosides for binding to cell membrane sites. If there were competition between potassium and digitalis for locus binding in the heart, the early peak myocardial glycoside concentration should be higher in the potassium-depleted rat than in the normal animal. Marcus and coworkers (1971) showed recently that there was no difference in the myocardial concentration of digoxin between comparable hypokalemic and normokalemic groups. The hypokalemic dogs infused with digoxin exhibited toxicity (three or more consecutive premature ventricular beats) after receiving an average of 39% less digoxin than the normokalemic animals. At that point, the myocardial concentration of digoxin was an average of 35% less than that of the normokalemic dogs. Therefore, myocardial sensitivity to digitalis during hypokalemia could not be due to enhanced cardiac uptake of digoxin. They also found that the hypokalemic dogs had a lower blood concentration of digoxin than the

normokalemic controls. This was not due to the excretion of digoxin in urine and bile. They suggested that it may be due to the increased tissue uptake of digoxin in the hypokalemic dogs, perhaps by the kidney. Dutta et al., (unpublished observation, 1970)could not demonstrate the correlationship between the total tissue uptake of cardiac glycoside and the positive inotropic effect in recent studies. Low-potassium medium decreased the cardiac activity, but increased the ouabain accumulation by threefold and addition of iodoacetic acid to this low-potassium medium caused profound depression of ouabain uptake, while cardiac activity was not changed.

These conflicting results may be explained by species differences, since Lefler et al., (1968) found that external potassium concentration did not influence the uptake of these glycosides by rat heart slices, but did affect that of the dog, rabbit and guinea pig heart slices.

These studies do not include the time course of myocardial uptake of digoxin and they do not provide information as to the onset or peak inotropic action during hypokalemia. Prindle et al., (1971) showed that a shorter time is required for maximum isometric tension development in cat papillary muscle exposed to digoxin in a Krebs-Henseleit solution with a potassium concentration of 1.5 mEq/l than in one exposed at a potassium concentration of 4.5 mEq/l. Muscles bathed in Krebs-Henseleit with a potassium concentration of 7.5 mEq/l took a longer time than controls to reach peak isometric tension. The rate of muscle accumulation of ^{3}H-digoxin was inversely related to the bath potassium concentration, suggesting that the change of contractility may have been due to a decreased uptake of digoxin by the heart.

However, the peak isometric tension and the concentration of digoxin at peak tension was the same in the presence of low, normal or high potassium. It was suggested that an increase in internal sodium is the factor responsible for the increased force of contraction (Miller, 1965). Since each beat of the heart leads to an entry of sodium, Glynn (1964) believed that the positive inotropic effect of cardiac glycoside was most likely due to inhibition of the sodium pump which increases the intracellular sodium concentration, and this in turn displaces calcium from the carrier and allows "free calcium" to exert its action on the contractile element. Harrison et al., (1969) investigated to determine whether sodium depletion influenced myocardial binding of cardiac glycosides in intact dogs and showed that depletion of serum sodium inhibits binding of digitalis glycosides to the myocardium during the first hour after injection. These results suggest that the binding of digoxin depends both on the myocardial and serum sodium concentration. Dutta and Marks (1969a) reported similar results in that the uptake of ^{3}H-ouabain by the guinea pig heart was directly related to the concentration of sodium ions in the perfusion medium. Palmer and Posey (1967) suggested that sodium ions may release calcium from cardiac sarcoplasmic vesicle.

c. The microsomal uptake of cardiac glycosides

Dutta and Marks showed that ^{3}H-digoxin (1968a) and other glycosides (1968b) 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 (1969b). Dutta and Marks (1968b; 1969a) 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 (see above). 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 is required and 10 mM KCl reduced the binding of cardiac glycosides by sarcoplasmic reticulum fragments. Dutta et al., (1968b) postulated that the potassium-dependent binding 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., (1968a), on the basis of kinetic studies with ^{3}H-ouabain, suggested that the digitalis receptor site may be located on the outer surface of the membrane. Their results suggest 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 (Dutta et al., 1968b).

2. Cardiac glycosides and calcium

Calcium ion is required for contraction of cardiac muscle (Ringer, 1883) and the presence of calcium ion is essential for the development of the inotropic effect to the cardiac glycosides (Clark,

1913; Loewi, 1918). Much evidence has accumulated in recent years indicating that calcium represents one of the major steps in excitation-contraction coupling (Sandow, 1965). It is thought that the wave of depolarization of the cell membrane is conducted deep within the cell by the transverse tubules (Huxley and Taylor, 1958). Depolarization of the transverse tubules causes calcium entry from this site (Niedergerke, 1957; Winegrad, 1962) but also causes the release of bound calcium from the sarcoplasmic reticulum (Lee, 1967; Inesi et al., 1964; Weber et al., 1967), which initiates muscular contraction. The ion is necessary for the interaction of myosin, actin and ATP which causes shortening of the contractile units. During repolarization, muscle relaxation is caused by the rebinding of calcium by the sarcoplasmic reticulum, a process requiring ATP. Thus, the sarcoplasmic reticulum is sometimes called the relaxing factor. It was found that cardiac glycosides increased the amount of free releasable calcium of sarcoplasmic reticulum fragments of dog heart (Klaus and Lee, 1969). This calcium releasing effect of cardiac glycosides was dose-dependent and had a correlation with the cardiotonic potency. They (Klaus and Lee, 1969) proposed that this calcium releasing effect of cardiac glycoside may be related to the mechanism of inotropic effect of cardiac glycosides. Lee et al., (1970) have shown recently that the cardiac glycosides cause the release of calcium not only from the sarcoplasmic reticulum but from the mitochondria as well. Both sources of calcium may contribute to the increased contractility after exposure to the cardiac glycosides. Dhalla et al., (1970) found that the mitochondrial fraction contains higher specific activity than sarcoplasmic reticulum in the isolated perfused rat heart and suggested that the free calcium

concentration in the heart is determined mainly by the mitochondria, sarcoplasmic reticulum and movements across the cell membrane. In this regard, it was demonstrated that the presence of calcium-activated-ATPase in the heart sarcolemma, which may be linked with the movements of calcium across cell membrane (Sulakhe and Dhalla, 1971).

The inotropic effect of the glycosides appears to be related to an increase in the rate of calcium exchange and not a change in the total tissue calcium content (Klaus and Kuschinsky, 1962; Klaus, 1963; Holland, 1966; Lüllman and Holland, 1962; Gersmeyer and Holland, 1963a,b), whereas toxic concentration of cardiac glycosides (those producing arrhythmias and/or contracture) cause an increase in both tissue calcium content and the exchangeable intracellular calcium fraction (Sekul and Holland, 1960; Thomas, 1960; Lee et al.,1961; Lüllman and Holland, 1962; Klaus, 1963). Bailey and Sures (1971) have investigated the effect of ouabain on the efflux and influx of calcium with a specific pool, Ca_{II}, known to be involved in the contractile process in the heart (Bailey and Dresel, 1968). They showed that ouabain significantly increased the rate of calcium accumulation and the quantity of calcium taken up by the heart. They suggested that ouabain increases the apparent volume of calcium distribution in the heart by increasing the bulk flow of calcium into the cell from the extracellular space. These effects of ouabain on calcium metabolism in the heart may be related to the positive inotropic effect of the drug. Other studies suggest that the cardiac glycosides increase calcium influx and exchange without directly affecting calcium efflux or content in rabbit atria. (Holland and Sekul, 1959; Govier and Holland, 1965), guinea pig atria (Lüllman and Holland, 1962), rat atria (Gersmeyer and Holland, 1963) and goat heart (Bailey and Harvey, 1969).

Recently Kuschinsky et al., (1968a) concluded that this effect of ouabain on calcium exchangeability was indirect and resulted from an interaction between the drug and receptor on the cell surface membrane. Lee and Choi (1966) and Carsten (1964, 1967) have proposed, however, that the ouabain-induced change in calcium exchangeability reflects its direct action on the sarcoplasmic reticulum and that this results in a significant diminution in the ability of the reticulum to accumulate calcium. Chipperfield and Nayler (1969) have reported that ouabain displaces calcium isolated from microsomes of perfused rabbit heart. These possibilities are seemingly supported by the results described by Dutta et al., (1968a,b), which showed that digoxin and ouabain bind to the microsomes of cardiac muscle cells.

The site of calcium binding in sarcoplasmic reticulum has not been identified. However, MacLennan and Wong (1971) have recently isolated a calcium-sequestering protein, calsequestrin, from sarcoplasmic reticulum of skeletal muscle.

3. Binding of digitalis glycosides to cardiac Na, K-ATPase

The mechanism by which digitalis inhibits the influx of potassium is still under investigation. However, it has been established that the active transport of sodium and potassium across the cell membrane is associated with the hydrolysis of adenosine triphosphate by an adenosine triphosphatase (Na, K-ATPase) (Skou, 1965). The Na, K-ATPase which seems to be located in the sarcolemma (Skou, 1965) is highly sensitive to inhibition by the digitalis glycosides, and it has been suggested that it might be the receptor responsible for the cardiotonic effect of these drugs (Repke, 1965; Schwartz et al., 1969). Such a hypothesis was suggested by in vitro findings that glycosides consistently

inhibited Na, K-ATPase activity in cardiac and other tissues (Schwartz, et al., 1962; Skou, 1965; Akera et al., 1969), that the inhibition was specific for cardioactive glycosides (Matsui and Schwartz, 1967; 1968; Albers et al., 1968; Schwartz et al., 1968), and that the concentrations causing inhibition were estimated to be within the range causing the positive inotropic effect (Repke, 1965). More recently it was demonstrated that when dog hearts were perfused with digoxin in situ, inhibition of Na, K-ATPase occurred concomitantly with an augmentation in myocardial contractility (Besch et al., 1970). Similar results were obtained from experiments with perfused frog heart (Brooken and Thomas, 1971).

The binding of ^{3}H-ouabain to the Na, K-ATPase appeared to be related to the inhibitory effect of ouabain on Na, K-ATPase (Schwartz, 1969). The binding required ATP and magnesium ion was increased by sodium, but was decreased by potassium. Active, unlabelled cardiotonic steroids were able to compete for binding to the Na, K-ATPase while non-cardiotonic steroids, such as cholesterol and prednisolone had no effect (Matsui and Schwartz, 1968). However, these workers concluded that the antagonism was not due to simple competition between the cardiac glycoside and potassium for the enzyme. As an alternative explanation they have suggested that the cardiac glycosides function as an allosteric inhibitor of the enzyme at a site not associated with the binding of potassium or sodium even though both sodium and potassium ion are required for cardiac glycoside action (Matsui and Schwartz, 1966, 1967, 1968; Schwartz and Matsui, 1967).

The Na, K-ATPase enzyme reaction sequence presumably involves phosphorylation and dephosphorylation steps. Sodium stimulates phosphory-

lation of the enzyme system by adenosine triphosphate (ATP) and dephosphorylation is potassium ion dependent. Both steps are inhibited specifically by the cardiac glycosides (Albers, 1967). Although it was postulated that cardiac glycoside combines with the phosphorylated enzyme to inhibit the potassium ion dependent dephosphorylation (Matsui and Schwartz, 1968), digitalis will bind to either phospho or dephospho form of the enzyme (Allen et al., 1971). Although earlier studies showed that binding of radioactive cardiac glycosides to preparations of Na, K-ATPase is irreversible (Matsui and Schwartz, 1968; Albers et al., 1968), Allen et al., (1971) and Allen and Schwartz (1970) have shown that the digitalis-enzyme interaction is reversible and is temperature dependent. When the drug was removed from the enzyme, Na, K-ATPase activity was completely restored (Allen et al., 1971).

Assuming that the Na, K-ATPase preparation contains the receptor for cardiac glycoside action on contractility (Repke, 1963, 1965), Schwartz et al., (1969) and Besch and Schwartz, (1970) proposed that cardioactive digitalis glycosides may induce a specific conformational change of Na, K-ATPase, which increases the affinity for calcium at the sodium binding sites, as well as decreases affinity for sodium at these sites (the sodium and calcium binding sites may be closely associated and interdependent rather than one and the same). The increased calcium affinity increases a labile calcium store at the inner surface of the sarcolemma and possibly the T-system, which is then available to participate in contractions of the heart muscle.

It is well known that calcium is a vital link in the excitation-contraction coupling process (Sandow, 1965), thus a process leading to an increase in the availability of intracellular calcium in

the vicinity of the contractile elements would lead to a positive inotropic effect. Glynn (1964) has suggested that the cardiac glycosides might act by increasing the liberation of calcium at the inner surface of the membrane and sodium and calcium are, in fact, competitively bound to an active site on a rat brain microsomal fraction (Alonso and Walser, 1968). In the frog heart, the force of contraction is determined by the ratio $(Ca^2)/(Na)^2$ in the external solution (Niedergerke, 1963) and Lüttgau and Niedergerke (1958) suggest that calcium and sodium ions compete for a carrier at the outer surface of the membrane, and that when the membrane is depolarized the calcium ion carrier complex moves inwards and releases calcium ions into the fibers. Results obtained by Langer and Brady (1968), Caprio and Farah (1967) and Talbot (1968) suggest that the increase in contraction of heart muscle by the cardiac glycosides may be caused by an elevation in membrane calcium concentration which is secondary to inhibition of active sodium transport. However, the relationship between the cardiac glycoside, membrane ATPase activity and contractility are still open to question. In fact, a number of investigators (Lee and Yu, 1963; Repke, 1963; Palmer and Nechay, 1964; Bonting et al., 1964; Brown et al., 1967) have suggested that an increase in Na, K-ATPase activity may be correlated with the positive inotropic effect of the cardiac glycosides.

4. Interation of cardiac glycosides with other drugs and their effect on the positive inotropic effect

a. Insulin

It is well known that insulin facilitates transmembrane sugar transport in adipose tissue and muscle, and can stimulate other transport processes, namely amino acids (Kipnis and Noall, 1958; Wool

and Krahl, 1959) or potassium (Zierler, 1960). The hormone is also known to modify other cellular processes, for example, insulin can antagonize hormone-stimulated lipolysis (Jungas and Ball, 1963; Mahler et al., 1964), and increases the incorporation of amino acid into protein (Penhos and Krahl, 1962). Cuatrecasas (1969) suggested that the interaction of insulin with superficial membrane structures alone may suffice to stimulate transport system and to alter metabolic process. Insulin is known to increase the uptake and pharmacological effects of a wide variety of drugs both in vivo and in vitro (Danysz and Wisniewski, 1965; Wisniewski, 1964; Danysz and Hoffman, 1966; Wisniewski and Zarebski, 1968). Pieroni and Levine (1969) have shown that insulin greatly increased the susceptibility of mice to endotoxin shock and increased the mortality rate of rats (Adamkiewicz, 1961). Keyl and Dragstedt (1954a,b) showed that concentrations of the cardiac glycosides which were toxic to chick embryos after development of the pancreas were inactive when given before the pancreas had developed. Addition of insulin to the embryos before the pancreas developed increased mortality to the cardiac glycosides. Travis et al., (1956) showed that dogs made diabetic by surgical removal of the pancreas were relatively resistant to a lethal dose of k-strophanthin. It was reported that highly purified insulin increases the rate of development of the positive inotropic effect of ouabain and k-strophanthidin in rabbit atria (Bailey and Dresel, 1969, 1971). Bailey and Dresel (1971) showed that a reduced inotropic response was observed in atria from alloxan-diabetic rabbits in the absence of insulin, however added insulin did not affect the rate of onset of the positive inotropic effect in these atria but restored the sensitivity of the atria

to ouabain. Bihler (1968) has shown that the stimulation of sugar uptake by the cardiac glycosides was enhanced after preincubation with insulin. In view of these and other results it has been postulated that the cardiac glycosides entered the cell by a sterospecific transport mechanism involved in the transport of sugar into the cell, and glucose transport and metabolism are required for the initiation of the positive inotropic response to the cardiac glycosides (Travis, et al., 1956). Dutta and coworkers (1969b, 1970) have recently reported that energy production from glucose and its metabolites is required for ouabain and digitoxin binding, and that dinitrophenol, iodoacetic acid and phloretin or phlorizin inhibited cardiac glycoside accumulation by the heart. This is consistent with the observations of Bailey and Dresel (1970) who have demonstrated that there may be a relationship between the glucose transport system and the inotropic activity of ouabain, since phloretin inhibits and insulin potentiates the positive inotropic response to ouabain. Regan et al., (1963) have shown that insulin antagonizes the positive inotropic effect of acetylstrophanthidin, probably due to enhanced potassium uptake in the presence of insulin.

b. Aldosterone

Aldosterone is known to stimulate the active transport of sodium across biological membrane in several tissues including skeletal muscle (Woodbury and Koch, 1957) and kidney tubules (Vander et al., 1958). In contrast to aldosterone, the cardiac glycosides are known to inhibit active cation transport as mentioned previously (Hajdu and Leonard, 1959). Since aldosterone and ouabain appear to exert opposite effects on ion transport, several investigators have postulated that aldosterone may antagonize the pharmacological actions of the cardiac glycosides.

For example, cation transport in rat kidney (Sulser, 1959), sodium transport across isolated frog skin (Bronstein et al., 1958), potassium loss from the isolated guinea pig heart (Kunz and Wilbrandt, 1963), tension development in isolated strips of rat aorta (Schatzmann, 1959). However, several investigators have failed to find such antagonism in cation transport across red blood cell membranes (Ashwini Kumar and Sheth, 1961; Glynn, 1957).

Aldosterone has been shown to exert a cardiotonic effect on the rat heart-lung preparation (Ballard et al., 1960). Lefer and Sayers (1965) and Tanz (1962, 1963) reported that aldosterone exerted a small but significant positive inotropic effect in isolated cardiac tissue. When aldosterone and ouabain were administered together, aldosterone antagonized the positive inotropic effect and toxic effects of ouabain in cat papillary muscle (Lefer and Sayers, 1965; Lefer, 1966). The positive inotropic effect to ouabain was blocked most effectively when aldosterone in the concentration range of 10^{-8} to 10^{-7} was administered 20 min prior to addition of the glycoside. Read et al., (1964) reported that ouabain decreased the accumulation of aldosterone by the heart and suggested that the two compounds compete for a binding site on the cell membrane. In a later study, ouabain was shown to increase ^{3}H-d-aldosterone levels in blood and fat, but to decrease the aldosterone levels in heart tissue (Read, et al., 1969). Fujino et al., (1969) demonstrated an anti-ouabain action of aldosterone in frog ventricle strips and have shown (Yorozuya and Fujino, 1967) that ouabain increased the uptake of calcium during contraction of frog ventricular muscle and that the increased calcium uptake was inhibited by several steroid hormones including aldosterone. In contrast, Levy (1969) and

Richards (1963a, 1963b, 1964) were unable to demonstrate that aldosterone antagonizes the positive inotropic effect of ouabain on rabbit atria under the condition used by Lefer (1966). Jørgensen (1969) has reported that repeated injection of aldosterone increased the activity of Na, K-ATPase in the microsomal fraction from kidneys of adrenalectomized rats.

c. Chlorpromazine

Chlorpromazine has been shown to be bound "non-specifically" to liver microsomes (Dingell et al., 1961). Idänpään-Heikkilä and co-workers (1968) have shown autoradiographically that the drug was concentrated by the heart. High concentrations of chlorpromazine have a cardiac depressant effect in isolated perfused rabbit heart (Melville, 1954) and in perfused rat heart (Langslet, 1970). Calcium associated with the cell membrane seems to be required for excitation-contraction coupling, and Kwant and Seeman (1969) have shown that chlorpromazine competitively displaced the plasma membrane bound calcium, and thus interferes with excitation-contraction coupling. Balzer et al., (1968), however, found that chlorpromazine did not affect the calcium storing capacity of sarcoplasmic reticulum. They found, however, that the rate of calcium exchange was reduced by the drug.

Freeman and Spirtes (1962) have shown that chlorpromazine protected erythrocytes against various forms of lysis. Seeman and Weinstein (1966) confirmed these findings and observed that protection of erythrocytes was associated with a reduction in potassium loss from the cell. It has recently been shown that chlorpromazine also reduces potassium efflux from isolated perfused rat hearts (Landmark, 1970).

Several investigators have attempted to demonstrate inhibition of a Na, K-ATPase by chlorpromazine. Most studies, however, have indicated that the Na, K-ATPase is rather insensitive to chlorpromazine (Judah and Ahmed, 1964; Davis and Brody, 1966). Recently Akera and Brody (1968, 1969, 1970) have found that a semi-quinone free radical of chlorpromazine is a potent inhibitor of brain microsomal Na, K-ATPase activity and that the free radical form of chlorpromazine, rather than chlorpromazine itself, is responsible for the inhibition of the enzyme system in vitro(Akera and Brody, 1968, 1969). They concluded that chlorpromazine free radical inhibits enzyme activity by interacting with sulfhydryl groups on the Na, K-ATPase, whereas ouabain is bound to a different site on the enzyme, thus chlorpromazine free radical does not prevent ^{3}H-ouabain binding on Na, K-ATPase, but only inhibits the enzyme activity.

Although high concentrations of chlorpromazine have a cardiac depressant effect in isolated hearts, we know of no report concerning the interference of chlorpromazine with the positive inotropic effect of digitalis, and we hoped that low concentrations of chlorpromazine might affect the binding of digoxin without affect on the pharmacological effect

B. Statement of Problem

According to a basic pharmacologic principle (Clark, 1937; Furchgott, 1964), the cardiac glycosides must exert their effect by first combining with the digitalis receptor specific for the positive inotropic effect. However, digitalis combines not only with the particular site for production of the positive inotropic response, but

also with non-specific sites in the heart. There may be other sites within a cell which may have specialized functions, for example, enzymatic removal, storage, or transport of the cardiac glycoside, which may modify the concentration of the agonist reaching the receptor. These sites may or may not be the receptor.

This investigation was directed mainly to the study of the subcellular distribution of the cardiac glycosides, and to relating the binding of digitalis glycosides to the various subcellular components to the positive inotropic effect. Unlike the previous experiments (Dutta et al., 1968a) the distribution of the glycosides was measured in the same experiment in which the pharmacological effect was determined.

Three agents, insulin, aldosterone and chlorpromazine were used as tools to interfere with the positive inotropic effect of digoxin. It was anticipated that these pharmacological interventions with the positive inotropic response unlike the changes in ionic concentrations examined by Dutta and Marks, probably would not cause major changes in the total tissue uptake of digoxin nor would the agents themselves have an intrinsic effect on cardiac contractility, but would only affect the cellular distribution of the glycosides. Glucose-free medium was also used to interfere with the positive inotropic effect and related to the digoxin uptake by the microsomal fraction. The effect of a variety of ions and other substances on the binding and release of the digoxin bound to microsomes was studied to determine the nature of this binding. Fractionation of the microsomal fraction by sucrose density gradient was also used in an attempt to characterize the digoxin binding site.

II METHODS

A. Experimental Preparation

Guinea pigs were selected as the experimental animal in this investigation primarily because they were sensitive to cardiac glycosides and because the majority of the previous work on digitalis uptake by the heart was done on this species (Dutta et al., 1968a). Rabbit hearts were used in a few preliminary experiments because the effect of highly purified insulin on the rate of development of the positive inotropic effect of ouabain was investigated by using rabbit atria (Bailey and Dresel, 1969). Dog hearts were employed in this study because they were sensitive to cardiac glycoside and because large quantities of the microsomal fraction could be obtained.

Guinea pigs and rabbits of either sex, weighing approximately 300 g and 1 kg respectively, were killed by a blow on the head, and their hearts were rapidly removed and placed in a beaker of cold ($4^{o}C$), oxygenated Krebs-Henseleit solution. This weight range was chosen since the size of heart may affect the uptake of glycoside (Dutta et al., 1968a) and because the hearts of young animals contain proportionally higher concentration of energy yielding lipids than do older animals (Noble et al., 1954). Anticoagulants and anesthetics were not employed because of the possible effect of tissue metabolism (Michajlik and Bragdon, 1960; Galla et al., 1962).

Immediately after extraneous tissue was removed from the heart, the aorta was cannulated. The hearts were perfused by the Langendorff technique (Langendorff et al., 1895) at a constant pressure of 50 mm Hg (65 cm H_2O). A constant pressure was maintained by Mariotte bottle placed approximately 65 cm above the heart (fig. 1). Reservoir A

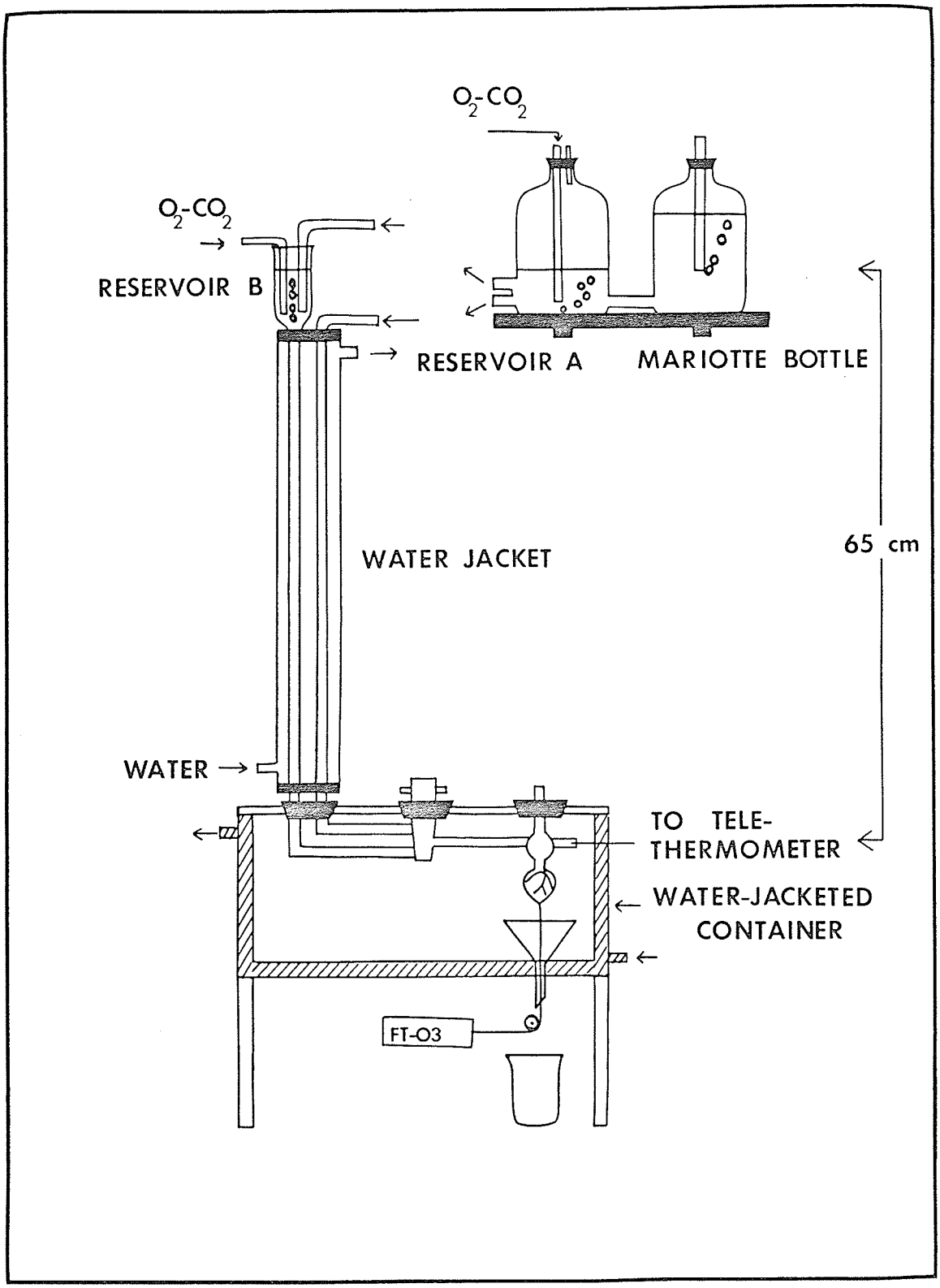


Figure 1.

Apparatus for perfusion

was connected to the Mariotte bottle. Reservoir A, therefore, had a constant level of perfusate during the experiments and reservoir A was gassed with 95% O_2 - 5% CO_2 throughout the experiments (fig. 1). From reservoir A, the perfusate was delivered to reservoir B, in which the level of perfusate was maintained constant, exactly the same as that in reservoir A. After pre-equilibration period of 60 min delivery of perfusate from reservoir A to reservoir B was disconnected and perfusate containing the given concentration of drugs were poured from a beaker into reservoir B manually. The volume of perfusate in reservoir B was approximately 40 ml. Reservoir B was gassed with 95% O_2 - 5% CO_2 throughout the experiments. The perfusate in the reservoir A was delivered to another channel of the water jacket column which was used only to wash the hearts with drug-free perfusate at the end of the experiments. The perfusate in the Mariotte bottle was preheated to $30^{o}C$. The temperature of the perfusion fluid was maintained at $30^{o}C$ by adjusting the water temperature of the column. The heart was enclosed in a water jacketed lucite box, and the atmosphere was saturated with water. The bulb of the tele-thermometer was inserted in one of the arms of the aortic cannula so that perfusion fluid flowed directly over the bulb of the thermometer and enabled the temperature to be monitored just before the fluid entered the heart (fig. 1). The composition of the modified Krebs-Henseleit (1932) solution is given in table 1.

TABLE 1.

The composition of the modified Krebs-Henseleit solution

	g/l	mM	
NaCl	6.9	118.05	
KCl	0.35	4.7	(5.8)*
$NaH_2PO_4.H_2O$	0.162	1.175	
$CaCl_2.6H_2O$	0.548	2.5	
$MgSO_4.7H_2O$	0.290	1.176	
$NaHCO_3$	2.20	26.2	
Glucose	2.0	11.2	

The perfusion medium was equilibrated with 95% O_2-5% CO_2 before used, and the pH was 7.4.

*This concentration of KCl was used only in experiment of spontaneously beating rabbit and guinea pig hearts perfused for 15 min.

Isometric contractile force was measured with a Grass FT-03 force displacement transducer attached by a stainless steel clip and nylon monofilament line to the apex of the heart. The nylon monofilament line attached to the hook on the apex of the heart was led around a pulley to the Grass transducer. When the heart contracted, the muscle shortened along the longitudinal axis of the ventricular chambers, and the deep fibers acted to compress the transverse dimensions of the ventricles. Hamilton and Rompf (1932) and Rushmer (1956) studied the movements of specific points on the heart walls, and concluded that ventricular ejection is primarily accomplished by a longitudinal shortening of the chambers. Thus, measurement of contractile force by our instrument was mainly based on the change of longitudinal axis of heart chambers. The force of contraction was recorded on a Grass Model 5D polygraph. The wire attached to the clip on the apex of the heart served as an indifferent electrode, and the hearts were stimulated at twice threshold voltage (1.5 to 2 volts) by a second stainless steel electrode attached to the right atrium of the heart.

In experiments of spontaneously beating guinea pig hearts and rabbit hearts perfused for 15 min with drug, resting tension was maintained at 1 g. In the remainder of the experiments the resting length was adjusted to produce a developed force of contraction which was 50% of maximum as determined by the length-tension relationship. This was about 3 g for the guinea pig hearts and 7 g for the rabbit hearts. The spontaneous heart rate ranged from 150 to 200/min, thus a stimulation rate of 216/min was selected to preclude irregular beating. In early experiments hearts beating spontaneously were used.

B. Perfusion Procedure

Both the rabbit and guinea pig hearts were perfused for 90 min with Krebs-Henseleit solution before exposure to either ouabain or digoxin (2×10^{-7} g/ml). The hearts were then treated with digoxin for either 15 or 30 min. Untreated hearts were defined as the hearts received digoxin or ouabain alone during perfusion. One-half the digoxin was either ^{3}H-digoxin (G) or 12 α -^{3}H-digoxin (0.93 mC/mg and 4.0 mC/mg digoxin, respectively). The perfusate from each heart was collected throughout each experiment.

After exposure to digoxin the hearts were washed with drug-free Krebs-Henseleit solution for four min to remove digoxin from the vascular and interstitial spaces. This period of washing was shorter than that used by Dutta et al., (1968a) and was based on the demonstration that ^{14}C-inulin (0.25 mc/100 mg) was removed rapidly from the hearts under the conditions of our experiments. Figure 2 shows the time course of the removal of inulin from the extracellular space of guinea pig hearts perfused under our conditions. The hearts had been perfused with inulin for 30 min after 90 min of equilibration. Approximately 95% of the marker was removed by four min of washout with the inulin-free medium. We therefore assumed that most of the digoxin which occupied the inulin spaces was removed by this procedure.

1. Treatment with agents which affect the positive inotropic effect.

Hearts were equilibrated by perfusion with Krebs-Henseleit solution for 60 min. Either recrystallized insulin (2 mU/ml) or aldosterone (2×10^{-7} g/ml) or chlorpromazine hydrochloride (2×10^{-7} g/ml) was added to the perfusate 30 min before exposure to digoxin

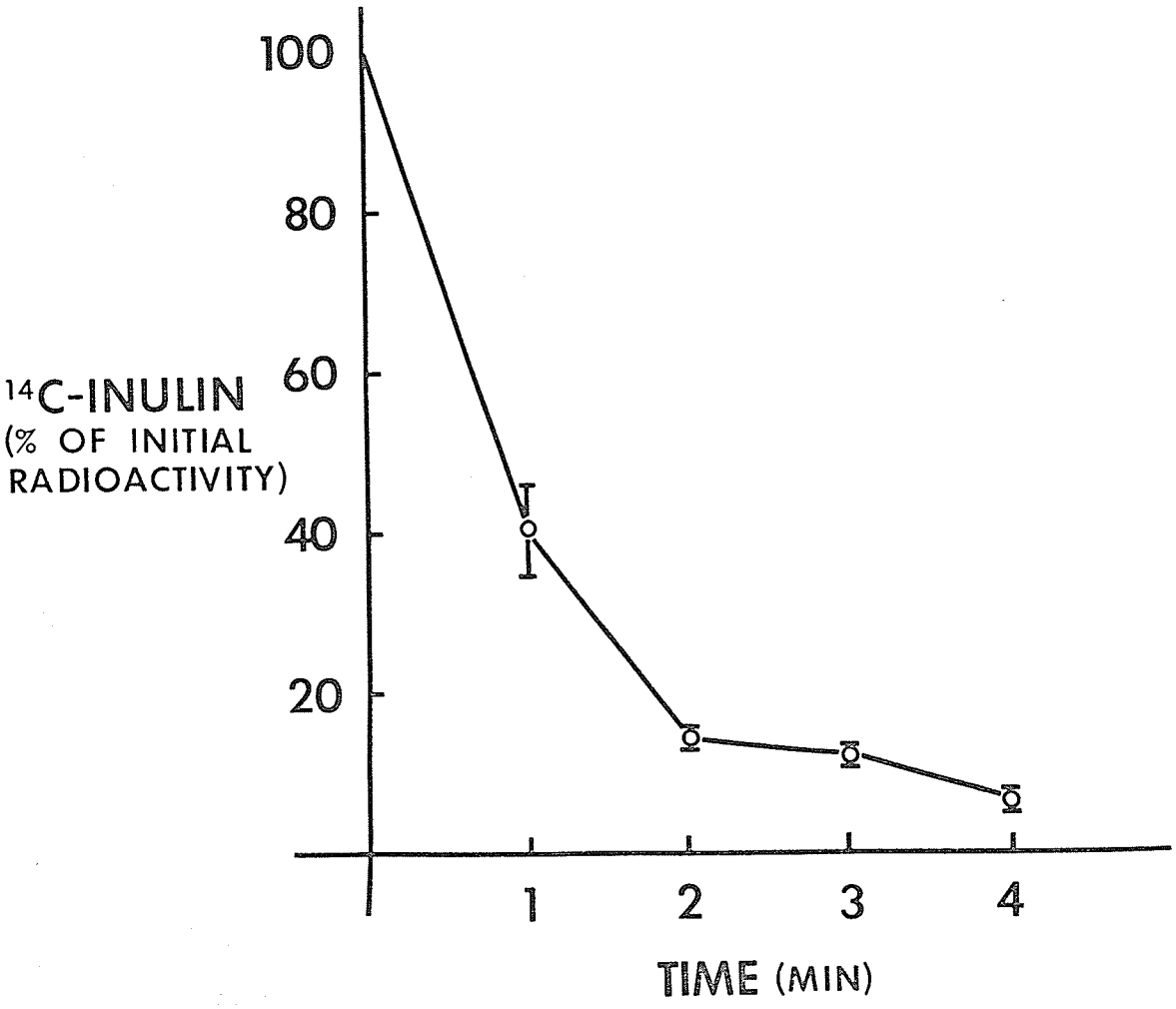


Figure 2.

Time-course of ^{14}C-inulin clearance in guinea-pig hearts. The hearts were perfused with a medium containing ^{14}C-inulin for 30 min. The values are expressed as percent of the amount present at zero time. Each point represents the Mean $\pm$ S.E. of 3 experiments.

and remained in the perfusate throughout the remainder of the experiments. Time control hearts were treated identically except that they did not receive any drug during the two hrs of perfusion.

2. Dose-response relationship

In another series of experiments, the hearts were perfused with the various concentrations of digoxin for 30 min after 90 min equilibration to establish the dose-response relationship. The concentrations of digoxin ranged from 5×10^{-8} to 1×10^{-5} g/ml.

3. Perfusion with substrate-free medium

Hearts were perfused for 60 min with Krebs-Henseleit solution containing 11 mM glucose. The hearts were then perfused with Krebs-Henseleit solution omitted glucose for 30 min before exposure to digoxin. Digoxin was added in the absence of glucose.

4. Treatment with ouabain

Hearts were perfused for 60 min with Krebs-Henseleit solution for equilibration. Recrystallized insulin (2 mU/ml) was added to the perfusate 30 min before exposure to ouabain (2×10^{-7} g/ml) and remained in the perfusate throughout the remainder of the experiment. One-half of the ouabain was ^{3}H-ouabain (G), (20 mC/mg ouabain).

C. Preparation of Subcellular Fractions

The hearts were removed from the cannula and blotted on filter paper to remove excess moisture. A sample of muscle from both ventricles weighing approximately 200 mg was homogenized in a glass homogenizer, extracted for 3 hrs with 2 ml of dioxane, and the radioactivity in the extract was counted on a Phillips Liquid Scintillation Spectrometer.

Dutta et al., (1968a) have shown that this procedure completely extracted digoxin from the tissue.

The remainder of the heart which consisted of both left and right ventricular tissue and the septum was weighed and fractionated by the method of Dutta et al., (1968a). The flow diagram of the procedure is shown in figure 3. Approximately 800 mg of the ventricle was minced in an ice bath and homogenized in 10 volumes of cold ($4^{o}C$) 0.33 M sucrose solution containing 0.001 M ethylenediaminetetraacetic acid (EDTA) by 7 gentle strokes of a glass Potter-Elvehjem homogenizer fitted with a motor driven Teflon pestle. The homogenate was spun in an International Preparative Ultracentrifuge (Model B-60) at 166,000 x g (45,000 rpm) for 60 min at $4^{o}C$ to separate the total particulate from the soluble supernatant fraction (1st supernatant). The total particulate was resuspended in 10 volumes of cold ($4^{o}C$) sucrose-EDTA solution and was centrifuged in a Servall refrigerated centrifuge (Head Type SS-1) at 700 x g (2500 rpm) for 10 min to remove nuclei, myofibrils and other cell debris. The sediment was used as a nuclear fraction, and the supernatant was centrifuged in the same centrifuge at 12,000 x g (12,000 rpm) for 15 min at $4^{o}C$. The resultant pellet was the mitochondrial fraction. The supernatant was centrifuged in the ultracentrifuge at 166,000 x g for 60 min at $4^{o}C$ yielding a 2nd supernatant and pellet. The pellet was the microsomal fraction (fig. 3). Each of the pellets was suspended in a 2 ml of distilled water and the ^{3}H-digoxin content of each subcellular fraction and the perfusion medium was measured in the liquid scintillation spectrometer. The scintillation mixture contained 5.3 g/l of PPO (2, 5-diphenyloxazole) and 66.7 mg/l of POPOP (1, 4-bis-2-(5-phenyl-oxazolyl)-benzene) dissolved in a toluene:Triton X-100 (2:1 v/v)

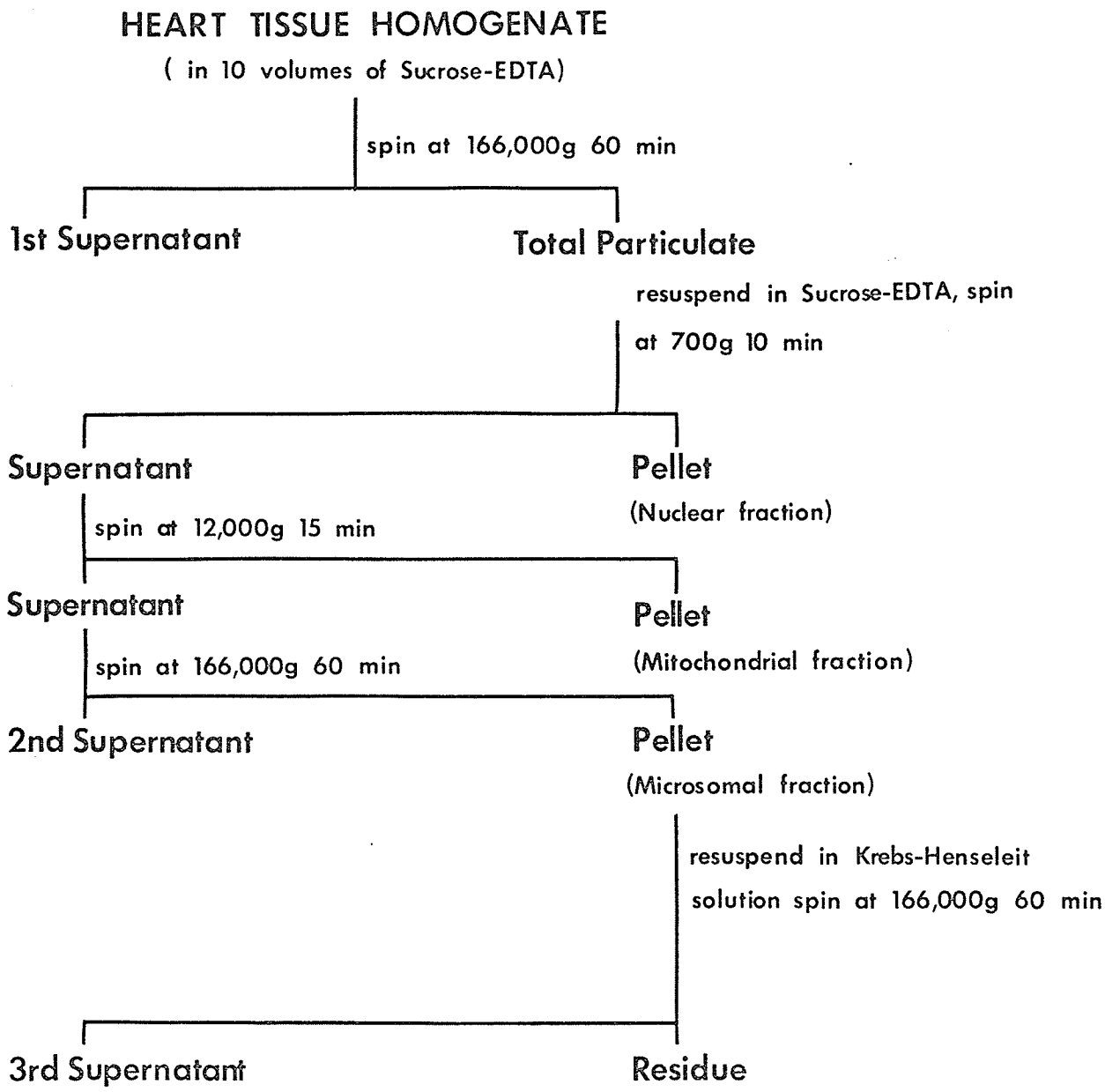


Figure 3.

Flow sheet for subcellular fractionation of guinea pig hearts to obtain the microsomal fraction. See text for details of procedures.

scintillation mixture (Patterson, 1965). The measured ^{3}H-digoxin represents unchanged digoxin, since various studies e.g. (Lage and Spratt, 1965; Kuschinsky et al., 1967) have demonstrated that guinea pig myocardium does not metabolize digoxin in vitro. In order to normalize the digoxin concentration for the purpose of comparison, the protein in an aliquot of each subcellular fraction was measured and the digoxin content was related to the protein content of each fraction. The protein concentration in these fractions was determined by the method of Lowry et al., (1951), modified by addition of 5% deoxycholate to each sample to solubilize the protein. Bovine serum albumin was used as the standard.

D. Determination of the Nature of Digoxin Binding

1. Sucrose density gradient

The distribution of digoxin in the various subfractions of guinea pig microsomes was determined by centrifugation on a continuous density gradient. A one ml aliquot of the microsomal resuspension Krebs-Henseleit solution was applied to the top of a sucrose gradient and was centrifuged for 60 min at 45,000 rpm. A SB-269 International rotor was used. The gradient consisted of 2 ml each 20%, 30%, 40%, 50% and 60% sucrose, layered a day in advance, which produced a gradual concentration gradient within the cellulose centrifuge tube. The microsomes spread into several bands in the tubes. A one ml aliquot of each sucrose layer was obtained from the tubes with a pasteur pipette. The protein concentration and radioactivity in each layer was determined by the methods described previously.

2. Loosely bound digoxin

To determine loosely bound digoxin, the microsomal fraction (fig. 3) was resuspended by shaking for 5 min with a Vortex Mixer in 2 ml Krebs-Henseleit solution in the cold room (4°C). The suspension was allowed to stand in the cold (4°C) for 40 min. The microsomal pellet was dispersed completely within 40 min in most cases. Aliquots were taken for the determination of protein and total microsomal digoxin concentration (as above) and the remainder was centrifuged in the ultracentrifuge at 166,000 x g for 60 min at 4°C and the radioactivity in the supernatant and pellet was counted. We have defined this as the 3rd supernatant. To investigate the nature of the binding processes for digoxin to the microsomal fraction, the microsomal fraction was resuspended in 2 ml of various media for 5 min by shaking with a Vortex Mixer and allowed to stand in the cold (4°C) for 40 min. The resuspension media are given in table 2.

Aliquots were taken for the determination of protein and the total microsomal digoxin concentrations and the remainder was centrifuged in the ultracentrigue at 166,000 x g for 60 min at 4°C. The radioactivity in the supernatant was measured. The ratio of the digoxin concentration in the supernatant was compared to the total microsomal digoxin concentration.

3. Gel filtration

To determine if the digoxin was bound to peptide or other macromolecule after release columns of Sephadex G-15, were prepared according to the manufacturer's recommendations. Twenty g of gel was suspended in 200 ml of distilled water in a beaker. After 12 hrs the water was replaced with an equal amount of 0.9% NaCl. A small amount

TABLE 2.

The various solutions used for resuspension of the guinea pig microsomal fraction.

Krebs-Henseleit solution
Zero Ca Krebs-Henseleit solution
Zero Mg Krebs-Henseleit solution

Distilled water
0.9% NaCl solution
0.33 M sucrose solution containing 1 mM EDTA

0.33 M sucrose solution containing 2.5 mM Ca

5 M Urea solution
5 M Urea solution containing 2.5 mM Ca

120 mM KCl solution in Imidazole buffer

of 0.9% NaCl solution was poured into a 1.5 x 22 cm column, the outlet was closed, and the column was filled with the G-15 gel slurry. When the gel in the column settled down to constant level, flow through the column was adjusted to 30 ml/hr. One ml of aliquot of the 3rd supernatant of the microsomal fraction was placed on the column without disturbing the gel, and eluted with 0.9% NaCl. Protein concentration and radioactivity was measured in one ml samples of the eluate.

E. Dog Heart Experiments

Dog hearts were also used in these investigations because large quantities of microsomal fraction could be obtained. Thus, by using microsomes obtained from dog heart the effect of various media on the *in vitro* release of digoxin from the microsomal fraction could be measured in the same heart.

1. Preparation of microsomal fraction

Hearts were removed from dogs anesthetized with sodium pentobarbital (30 mg/kg) and the coronary vessels were perfused with cold ($4^{o}C$) 0.9% NaCl solutions. The atria, valves, great vessels and most of the epicardial fat were removed. The ventricular muscle was then cut into small pieces and homogenized in a Waring Blender for 45 sec in 0.33 M sucrose solution containing 1 mM EDTA (1:2, w/v), (fig. 4). The homogenate was filtered through 4 layers of gauze and centrifuged. After a centrifugation at 15,000 x g for 20 min in the International Preparative centrifuge (Head Model B-60) at $0^{o}C$, the supernatant was filtered through 4 layers of gauze to remove floating fat layers and was centrifuged at 40,000 x g for 90 min in the same head of the International Ultracentrifuge (first cycle of centrifugation). This

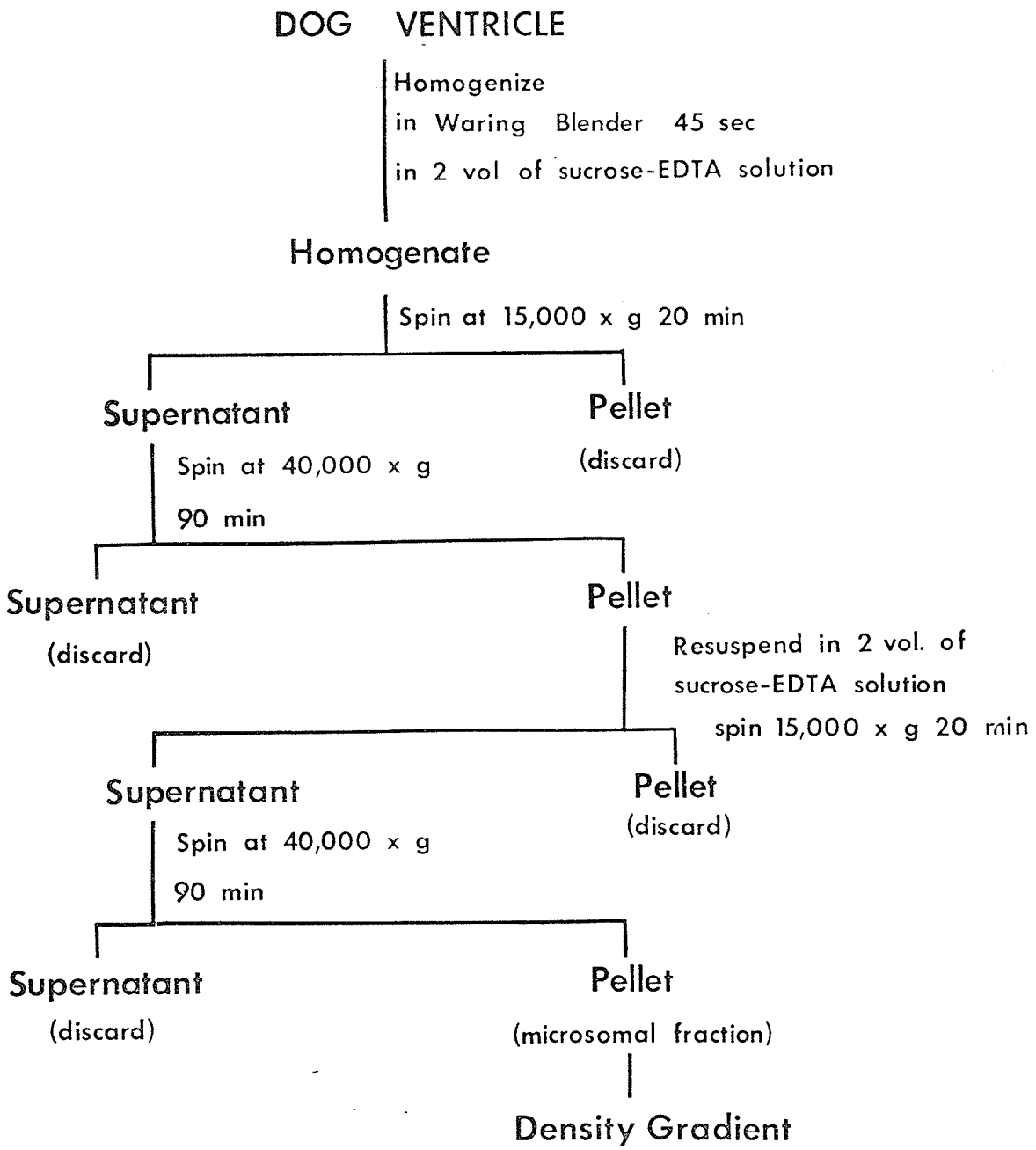


Figure 4.

Flow sheet for subcellular fractionation of dog hearts to obtain the microsomal fraction. See text for details.

sediment was resuspended in 0.33 M sucrose solution containing 1 mM EDTA. Centrifugation at both 15,000 x g and 40,000 x g were then repeated (second cycle of centrifugations), (Pretorius et al., 1969). The pellet (microsomal fraction) was resuspended in the buffer medium (table 3) and incubated with tritiated digoxin (2×10^{-7} g/ml) in polypropylene tubes at 30°C for 30 min in a volume of 2.5 ml. The phosphate buffer composition is given in table 3 and is identical to that used by Dutta et al., (1968b). After incubating the reaction mixture was centrifuged immeidately for 15 min at 54,000 x g. The pellet and tube wall were rinsed twice with cold distilled water and the supernatant was used for the determination of the medium concentration.

The labelled pellet (microsomal fraction) was resuspended in various media (table 4) and shaken for 5 min on the Vortex Mixer as in the experiments with guinea pig hearts.

The radioactivity in the total microsomal fraction and in the supernatant were measured by the methods previously described for the experiments with the guinea pig hearts. Microsomal protein concentration was determined by the method of Lowry (1951).

2. Density gradient centrifugation

We have used six resuspension media to determine which subfraction was affected by Ca ions (table 4). Microsomal pellets obtained from dogs were incubated with ^{3}H-digoxin and were resuspended in the cold (4°C) in 2 ml of one of the six solutions for 5 min. A one ml aliquot of each microsomal resuspension which contained about ten mg of protein was applied to the top of a sucrose gradient and was centrifuged for 2 hrs at 25,000 rpm in an SB-269 International rotor.

TABLE 3.

Buffer medium for incubation of dog microsomal pellet

	mM	
NaCl	100	
$MgCl_2$	2	
NaH_2PO_4	5	
$CaCl_2$	0.03	
Tris-ATP	2	
Tris-maleate	20	at pH 6.5

TABLE 4.

The various solutions used for resuspension of the dog microsomal fraction

Ca-free Krebs Henseleit solution
0.33 M sucrose solution containing 1 mM EDTA
5 M urea solution
Krebs-Henseleit solution
0.33 M sucrose solution containing 2.5 mM Ca
5 M urea solution containing 2.5 mM Ca

The 5 M urea resuspension was found to diffuse into the least dense sucrose gradient. To prevent this, an 0.5 ml aliquot of 5 M urea resuspension was diluted with 1.0 ml distilled water and the diluted microsomal resuspension was added on the top of the sucrose gradient. The gradient (usually) consisted of 4 ml each 35%, 45% and 60% sucrose, layered 1 hr before in a cellulose centrifuge tube. EDTA (0.5 mM) was added to each concentration of sucrose solution to remove free calcium in the sucrose. Three layers were obtained at the interfaces between the sucrose concentration. The centrifuge tubes were punctured with a needle and the layers were removed. (Katz and Rapke, 1967).

The protein concentration and radioactivity in each interface were determined by the methods described previously.

3. Electronmicroscopic examination of microsomal fraction of dog

Pellets obtained from each layer of the density gradient (see above) separation of the microsomal fraction of dog were initially fixed for 1 hr in 2% of glutaraldehyde prepared in 0.1 N Na cacodylate brought to pH 7.3 with concentration HCl. The samples were washed in 0.1 N Na cacodylate-HCl buffer containing 7% sucrose and sliced into blocks no larger than 1 mm^3. The blocks were postfixed for 1 hr in Zettergvist's osmic acid (Sjöstrand, 1967), stained in 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon. Sections were cut using a Reichert OmU_2 ultramicrotome, mounted on uncoated copper grids, and stained with Reynold's lead citrate (Sjöstrand, 1967). Specimens were examined with an Hitachi HS 8 electronmicroscope.

F. Purity of ^{3}H-Digoxin

To avoid the erroneous results because of the contamination

of impure radioactive materials, samples of the ^{3}H-digoxin were tested for purity by thin layer chromatography on Eastman Silica Gel G chromatoplates. Labelled digoxin was mixed with unlabelled digoxin and the mixed digoxin was spotted on the plate. The plate was developed with a solvent of Benzene-95% ethanol (7:3 v/v) for approximately 3 1/2 hrs and developed by the method of Johnston and Lacobs (1966). Only a single spot was detected. To identify the contamination of impure radioactive material, five mm zones of the spot were scraped into counting vials and the ^{3}H-digoxin activity was measured. Only a single radioactive compound corresponding to standard digoxin was detected.

G. Drugs

All drugs and reagents were of analytical grade.

Digoxin	Sigma Chemical Company, St. Louis, Mo.
d-aldosterone	Sigma Chemical Company, St. Louis, Mo.
Chlorpromazine hydrochloride	May and Baker, Ltd., Dagenham, England.
Insulin, recrystallized	Connaught Laboratories (27U/mg).
EDTA sodium salt	British Drug Houses Ltd., England.
Calcium chloride. $6H_2O$	Fisher Scientific Company, N.J.
PPO	Sigma Chemical Company, St. Louis, Mo.
POPOP	Sigma Chemical Company, St. Louis, Mo.
Triton X-100	Sigma Chemical Company, St. Louis, Mo.
Pentobarbital sodium	British Drug Houses Ltd., England.

Since ^{3}H-digoxin (New England Corp., Boston, Mass.) diluent, 90% ethanol in benzene, had a depressant effect on myocardial contractility, the diluent was evaporated under nitrogen and the tritiated digoxin was then dissolved in 90% alcohol. Unlabelled digoxin also dissolved in 90% alcohol. Alcohol amounts which were used in our

investigation did not affect the contractility. D-aldosterone was dissolved in 95% ethanol and chlorpromazine hydrochloride was dissolved in distilled water. Insulin was dissolved in distilled water and acidified with IN-HCl to pH 3.5-4.0 and was diluted to 0.4U/ml with distilled water. The final concentration of insulin in the perfusate was 2 mU/ml.

H. Statistical Analyses

Tests of significance between means were evaluated by student's unpaired *t* test. Test for significance of differences between coefficients of correlation, r, were evaluated by the method described in Steel and Torrie (1960). Correlation analyses between digoxin uptake by the various subcellular fractions was done by the method of least squares. A significant correlation was assumed to exist between the two variables when statistical analysis showed that the coefficient of correlation, r, was greater than zero. In all analyses a probability of 0.05 was preselected as the criterion of statistical significance.

III RESULTS

A. The Positive Inotropic Effect, the Tissue Uptake and Subcellular Distribution of ^{3}H-Digoxin

Insulin is known to increase the uptake and pharmacological effect of a wide variety of drugs *in vivo* (Travis *et al.*, 1956; Wisniewski *et al.*, 1964, 1965, 1966, 1968). Bailey and Dresel (1969) have shown that highly purified insulin increased the rate of development of the positive inotropic effect of ouabain in rabbit atria. It seemed possible that an increase in the response to ouabain *in vitro* might be related to an increase in the tissue uptake of the drug. We, therefore, have used insulin to test for the correlation between the subcellular distribution of ^{3}H-digoxin and its effect on contractility.

Spontaneously beating rabbit and guinea pig hearts and electrically driven guinea pig hearts were perfused for 60 min with Krebs-Henseleit solution and insulin was added after this equilibration. Perfusion with insulin (2 mU/ml) increased contractile force and the inotropic effect was maximum after 7 to 8 min exposure to insulin, but the increased contractility gradually returned to initial contractile force within 30 min (fig. 5). We have shown in work not reported here that this effect of insulin is probably due to release of histamine.

1. Spontaneously beating hearts

a. Effect of insulin on the positive inotropic effect and the subcellular distribution of ^{3}H-digoxin in rabbit hearts.

Rabbit hearts were chosen since it has been shown that insulin increased the rate of development of the positive inotropic effect of ouabain in rabbit atria (Bailey and Dresel, 1969). All

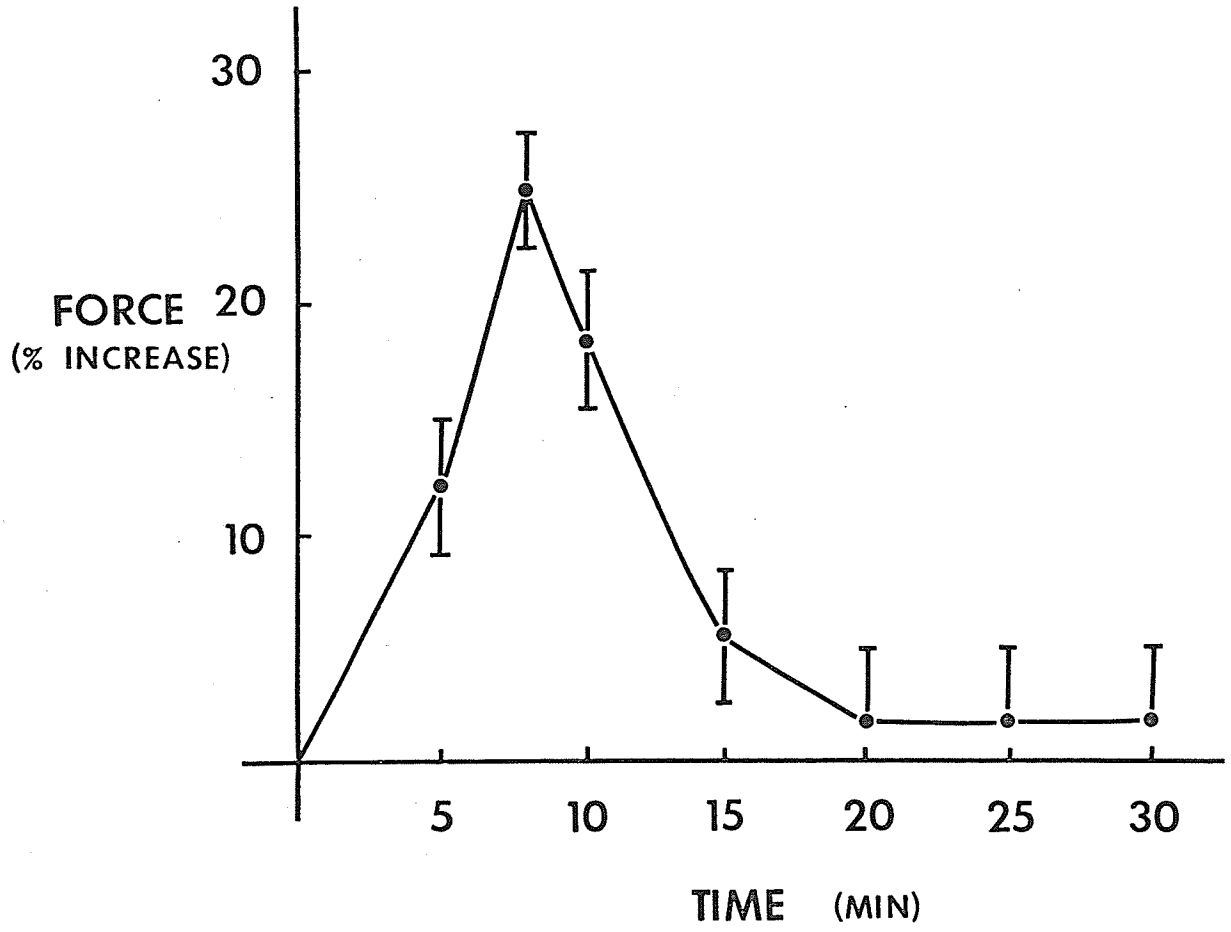


Figure 5.

The effect of insulin (2 mU/ml) on contractile force in perfused guinea pig hearts. Bars represent Mean ± S.E. Contractile force expressed as the percent change from control, measured after 60 min equilibration.

hearts were perfused for 90 min with Krebs-Henseleit solution before exposure to digoxin (2×10^{-7} g/ml) for 15 min. Insulin was added to the perfusate 30 min before the exposure to digoxin and remained in the perfusate throughout the remainder of the experiments.

Dutta et al., (1968a) have shown that there was no arterio-venous digoxin concentration difference after 4 min perfusion with digoxin in the guinea pig heart and the radioactivity of the perfusate between 4 and 64 min had not changed. They also reported that considerable amounts of digoxin were accumulated in the tissue and the subcellular fractions in the 16 min perfused hearts.

Preliminary experiments showed that recirculation of perfusate through the heart caused the contractility to decrease for unknown reasons, perhaps by the formation of microcrystal of $Ca_3 (PO_4)_2$ (Young, 1968). Therefore, the hearts were perfused with the medium only once and the perfusate discarded.

The effect of insulin on the positive inotropic effect after 15 min exposure to ^{3}H-digoxin is shown in table 5. Digoxin alone (7×10^{-7} g/ml) increased contractile force by 10.3 $\pm$ 3.4% (Mean $\pm$ S.E.). The positive inotropic effect was expressed as percent increase from initial contractile force. Contractile force was decreased in hearts treated with insulin, but not significantly ($P > .05$). This was not consistent with the results observed in the atria by Bailey and Dresel (1969). The uptake of digoxin by the microsomes in the rabbit hearts treated with insulin was increased, but this change again was not significant ($P > .05$).

b. Effect of insulin on the positive inotropic effect and the subcellular distribution of ^{3}H-digoxin in guinea pig hearts.

TABLE 5

The effect of insulin on the uptake and positive inotropic effect of ^{3}H-digoxin in the rabbit hearts perfused for 15 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
^{3}H-digoxin 7×10^{-7} (g/ml) (5)[a]	10.29[b,c] ± 3.37	1.26 ± 0.16	0.38 ± 0.03	266.0 ± 21.0	0.78 ± 0.03	0.68 ± 0.05	1.26 ± 0.15
^{3}H-digoxin PLUS Insulin (2mU/ml) (5)	6.3 ± 1.31	1.31 ± 0.09	0.43 ± 0.02	301.0 ± 14.0	0.95 ± 0.07	0.77 ± 0.05	1.88 ± 0.29

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

[c] Expressed as the percent increase from initial contractile force.

All hearts were perfused for 90 min with Krebs-Henseleit solution before exposure to digoxin (2×10^{-7} g/ml) for 15 min. Insulin was added to the perfusate 30 min before the exposure to digoxin and remained in the perfusate throughout the remainder of the experiments. The positive inotropic effect in the insulin treated or untreated hearts was variable. Contractile force increased by 57% out of three untreated hearts but was unchanged in the other. There was no change in contractile force in 2 of 4 insulin treated hearts. The other 2 hearts showed an increase of approximately 10%. This result showed that insulin seemingly blocked the positive inotropic effect of digoxin, but this was not significant statistically.

The effect of insulin on the subcellular distribution of ^{3}H-digoxin in the spontaneously beating hearts after 15 min exposure to ^{3}H-digoxin is shown in table 6. Total tissue, nuclear, mitochondrial and microsomal uptake of ^{3}H-digoxin in the guinea pig hearts were not significantly affected ($P > .05$) in the insulin treated hearts. The relation between two variables, such as the subcellular uptake of ^{3}H-digoxin and the change on contractile force due to digoxin infusion was examined by using a regression analysis. The inotropic effect of ^{3}H-digoxin was not correlated with ^{3}H-digoxin contents in any subcellular fractions in the hearts perfused for 15 min.

The experimental procedure was then modified as follows: First, the potassium concentration in the Krebs-Henseleit solution was reduced from 5.8 mM to 4.7 mM because potassium may interfere with the binding of digoxin to the tissue (Dutta et al., 1968b). Secondly, nylon monofilament line was attached to the hook on the apex of the heart and was led around a pulley to the Grass FT-03 transducer; this

TABLE 6

The effect of insulin on the uptake and pharmacological effect of ^{3}H-digoxin in the spontaneously beating guinea pig hearts perfused for 15 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Digoxin 2×10^{-7} g/ml (3)[a]	19	0.60[b] ± 0.08	0.98 ± 0.16	196.0 ± 32.0	0.72 ± 0.06	0.77 ± 0.15	1.73 ± 0.40
Digoxin PLUS Insulin 2mU/ml (4)	4.90 ± 2.81	0.71 ± 0.07	0.95 ± 0.06	190.0 ± 12.0	0.67 ± 0.07	0.67 ± 0.01	2.15 ± 0.29

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

replaced the rigid connection between the transducer and the heart used formerly. Thirdly, the resting tension was adjusted to produce a developed tension which was 50% of maximum as determined by the length-tension relations (see methods). Resting tension had been maintained at 1 g in the previous experiments. Finally, the hearts were perfused for 30 min to permit the positive inotropic effect to reach a steady level. This experimental procedure was followed in all subsequent experiments.

The effect of insulin on the positive inotropic effect of ^{3}H-digoxin is shown in table 7. Mean contractile force increased by 27.1 $\pm$ 3.8% (Mean $\pm$ S.E.) after 30 min exposure to digoxin (2×10^{-7} g/ml) in the absence of insulin. Contractile force in the hearts treated with insulin increased by 21.5%. The change was not significant ($P > .05$). The binding of ^{3}H-digoxin to each subcellular fraction was not changed significantly by insulin treatment, whereas total tissue uptake of ^{3}H-digoxin was decreased significantly ($P < .05$) in the insulin treated hearts. On the basis of these results, there was no correlation between the positive inotropic effect and the subcellular distribution or the total tissue uptake of ^{3}H-digoxin. Perhaps the reason is that earlier experiments used driven atria (Bailey and Dresel, 1969; 1971), whereas the present series did not.

The heart rate was measured during all perfusion procedures in a few experiments. As can be seen in figure 6, contractile force in spontaneously beating hearts increased significantly ($P < .05$) after 15 min exposure to digoxin, then decreased gradually after 30 min of perfusion. The heart rate was not increased significantly during the first 15 min of perfusion, but increased significantly after 30 min

TABLE 7

The effect of insulin on the uptake and pharmacological effect of ^{3}H-digoxin in the spontaneously beating guinea pig hearts perfused for 30 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Digoxin 2×10^{-7} (g/ml) (4)[a]	27.1[b] ± 3.82	0.41 ± 0.02	1.95 ± 0.04	390.0 ± 40.0	1.96 ± 0.14	1.83 ± 0.02	4.46 ± 0.33
Digoxin PLUS Insulin 2mU/ml (4)	21.5 ± 4.63	0.41 ± 0.02	1.69[c] ± 0.06	338.0 ± 12.0	1.83 ± 0.13	1.77 ± 0.04	4.90 ± 0.43

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

[c] Significant increase from values measured in hearts treated with digoxin alone ($P < .05$).

Figure 6.

Change in contractile force and heart rate in isolated spontaneously beating guinea pig heart during exposure to digoxin for 30 min. Each dot represents the average of two experiments.

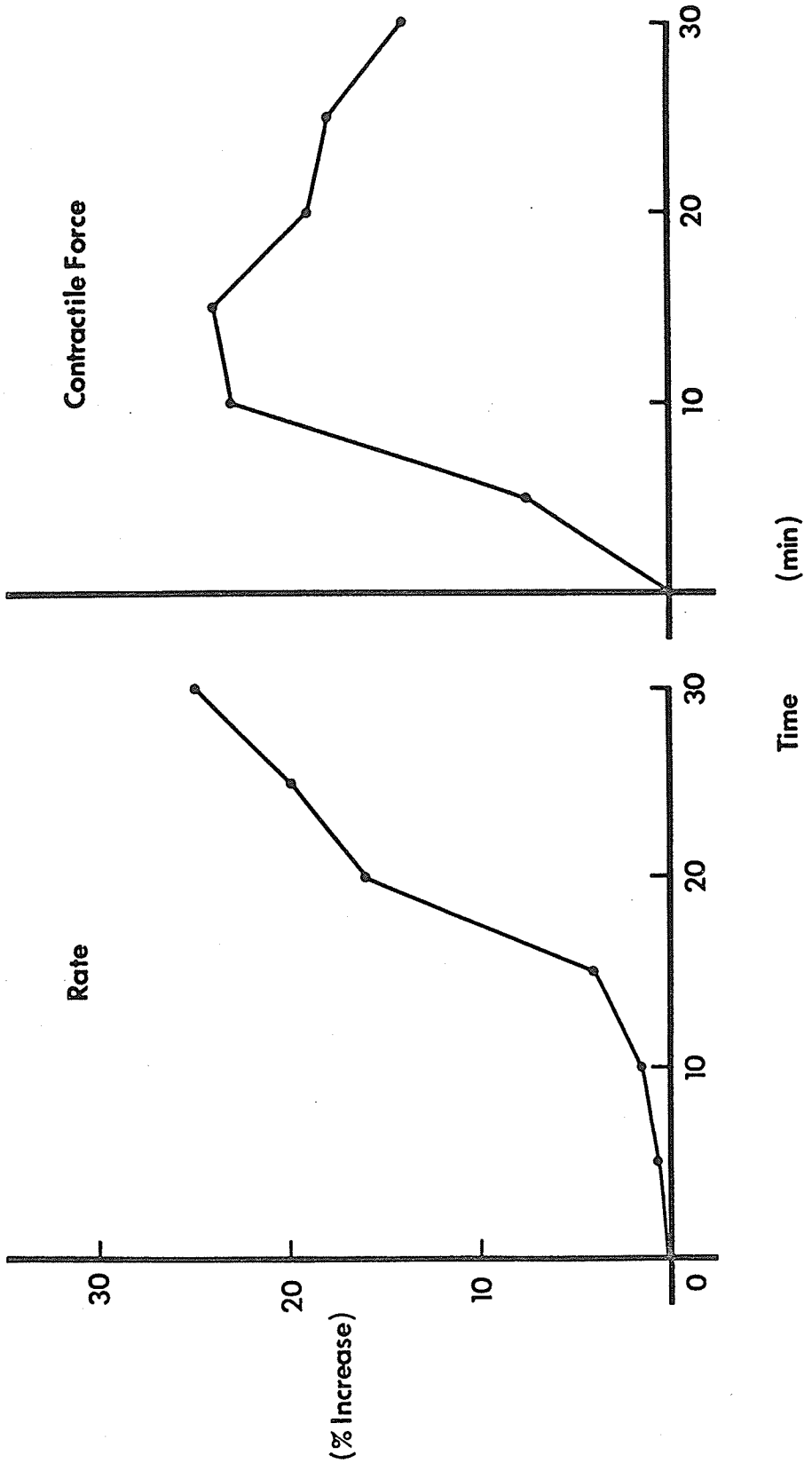


Figure 6.

exposure to digoxin. Thus, there exists an inverse relationship between heart rate and contractile force in spontaneously beating hearts. This is difficult to explain. The changes in rate should not be the cause of the changes in contractility because it has been known since the work of Blinks and Koch-Weser, (1963) that contractile force is not dependent on the change of frequency in atria treated with ouabain.

2. Electrically driven guinea pig hearts

a. Untreated hearts.

Untreated hearts were defined as hearts treated with digoxin alone. It was not possible to relate the subcellular distribution of digoxin to the positive inotropic effect in isolated sponteneously beating hearts because the increase in heart rate caused by digoxin (decrease in interval) effectively masked the magnitude of the positive inotropic response. Therefore, all subsequent experiments were done on electrically driven guinea pig hearts.

Contractile force in hearts stimulated at a constant rate of 216/min did not change significantly during 120 min in the absence of digoxin (cf Control heart, fig. 7). Changes in contractile force that occurred during the 60 min pre-equilibration period are not shown in figure 7. Addition of digoxin (2×10^{-7} g/ml), after 90 min perfusion, increased the contractile force significantly ($P < .05$) in these hearts (fig. 7). Approximately 90% of the maximum inotropic effect to digoxin occurred during the initial 15 min of perfusion (fig. 7) and the inotropic effect to digoxin was not further increased significantly (P .05) after 30 min perfusion with this concentration of digoxin. In the following experiments, the hearts were exposed to digoxin for either 15 or 30 min.

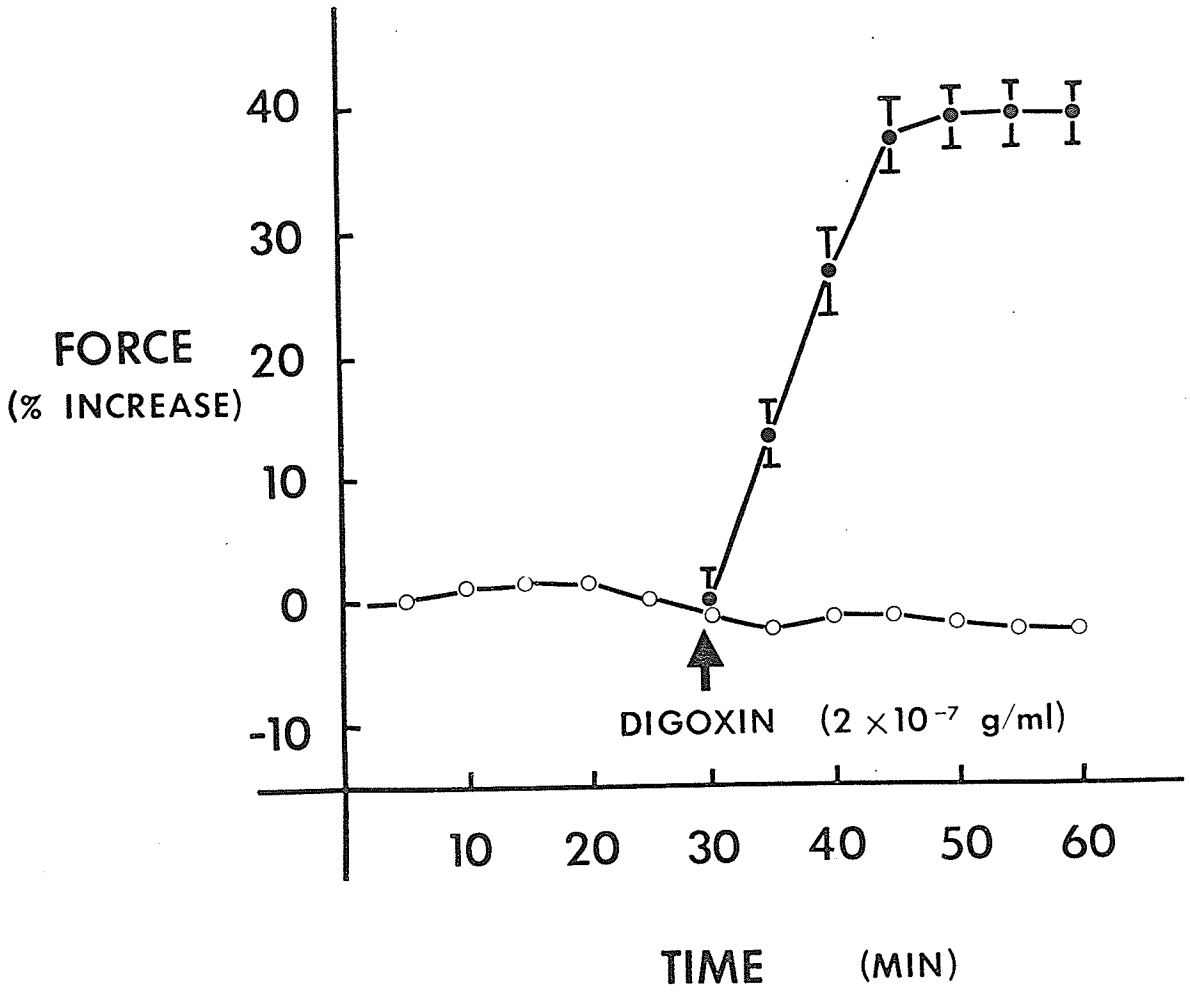


Figure 7.

Time course of contractility changes in digoxin treated, ● - ● and two control hearts o - o. Bar represents Mean ± S.E. Contractile force expressed as the percent change measured after 60 min equilibration.

b. The effect of insulin on the positive inotropic effect and the subcellular distribution of ^{3}H-digoxin in guinea pig hearts.

The effect of insulin on the uptake and pharmacological effect of ^{3}H-digoxin in hearts treated for 15 min are shown on table 8. Mean contractile force increased by 34.4 $\pm$ 3.7% after 15 min exposure to digoxin (2×10^{-7} g/ml) in the absence of insulin. Contractile force in the hearts treated with insulin increased by 25.2 $\pm$ 4.0%. There was no change in total tissue uptake or in the tissue:medium digoxin ratio in the insulin treated hearts after 15 min exposure to digoxin nor was uptake by the mitochondrial and nuclear fraction affected by treatment with insulin (table 8). The concentration of ^{3}H-digoxin in the microsomal fraction was 3.27 $\pm$ 0.04 ng/mg protein in untreated hearts. Insulin decreased the concentration of digoxin in the microsomal fraction to 2.88 $\pm$ 0.12 ng/mg protein. This decreased uptake of ^{3}H-digoxin by the microsomal fraction was statistically significant ($P < .05$).

The effect of insulin on the positive inotropic effect of ^{3}H-digoxin in hearts treated for 30 min is shown in table 9. Mean contractile force increased by 39.0 $\pm$ 2.6% (Mean $\pm$ S.E.) after 30 min exposure to digoxin (2×10^{-7} g/ml). Contractile force in the hearts treated with insulin increased by 50.0 $\pm$ 4.7%, which was significantly more than that measured in the untreated hearts ($P > .05$) (table 9). The change in total tissue uptake and tissue:medium ratio were not statistically significant (table 9). The tissue:medium ratio was approximately 2.0. Total tissue uptake was not significantly affected by treatment with insulin (table 9).

A homogenate of ventricular tissue was separated into

TABLE 8

The effect of insulin on the uptake and pharmacological effect of ^{3}H-digoxin in electrically driven guinea pig hearts after perfusion for 15 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Digoxin 2×10^{-7} g/ml (4)[a]	34.45[b] ± 3.70	0.53 ± 0.04	1.54 ± 0.11	309.40 ± 21.65	1.30 ± 0.08	1.60 ± 0.15	3.27 ± 0.04
Digoxin PLUS Insulin 2mU/ml (4)	25.20 ± 4.04	0.54 ± 0.03	1.50 ± 0.05	299.09 ± 10.53	1.23 ± 0.10	1.27 ± 0.09	2.88[c] ± 0.12

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

[c] Significant change from values measured in hearts treated with digoxin alone ($P < .01$).

TABLE 9

The effects of insulin on the uptake and pharmacological effect of ^{3}H-digoxin in electrically driven guinea pig hearts perfused for 30 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Digoxin 2×10^{-7} g/ml (4)[a]	39.0[b] ± 2.6	0.43 ± 0.01	2.07 ± 0.06	414.50 ± 12.19	1.80 ± 0.10	2.09 ± 0.10	4.63 ± 0.26
Digoxin PLUS Insulin 2mU/ml (4)	50.0[c] ± 4.7	0.39 ± 0.02	1.94 ± 0.13	386.75 ± 25.71	2.04 ± 0.01	2.29 ± 0.14	5.40[c] ± 0.30

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

[c] Significant increase from values measured in hearts treated with digoxin alone ($P < .05$).

supernatant and pellet. The distribution of digoxin in the total pellet and 1st supernatant changed significantly as a function of the time of perfusion in the electrically driven guinea pig hearts. Figure 8 shows the supernatant:pellet ratio decreased as the perfusion time was extended from 15 to 30 min. This was due to the fact that the digoxin concentration in the pellet was increased, whereas the digoxin concentration was not changed in the supernatant. The ratio in the hearts treated with insulin for 15 or 30 min were not significantly different from those of untreated hearts. Uptake by the mitochondrial and nuclear fraction was also not affected significantly ($P > .05$) by treatment with insulin (table 9). However, the uptake of digoxin by the microsomes in the hearts treated with insulin for 30 min was significantly greater than that of untreated hearts (table 9). The relation between two variables, such as the microsomal uptake of ^{3}H-digoxin and the change in cardiac contractile force due to digoxin infusion was examined by using a regression analysis. The correlation between the microsomal uptake of ^{3}H-digoxin and its inotropic effect in electrically driven guinea pig hearts is shown in figure 9. Hearts, insulin treated and untreated, perfused for 15 and 30 min are included in the correlation analysis. The coefficient of correlation is significantly greater than zero ($r = .68$, $P < .01$). These results suggest that the positive inotropic effect may be related to the uptake of ^{3}H-digoxin by the microsomal fraction.

c. Effect of aldosterone and chlorpromazine on the positive inotropic effect and the subcellular distribution of ^{3}H-digoxin.

We have shown that insulin increased the positive inotropic

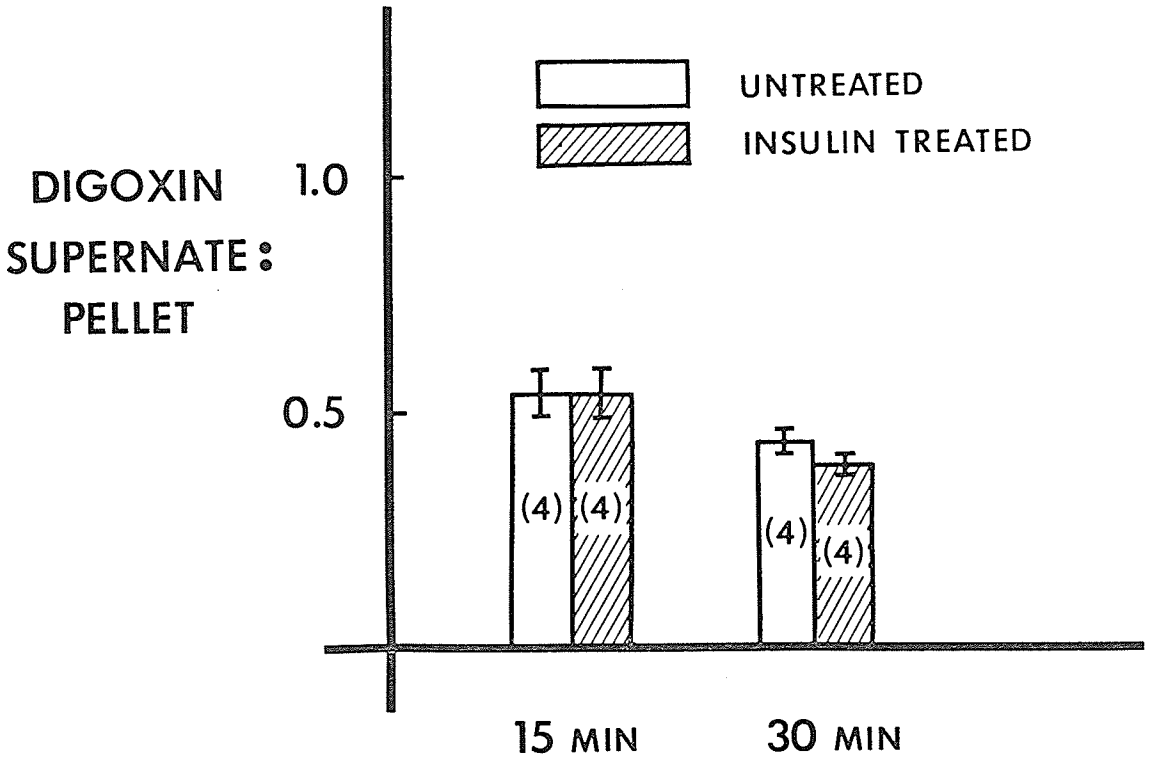


Figure 8.

The effect of insulin on the ratio of the digoxin concentration in the supernatant to that in pellet fraction of guinea pig hearts after 15 and 30 min perfusion with digoxin.

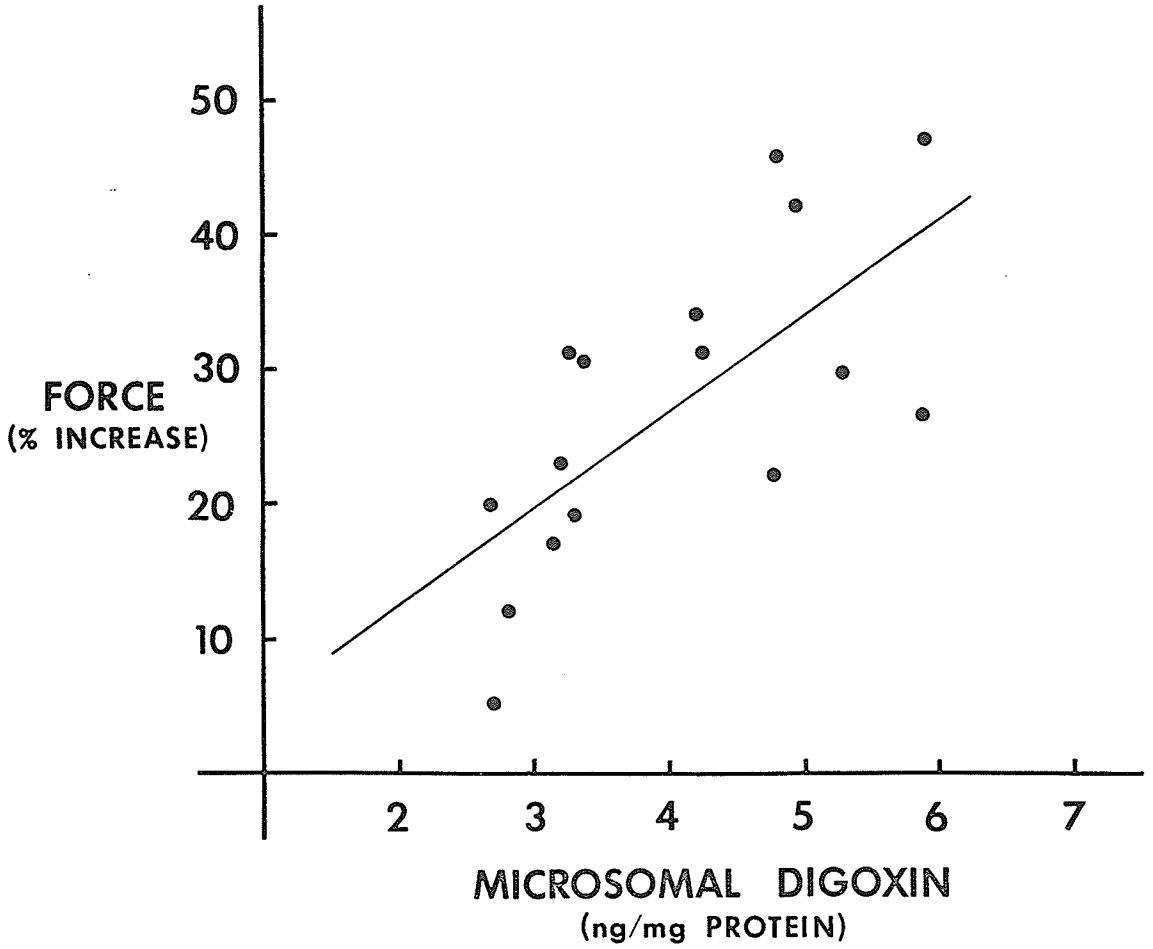


Figure 9.

The relationship between the microsomal uptake of ^{3}H-digoxin and the inotropic effect. Each dot represents a heart perfused for either 15 or 30 min and either with or without insulin (2 mU/ml).

effect of ^{3}H-digoxin in electrically driven-guinea pig hearts and that this 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 digoxin in tissue:medium ratio or to the binding of digoxin to the nuclear and mitochondrial fractions. The objective of the following work was to attempt to interfere with the positive inotropic effect of digoxin or with its binding to various subcellular fractions by agents which would not themselves have an intrinsic effect on cardiac contractility, but which would affect the subcellular distribution of the glycosides. All hearts were perfused for 90 min with Krebs-Henseleit solution before exposure to digoxin (2×10^{-7} g/ml) for 30 min. Aldosterone or chlorpromazine was added to the perfusate 30 min before the exposure to digoxin and remained in the perfusate throughout the remainder of the experiments.

The effects of aldosterone and chlorpromazine on the positive inotropic effect to ^{3}H-digoxin are shown in table 10. The contractility of control hearts did not change significantly during the duration of these experiments (fig. 7). Digoxin (2×10^{-7} g/ml) alone caused an increase in contractile force in this series of experiments of $47.9 \pm 2.4\%$ (Mean $\pm$ S.E.) after 30 min perfusion. It should be pointed out that this increased contractile force to digoxin in untreated hearts is different from the results of previous insulin experiments. This discrepancy may be due to the fact that different batch of isotope was used in these experiments and that they were carried out in different season. Perfusion with aldosterone (2×10^{-7} g/ml) for 30 min caused contractile force to increase approximately 7.4% ($P < .05$) (fig. 10). The positive inotropic response to digoxin was reduced to

TABLE 10

The effect of aldosterone and chlorpromazine on the positive inotropic effect and the uptake of ^{3}H-digoxin in electrically driven guinea pig hearts.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Digoxin Uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Digoxin 2×10^{-7} g/ml (4)[a]	47.9[b,c] ± 2.5	0.35 ± 0.02	2.23 ± 0.17	445.73 ± 30.4	2.84 ± 0.41	2.65 ± 0.13	6.12 ± 0.23
Digoxin plus Chlorpromazine 2×10^{-7} g/ml (4)	32.4* ± 1.5	0.37 ± 0.02	2.06 ± 0.04	410.25 ± 8.85	2.47 ± 0.21	2.47 ± 0.18	4.60* ± 0.22
Digoxin plus Aldosterone 2×10^{-7} g/ml (4)	17.7* ± 2.0	0.40 ± 0.01	2.13 ± 0.10	426.69 ± 21.06	2.09 ± 0.20	2.17 ± 0.08	4.34* ± 0.21

[a] Number in parenthesis indicates the number of experiments.

[b] Mean ± S.E.

[c] The positive inotropic effect expresses the increase in contractile force as percent of initial contractile force.

* Significant reduction from values measured in hearts treated with digoxin alone ($P < 0.01$).

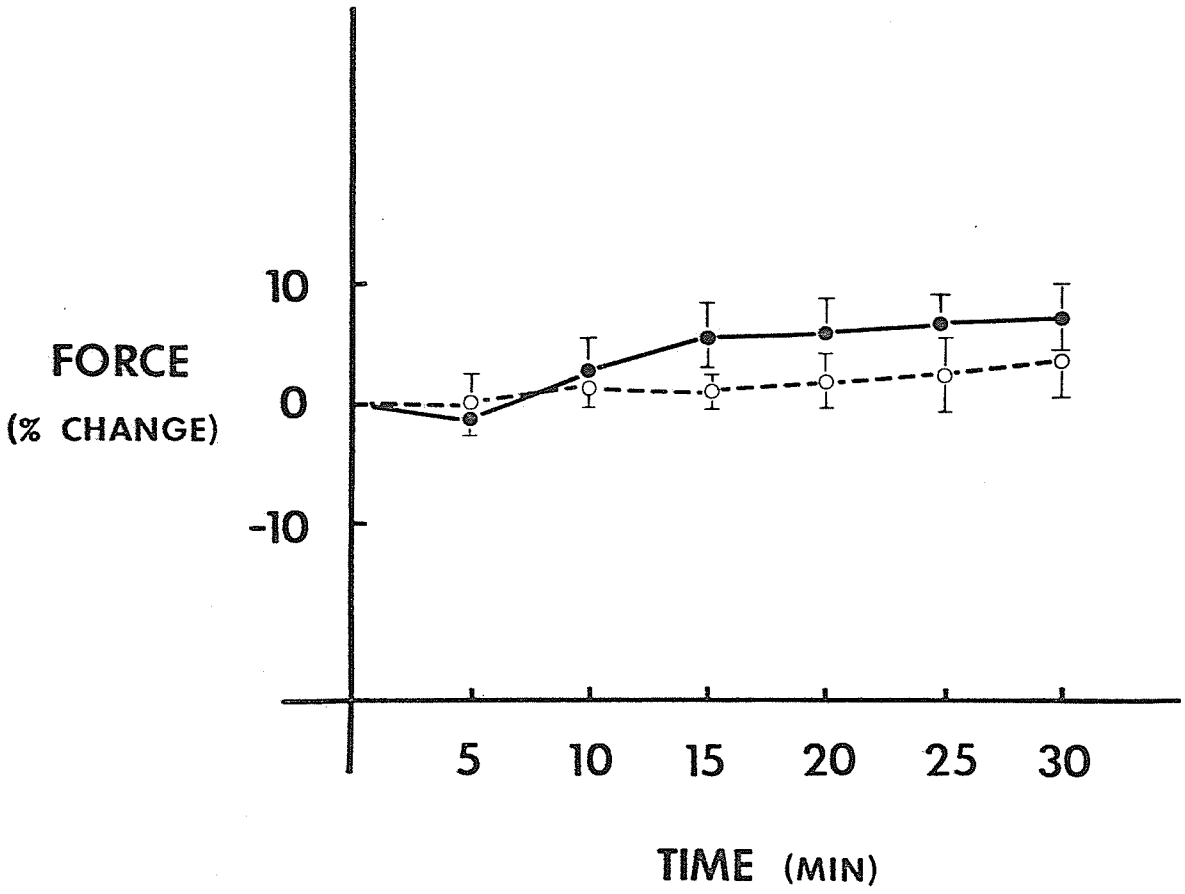


Figure 10.

Changes of contractile force in guinea pig hearts during perfusion with aldosterone (2×10^{-7} g/ml) o----o or chlorpromazine (2×10^{-7} g/ml) o----o. Contractile force was expressed as percent change after 60 min equilibration.

17.7% when aldosterone was administered 30 min prior to and throughout the exposure to digoxin ($P < .05$), which represent 63% reduction of the untreated response. Addition of 95% ethanol in amounts corresponding to those added with aldosterone did not affect contractility nor the response to digoxin.

Perfusion of chlorpromazine (2×10^{-7} g/ml) for 30 min caused approximately 4% increase in contractile force (fig 10), this increase was not significant ($P > .05$). However, the positive inotropic effect of digoxin was reduced significantly ($P < .01$) when chlorpromazine was given 30 min prior to and throughout the exposure to digoxin, but not to the extent seen after aldosterone pretreatment (table 10).

The total tissue uptake of digoxin by the control hearts was 445.7 ± 30.4 ng/g tissue and the tissue:medium digoxin radioactivity ratio was 2.23 ± 0.17 (table 10). Neither of these measurements were affected significantly by treatment with aldosterone or chlorpromazine. The two drugs decreased slightly the concentration of ^{3}H-digoxin in the nuclear and mitochondrial fractions from that measured in hearts treated with digoxin alone, but these changes were not statistically significant (table 10). The 1st supernatant:pellet ratio was increased from 0.35 ± 0.02 to 0.37 ± 0.02 and 0.40 ± 0.01 in chlorpromazine and aldosterone treated hearts, respectively, suggesting that either the digoxin content in the 1st supernatant was increased slightly or the digoxin content in pellet was decreased, although these changes were not significantly different ($P > .05$). Total digoxin concentration in the total pellet, 1st supernatant and 2nd supernatant (see Methods) was measured in the hearts treated with aldosterone, chlorpromazine and untreated hearts. The digoxin concentration in the total pellet

was 2.54 $\pm$ 0.15 x 10^{-7} g/ml in the untreated hearts. The pellet digoxin concentration in the aldosterone and chlorpromazine treated hearts was reduced to 2.04 and 2.37 x 10^{-7} g/ml, respectively (table 11), the former decrease being statistically significant. On the other hand, the digoxin concentration in the 1st supernatant was not changed significantly by either treatment. These results suggest that aldosterone and chlorpromazine may interfere with binding of digoxin to the subcellular fractions. The digoxin concentration in the 2nd supernatant was 0.29 $\pm$ 0.01 x 10^{-7} g/ml in the untreated hearts, but the digoxin concentration in this fraction was decreased significantly in the aldosterone and chlorpromazine treated hearts, suggesting that the digoxin was released proportionally to the amounts of digoxin bound to the pellet in these hearts. The right-hand column of table 11 indicates that approximately 10% of the bound digoxin was released.

In four untreated hearts, the concentration of ^{3}H-digoxin in the microsomal fraction (6.12 $\pm$ 0.23 ng/mg protein) was at least twice the concentration measured in the mitochondrial and nuclear fractions. Both aldosterone and chlorpromazine significantly decreased the concentration of digoxin in the microsomal fraction (table 10) to 4.34 $\pm$ 0.23 and 4.60 $\pm$ 0.22 ng/mg protein, respectively ($P < .01$) without affecting the uptake by the mitochondrial and nuclear fractions. The difference in microsomal uptake of ^{3}H-digoxin between the two groups of treated hearts was not significant statistically ($P > .05$), although the difference in the inotropic effect observed was significant ($P < .05$). Despite this apparent dissociation of the inotropic effect from total microsomal uptake as concluded from the data in table 10, we were able to show that a relationship exists between the two parameters.

TABLE 11

Comparison of digoxin concentration in the pellet and supernatant between the hearts treated with aldosterone and chlorpromazine and untreated hearts.

Treatment	Digoxin Concentration ($\times 10^{-7}$/ml)			% released into 2nd supernatant
	Total Pellet	1st Supernatant	2nd Supernatant	
Digoxin 2×10^{-7} g/ml (4)[a]	2.54[b] $\pm$ 0.15	0.92 $\pm$ 0.04	0.29 $\pm$ 0.01	12.0
Digoxin plus Chlorpromazine 2×10^{-7} g/ml (4)	2.37 $\pm$ 0.07	0.88 $\pm$ 0.03	0.21* $\pm$ 0.02	9.0
Digoxin plus Aldosterone 2×10^{-7} g/ml (4)	2.04* $\pm$ 0.10	0.83 $\pm$ 0.03	0.19* $\pm$ 0.03	9.0

[a] Number in parenthesis indicates the number of experiments.

[b] Mean $\pm$ S.E.

* Significant reduction from value measured in hearts treated with digoxin alone ($P < .05$).

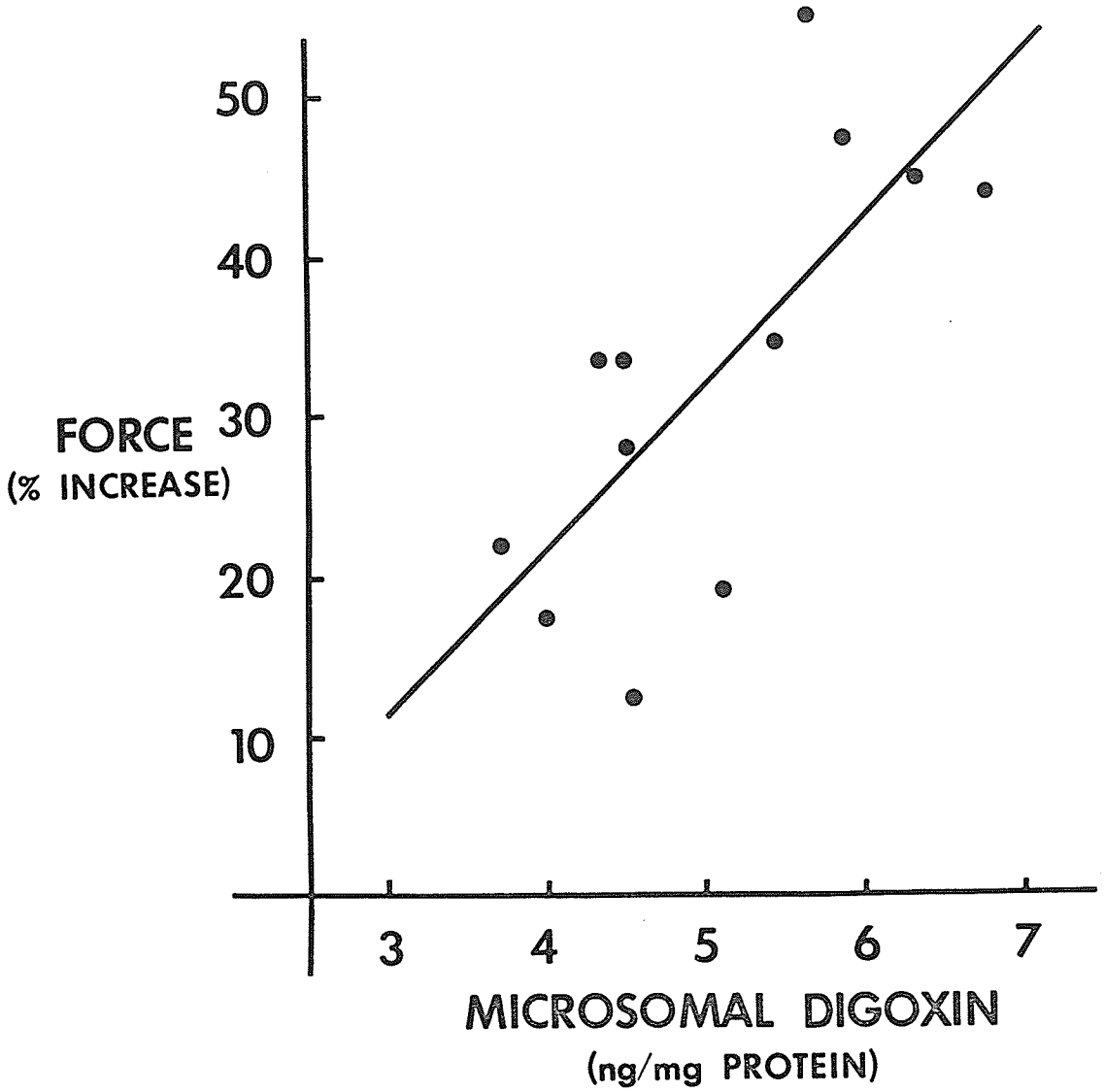


Figure 11.

Correlation between the uptake of ^{3}H-digoxin and the changes in contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 min with ^{3}H-digoxin alone or in the presence of aldosterone (2×10^{-7}g/ml) or chlorpromazine (2×10^{-7}g/ml).

Figure 11 shows this relationship measured in the 12 experiments contained in table 10. The two measurements were correlated significantly with a coefficient of correlation, r, of 0.73 ($P < .01$). When the regression line was extrapolated to the digoxin concentration where no positive inotropic effect would be expected to occur, 2.0 ng digoxin/mg protein, or 32% of the total digoxin taken up by the microsomes of the control hearts would be bound before there would be any evidence of a positive inotropic effect. The threshold concentration is defined as the level of digoxin at which a concentration just produces a change in contractility detectable by our instrumentation.

d. Effect of insulin on the positive inotropic effect and the subcellular distribution of ^{3}H-ouabain.

Ouabain (2×10^{-7} g/ml) was used to examine the correlation between the positive inotropic effect and the binding of ^{3}H-ouabain by the microsomal fraction. The force of contraction was increased by 45.73% by ouabain (table 12). The positive inotropic effect to ouabain was not changed by insulin in these experiments which were of 30 min duration (table 12). There was no significant change in the tissue: medium ratio and the subcellular distribution of ouabain attributable to insulin treatment (table 12). Correlation analysis in this group of 6 hearts did not indicate a significant relation of microsomal binding to the positive inotropic effect.

B. Binding Properties of ^{3}H-digoxin to Microsomes

1. Density gradient centrifugation

We attempted, because the microsomal fraction is probably not homogeneous (Katz et al., 1970), to determine the distribution

TABLE 12

The effects of insulin on the uptake and pharmacological effect of ^{3}H-ouabain in the guinea pig hearts perfused for 30 min.

Treatment	Force (% Increase)	1st supernate: Pellet	Tissue: Medium	Ouabain uptake			
				Total tissue (ng/g tissue)	Nuclear Fraction (ng/mg protein)	Mitochondrial Fraction (ng/mg protein)	Microsomal Fraction (ng/mg protein)
Ouabain 2×10^{-7} g/ml (3)[a]	45.73[b] ± 8.77	0.18 ± 0.02	1.99 ± 0.23	398.0 ± 46.0	1.95 ± 0.15	1.44 ± 0.07	3.29 ± 0.16
Ouabain plus Insulin 2mU/ml (3)	43.67 ± 11.09	0.15 ± 0.02	2.12 ± 0.34	424.0 ± 68.0	2.41 ± 0.67	1.65 ± 0.28	3.78 ± 0.57

[a] Number in parenthesis indicates the number of experiments

[b] Mean ± S.E.

of digoxin in various subfractions of guinea pig microsomes by centrifugation on a continuous sucrose density gradient. The microsomal fraction was resuspended in Krebs-Henseleit solution and layered on the top of the gradients which consisted of 20 to 60% of sucrose solution. These experiments were unsuccessful because a major portion of the radioactivity remained at the top of the gradient and only a small amount of radioactivity was detected on the lower portion layers, which indicated that the radioactivity had become dissociated from the particulate fraction as a result of the resuspension. It should be mentioned here that commercial sucrose was used without purification for density gradients.

2. Loosely bound digoxin

The results in (1) above led to a systematic examination of the loose binding of digoxin. Microsomal pellets were resuspended in Krebs-Henseleit solution under standardized conditions (see Methods). Aliquots of the microsomal resuspension were analyzed for protein concentration and radioactivity to yield the data shown in table 13. The remainder of the suspension was centrifuged at 166,000 x g for 60 min. We have called the resultant supernatant the 3rd supernatant. It contains the digoxin which was been removed from the microsomes by the resuspension procedure. We have defined this as "loosely bound" digoxin.

As shown in table 13, the mean amount of loosely bound digoxin in the control hearts was 1.96 $\pm$ 0.08 ng/mg digoxin microsomal protein. Aldosterone reduced this to 1.17 $\pm$ 0.08 ng digoxin/mg microsomal protein ($P < .01$). The mean quantity of loosely bound digoxin in chlorpromazine treated hearts did not differ significantly

TABLE 13

The effect of aldosterone and chlorpromazine on the positive inotropic effect of ^{3}H-digoxin and the concentration of ^{3}H-digoxin in the cardiac microsomal fraction and 3rd supernatant obtained from the microsomal fraction by shaking with K-H media for 5 min.

Treatment	Force % Increase	Uptake (ng/mg microsomal protein)		
		total microsome	3rd supernate	residual digoxin
Digoxin 2×10^{-7} g/ml (4)[a]	47.9 ± 2.5 [b,c]	6.12 ± 0.23	1.96 ± 0.06	4.16 ± 0.22
Digoxin plus Chlorpromazine 2×10^{-7} g/ml (4)	32.4 ± 1.5*	4.60 ± 0.22*	1.74 ± 0.17	2.86 ± 0.27
Digoxin plus Aldosterone 2×10^{-7} g/ml (4)	17.7 ± 2.0*	4.34 ± 0.21*	1.17 ± 0.08*	3.17 ± 0.32

a Number in parentheses indicates the number of experiments.

b Mean ± S.E.

c The positive inotropic effect is expressed as percent increase in contractile force of initial contractile force.

* Significant reduction from values measured in hearts treated with digoxin alone ($P < 0.01$).

from that in the controls.

The coefficient of correlation was calculated for the relationship between the positive inotropic effect and the content of the two sub-fractions of the total microsomal digoxin content, i.e., the loosely bound digoxin and the residual digoxin which remained bound to the microsomes after the resuspension procedure. Figure 12 shows that loosely bound digoxin is highly correlated with the positive inotropic effect. The coefficient of correlation, r, of .84 ($P < .01$), is considerably larger than that associated with the correlation of total microsomal uptake shown in figure 11 ($r = .73$), but this difference did not reach statistical significance. However, the amount of digoxin which remained bound after the resuspension procedure was not correlated at all with the inotropic effect. The coefficient of correlation, $r = .37$, was not significantly different from zero ($P > 0.05$) and differed significantly ($P < .05$) from $r = .84$, the value for the data shown in figure 12. When the regression line was extrapolated to the digoxin concentration where no positive inotropic effect was observed, 0.44 ng digoxin/mg protein must be bound loosely before there would be any evidence of a positive inotropic effect. Since 2.0 ng digoxin/mg protein must be bound to the microsomes, approximately 0.44 ng digoxin/ 2.0 ng digoxin x 100 = 22% of the total bound digoxin must be associated with the positive inotropic effect.

3. The effect of perfusion with drug-free medium on the positive inotropic effect and microsomal digoxin binding

In view of the fact that the positive inotropic effect of the cardiac glycosides disappeared during perfusion with drug-free medium

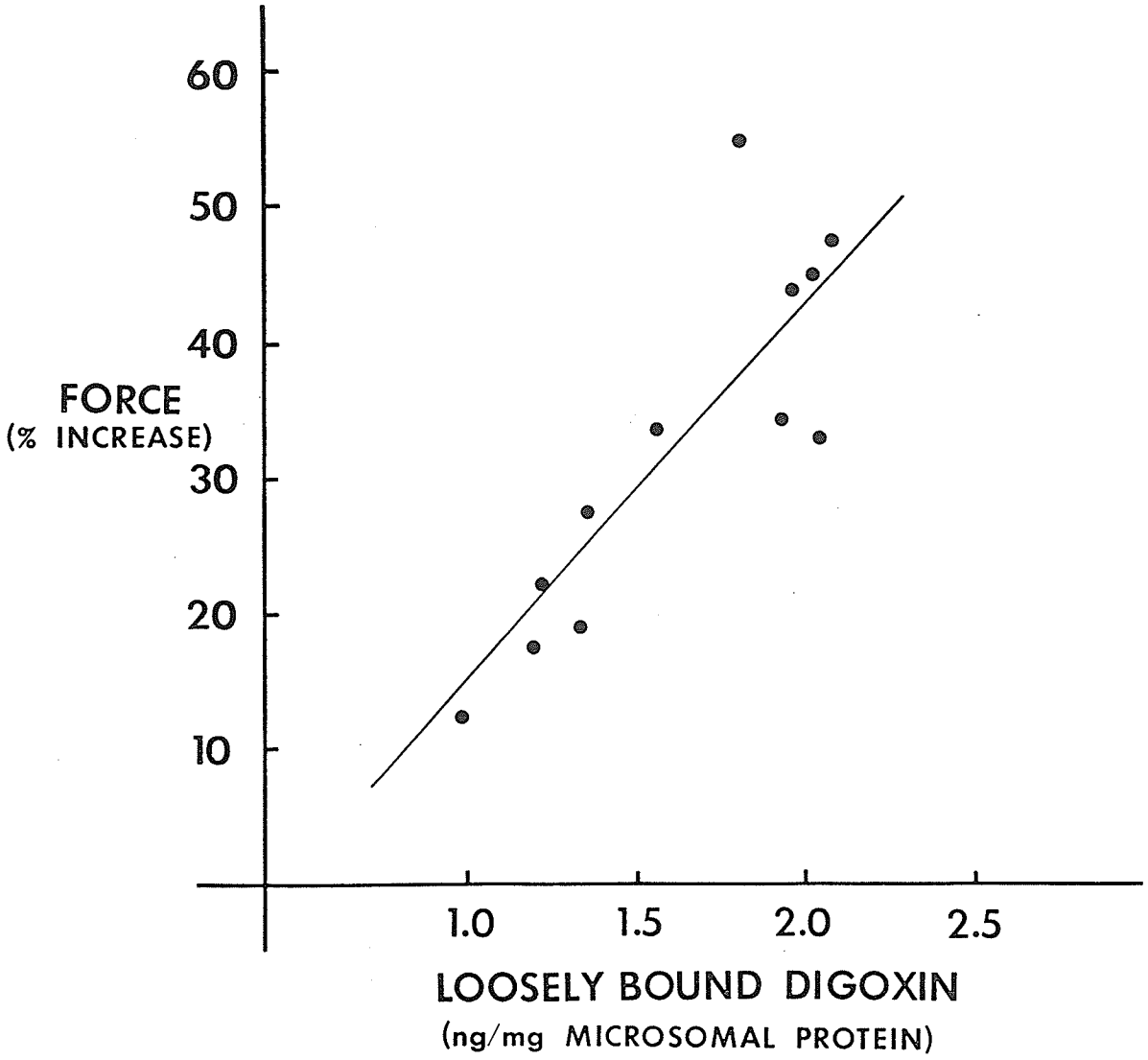


Figure 12.

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 min with ^{3}H-digoxin alone, or in the presence of aldosterone (2×10^{-7}g/ml) or chlorpromazine (2×10^{-7}g/ml).

within a very short period of time in guinea pig atria (Lüllmann et al., 1969), it was of interest to follow the disappearance of the radioactivity in the microsomal fraction and loosely bound digoxin after they have been washed with a drug-free solution until the pharmacologic effect of digoxin has been removed.

The positive inotropic effect of digoxin disappeared after 20 to 30 min washout with drug-free Krebs-Henseleit solution in isolated hearts. These results are similar to those of Lüllmann et al., in guinea pig atria (1969). Approximately 52% of total microsomal digoxin and 65% of digoxin from the loosely bound site were removed during 20 min washout period and yet a considerable amount of microsomal and loosely bound digoxin remained in each fraction of the hearts, 2.21 and 0.70 ng/mg microsomal protein, respectively. This is shown in table 14. These observations tend to confirm the conclusion reached by extrapolation of the regression line to zero (figs. 11, 12), i.e, there is a threshold concentration of digoxin required to activate the inotropic process.

Approximately 1.0 ng digoxin/mg microsomal protein remained after washout for 60 min and is probably not related to the positive inotropic effect in the guinea pig heart (table 15).

It is also interesting to mention here that ventricular tissue has been found to have a higher digoxin uptake than atrial tissue (table 16). The ratio of total digoxin concentration in the atria to ventricle was approximately .72. When the hearts were perfused with digoxin for 30 min and then washed with drug-free Krebs-Henseleit solution for 1 hr, the disappearance of digoxin from the atria was parallel to that from the ventricles. Namely, the ratio of total

TABLE 14

Microsomal ^{3}H-digoxin concentration after washout for 20 min with Krebs-Henseleit solution.

Before Washout n = 2		After Washout n = 2	
Microsomal digoxin	Loosely bound digoxin	Microsomal digoxin	Loosely bound digoxin
(ng/mg protein)		(ng/mg protein)	
4.68	1.99	2.21	0.70

TABLE 15

The subcellular distribution of ^{3}H-digoxin in guinea pig hearts perfused for 30 min and washout for 1 hr with drug-free Krebs-Henseleit solution.

Treatment	Digoxin uptake (ng/mg protein)			1st Supernatant: pellet	Tissue: Medium	Total tissue (ng/g tissue)	Atria: Ventricle uptake ratio
	Nuclear Fraction	Mitochondrial Fraction	Microsomal Fraction				
Digoxin 2×10^{-7} (g/ml) (2)[a]	0.34[b]	0.33	0.96	0.16	0.29	56.5	0.72

a. Number in parenthesis indicates the number of experiments.

b. Mean.

TABLE 16

The uptake of ^{3}H-digoxin in guinea pig hearts perfused for 30 min.

Treatment	Digoxin uptake (ng/g tissue)		
	Ventricle	Atrium	Ratio Ventricle:Atrium
Digoxin 2×10^{-7} g/ml (3)[a]	387.64[b] ± 36.27	279.15[c] ± 9.93	72.74

a. Parenthesis indicate the number of hearts

b. Mean ± S.E.

c. Digoxin content significantly less ($P < .05$) than ventricle.

digoxin content in ventricle to atria after 60 min washout was again approximately 72% (table 15).

4. The effect of resuspension media on the release of bound digoxin

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 hearts, but was more closely related to the fraction of digoxin released by resuspension of the microsomes in Krebs-Henseleit solution and was not related to remainder. These results suggest that the digoxin responsible for the positive inotropic response is the labile fraction of the total digoxin bound to the microsomes. The following experiments, therefore, were designed to determine if any particular components of Krebs-Henseleit solution might be responsible for the lability of bound digoxin. The resuspension media used in these experiments are given in table 17.

The resuspension of the microsomal pellet in Krebs-Henseleit solution released 46.8 $\pm$ 4.8% of the total microsomal digoxin into the supernatant (table 17, fig. 13). The omission of Mg from the Krebs-Henseleit solution did not affect the release of bound digoxin (table 17). Resuspension of the microsomal pellet in calcium-free Krebs-Henseleit solution released 38.8 $\pm$ 0.73%. However, this difference was not statistically significant ($P > .05$) from the results of Krebs-Henseleit solution. When the microsomal pellet was resuspended in sucrose solution containing EDTA, 17.8% of the total microsomal digoxin was released and resuspension in distilled water or isotonic sodium chloride released 30.3% of the bound digoxin, respectively.

Addition of 2.5 mM calcium to the sucrose resuspension media

TABLE 17

The effect of various ion on the release of bound digoxin from the microsomal fraction of the guinea pig hearts

Medium	% of total microsomal digoxin (per mg protein) removed by resuspension procedure in guinea pig hearts.	
Krebs	46.80 ± 4.8[b]	(4)[a]
Zero Ca Krebs	38.75 ± 0.73	(3)
Zero Mg Krebs	45.63	(1)
KCl (120 mM) solution	34.0	(2)
Distilled water	30.30	(2)
0.9% NaCl	28.90	(2)
Sucrose plus EDTA	17.84	(2)
Sucrose plus 2.5 mM Ca	42.96	(2)
5 M Urea	75.30 ± 0.23	(3)
5 M Urea plus 2.5 mM Ca	100 ± 7.35	(3)

a. Number in parenthesis indicates the number of experiments.

b. Mean ± S.E.

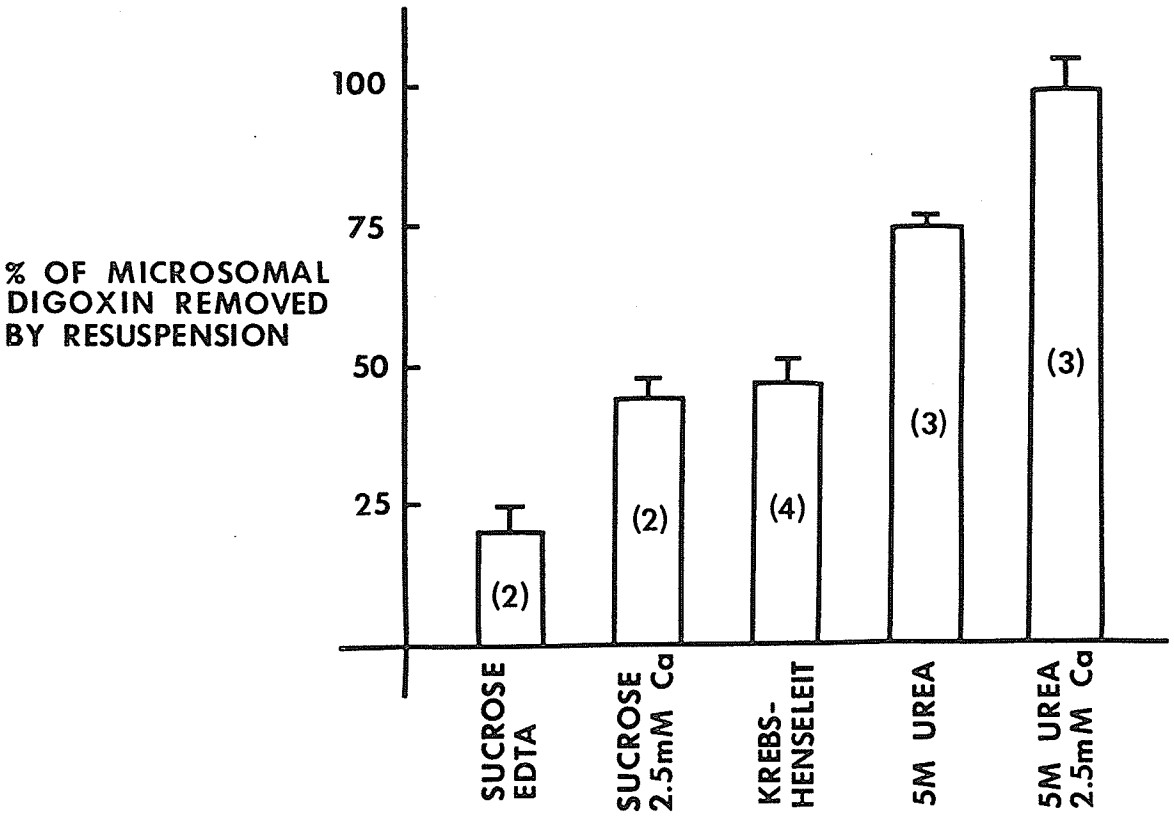


Figure 13.

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

released comparable amounts of digoxin from the microsomal fraction as did the Krebs-Henseleit solution (table 17, fig. 13), that is, 43.0%. Calcium ion caused release of a further 25% of the bound digoxin. This suggests that calcium may be important in the release of digoxin from the microsomal fraction.

The microsomal fraction was resuspended in the medium containing 120 mM potassium. The high potassium solution released 34.0% of bound digoxin. These amounts are comparable to the results obtained from zero calcium Krebs-Henseleit solution, but different from the results of Krebs-Henseleit solution.

The microsomal fraction was resuspended in 5 M urea solution to determine if the binding of digoxin to microsomes is due wholly, or in part, to hydrogen bonding. A 5 M urea solution containing no calcium released 75.3% of the total microsomal digoxin. Addition of 2.5 mM calcium to the urea solution caused the release of a further 25% of the bound digoxin, i.e., 100% of the bound digoxin was now released. The addition of calcium to the urea solution released a quantity of hitherto bound digoxin similar to that observed with the addition of calcium to the sucrose solution.

The results suggest that the digoxin bound to microsomes may be categorized into three subfractions. A fraction, consisting of about 25% of the bound digoxin, which is released by resuspension in distilled water, sucrose solution containing EDTA or isotonic NaCl, a second fraction, removed by resuspension in 5 M urea comprising an additional 50% of the bound digoxin, and a final fraction of approximately 25% of the total microsomal bound digoxin which can be removed when the resuspension medium either sucrose or urea, contains calcium ion.

Since calcium-free urea did not release, the digoxin in the calcium-labile fraction is probably not bound to the microsomal fraction by hydrogen-bonds. It should be noted however, that solutions containing K^+ ions, i.e. Ca-free Krebs of 120 mM KCl, released more digoxin than did either sucrose-EDTA, isotonic NaCl or distilled water. We have not yet determined whether this additional release was from the calcium-labile or the urea-labile pool, or both. If future experiments confirm the values shown in table 17, it would appear that EDTA must be present to minimize the loss of digoxin from the microsomes.

5. Calcium-labile fraction

The known interactions between digitalis and calcium in the microsomal fraction in the heart (Lee and Choi, 1966) suggest that the calcium-labile fraction may be related to the positive inotropic effect and may contain the digitalis receptor. If this hypothesis is correct, then the inotropic effect should be directly related not only to the concentration of digoxin in the perfusate, but moreover to the absolute amounts of digitalis on the calcium-labile fraction.

To determine if a relationship exists between the positive inotropic effect and the glycoside concentration in the calcium-labile fraction, the following criteria must be satisfied:

a) Contractility must be dependent on the glycoside concentration in the medium

b) The positive inotropic effect must be related to the digoxin concentration of the calcium-labile fraction

c) Inotropic interventions must alter the digitalis

concentration in the calcium-labile fraction

d) The calcium-labile fraction should be saturable at the upper limit of the therapeutic range of digoxin concentrations.

a. Dose-response relationship

Hearts were perfused for 30 min with various digoxin concentrations, that is, 5×10^{-8}, 1×10^{-7}, 2×10^{-7}, 4×10^{-7}, 5×10^{-7}, 1×10^{-6} and 1×10^{-5} g/ml. The lowest concentration did not affect contractile force. The increase in contractile force was dose-dependent in the range of 1×10^{-7} to 5×10^{-7} g/ml.

Positive inotropic effect was plotted as a function of the logarithm of digoxin concentration in the perfusate (fig. 14). A straight line relationship between the concentration and positive inotropic effect was obtained ($r = 0.99$, $P < .05$). When the regression line was extrapolated to the digoxin concentration where no positive inotropic effect would be expected to occur, 7.3×10^{-8} g/ml digoxin would be threshold concentration for the positive inotropic effect. In the majority of experiments, 5×10^{-7} g/ml caused contracture after 30 min exposure. The highest concentrations (1×10^{-6} and 1×10^{-5} g/ml) showed a marked positive inotropic effect, which was immediately followed by the development of toxic effects, i.e. contracture. Arrhythmias also occurred. The effect of digoxin on the contractility in the concentration used in our investigation have been summarized in table 18. The tissue to medium radioactivity ratio after 30 min perfusion with digoxin decreased as the concentration of digoxin in the perfusate was increased.

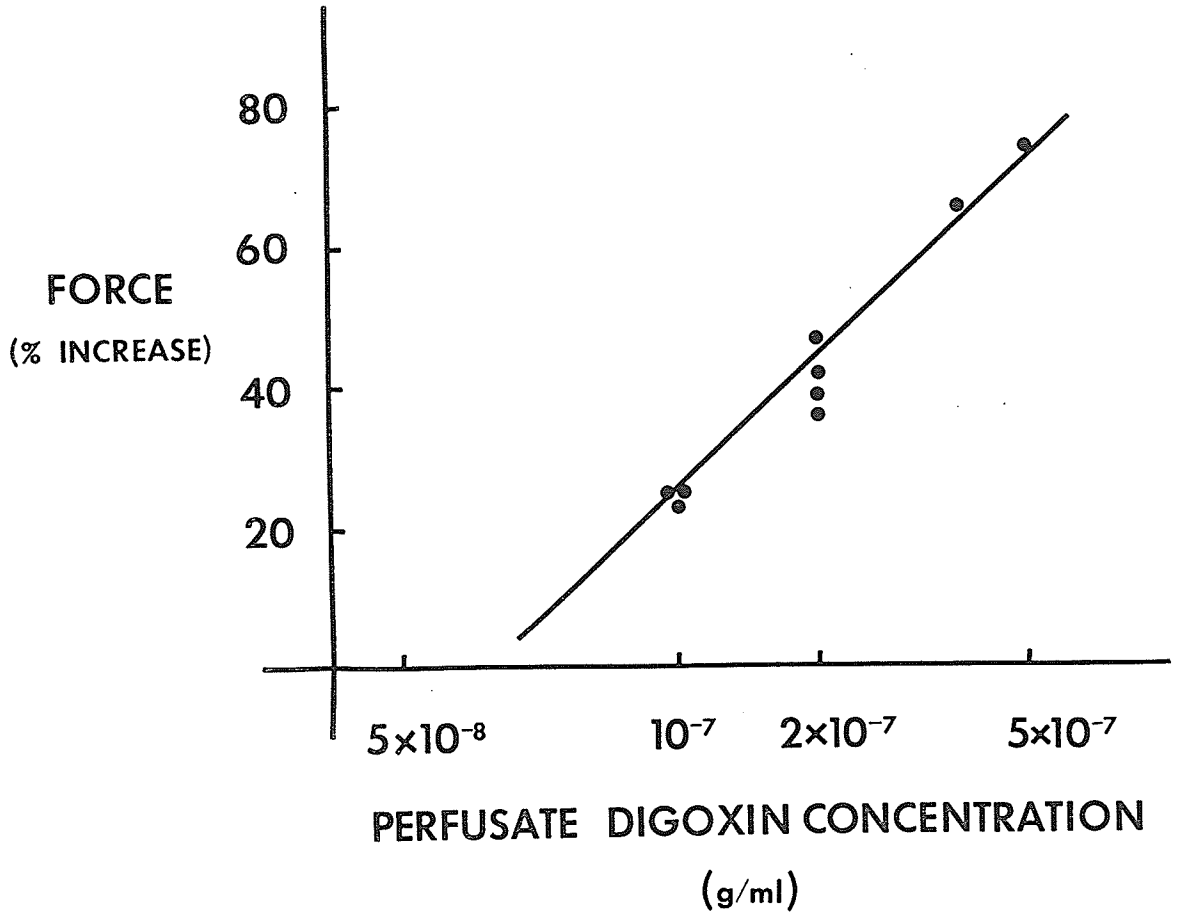


Figure 14.

Dose response curve for the positive inotropic effect of digoxin in isolated guinea pig hearts. Each guinea pig heart was perfused with a single concentration of digoxin for 30 min. The increase in contraction is expressed as the percent increase over initial contractility.

TABLE 18

Effect of various concentrations of digoxin on the positive inotropic effect and tissue uptake in the guinea pig heart perfused for 30 min with digoxin.

Digoxin concentration (g/ml)	Contractile force (% increase of the initial value)	Tissue : Medium radioactivity ratio
5×10^{-8}	-----	3.04
1×10^{-7}	23.6	2.12
2×10^{-7}	42.05	2.14
4×10^{-7}	65.0	1.92
5×10^{-7}	73.0	1.51
1×10^{-6}	Contracture and arrhythmia	0.87
1×10^{-5}	Contracture and decreased tension	0.86

The correlation between the microsomal digoxin and calcium labile digoxin and the change in cardiac contractile force are shown in figures 15 and 16.

The amounts of digoxin on the calcium labile fraction was obtained from the microsomal digoxin remaining on the pellet after resuspension in the 5 M urea solution. Significant correlations ($r = .87$, $P < .01$ for the data contained in figure 15; $r = .97$, $P < .001$ for the data in figure 16) were observed when the digoxin amount on each fraction was plotted against the corresponding enhancement of cardiac contractile force after digoxin perfusion for 30 min. As shown in figure 20A, total tissue uptake of ^{3}H-digoxin was also correlated well with the change in contractile force ($r = .99$, $P < .01$) in these dose-response experiments.

The inotropic response was observed with microsomal contents of digoxin from 2.34 to 9.35 ng/mg microsomal protein and from 0.46 to 1.89 ng/mg microsomal protein of digoxin on the Ca-labile sites. The amount of digoxin on the calcium-labile fraction corresponds to 20 to 24% of total microsomal digoxin. Inotropic effects were not observed in two hearts perfused with a digoxin concentration of 5×10^{-8} g/ml, but the microsomal digoxin contents and the digoxin on the calcium-labile fraction were 1.95 ± 0.02 and 0.41 ± 0.01 ng/mg microsomal protein, respectively. The microsomal digoxin contents of 1.95 ng/mg microsomal protein tend to confirm again the conclusion reached by extrapolation of the regression line to zero in the microsomal fraction (fig. 11). That is, there is a threshold concentration of digoxin required to activate the inotropic processes. In these experiments, 0.41 ng digoxin/mg microsomal protein was bound on the calcium labile

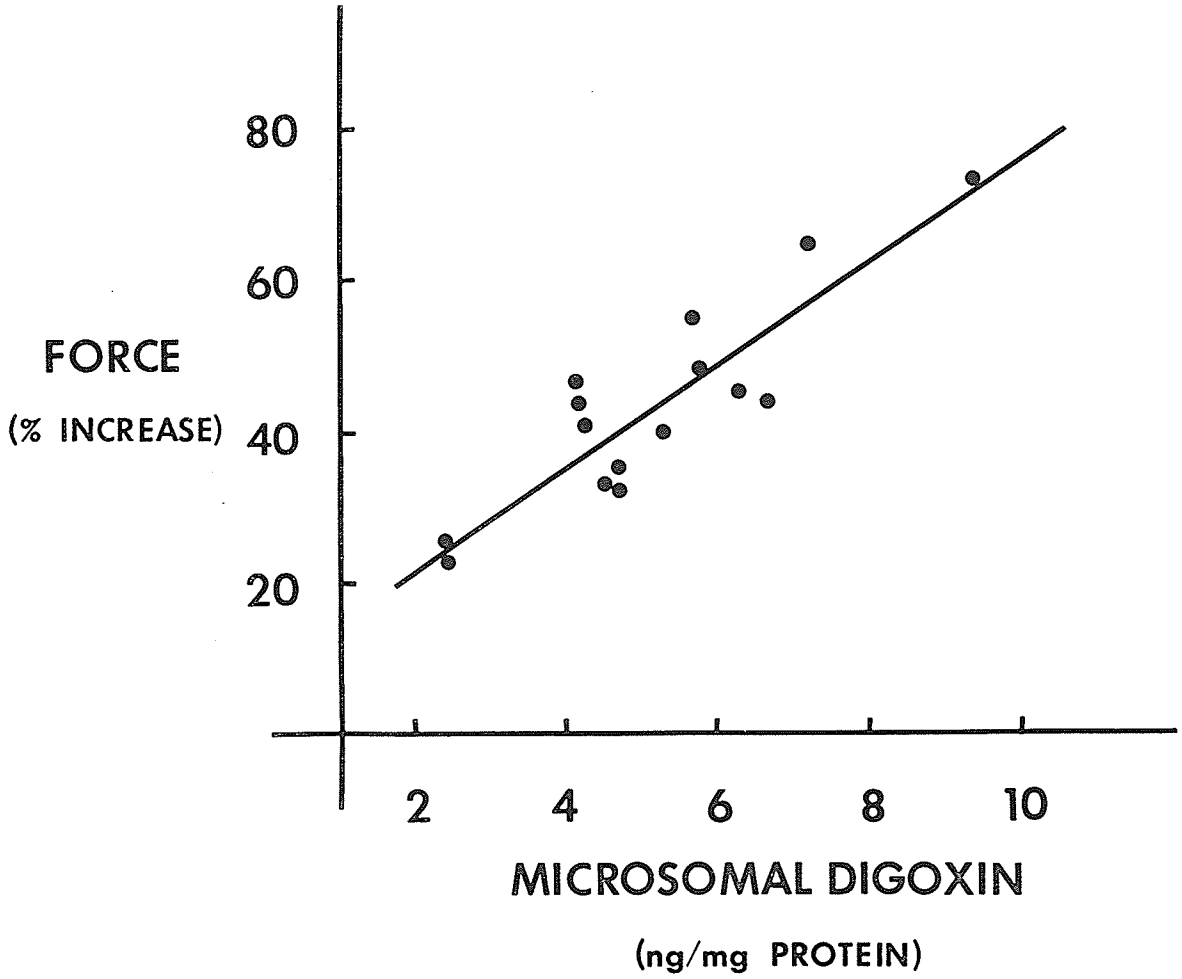


Figure 15.

Relationship between the total microsomal uptake of ^{3}H-digoxin and the changes in contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 min with ^{3}H-digoxin. The digoxin concentrations ranged from 1×10^{-7} to 5×10^{-7} g/ml.

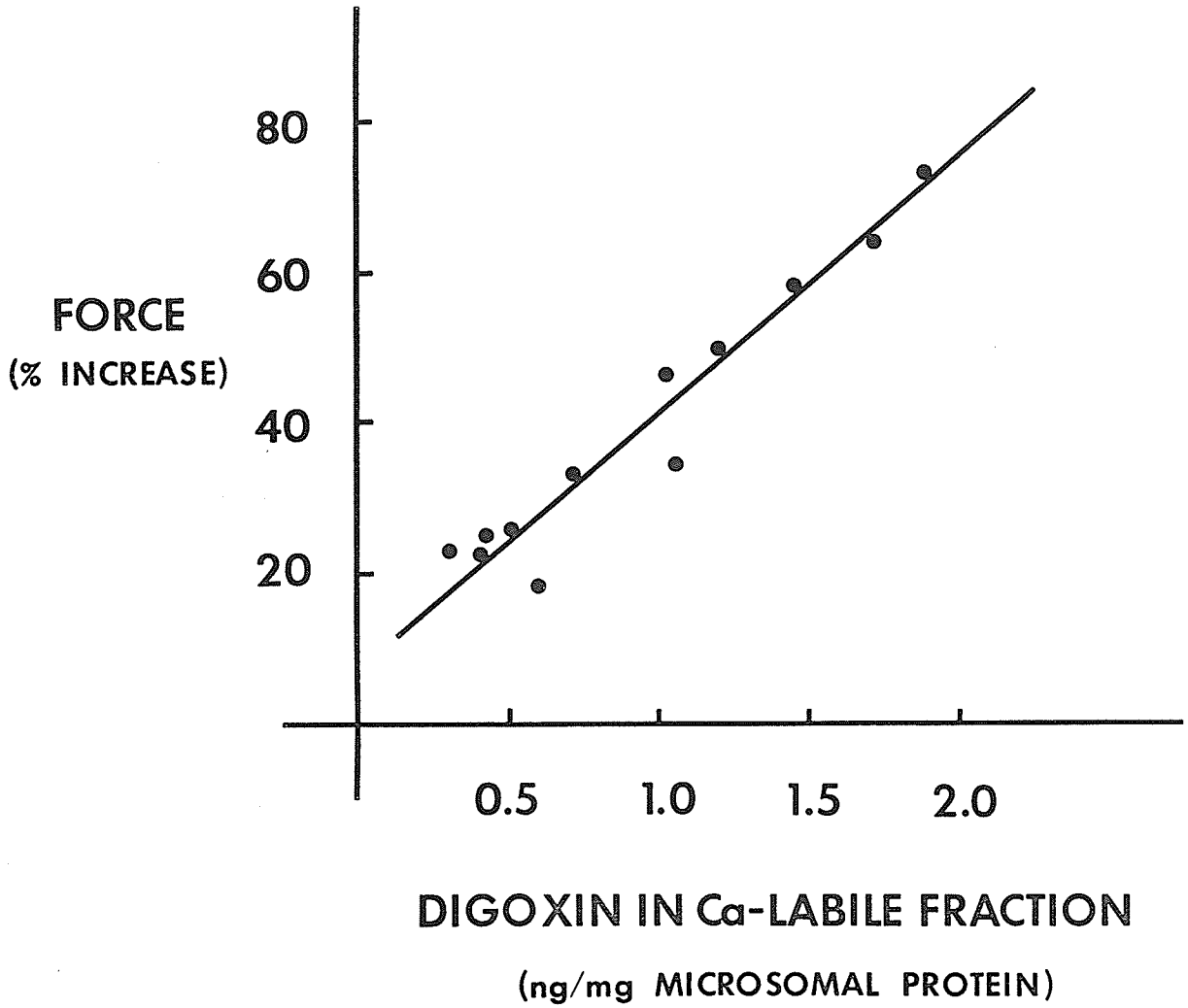


Figure 16.

Relationship between the digoxin in the Ca-labile fraction released from the microsomal fraction by resuspension and the changes in contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 min with ^{3}H-digoxin. The digoxin concentration ranged from 1 x 10^{-7} to 5 x 10^{-7}g/ml.

fraction without showing a positive inotropic effect. In figure 16, it was shown that 0.46 ng/mg microsomal protein is the digoxin amounts responsible for the minimum inotropic effect. Therefore, it is probable that 0.4 to 0.5 ng digoxin/mg microsomal protein is the range of threshold concentration for the minimum inotropic effect.

Table 19 shows that digoxin binding by the microsomal fraction and by the calcium-labile fraction in the heart perfused with glucose-free Krebs-Henseleit solution. Digoxin did not have a positive inotropic effect in this medium but rather decreased contractility. The digoxin content on the calcium-labile fraction was 0.46 ng/mg microsomal protein. This result again confirms that there is a threshold concentration of digoxin on the calcium-labile fraction.

b. The effect of the inotropic interventions on the calcium-labile fraction.

The effect of insulin and aldosterone on the digoxin binding on the calcium-labile site was investigated. It was shown previously that aldosterone decreased the positive inotropic effect by decreasing the quantity of the digoxin bound loosely to the microsomes (see above). Insulin was also used to increase the positive inotropic effect by increasing the microsomal uptake of digoxin. It should be emphasized that these measurements were made at a single digoxin concentration, 2×10^{-7} g/ml.

2 mU/ml of insulin and 2×10^{-7} g/ml of aldosterone were given 30 min prior to and throughout the exposure to digoxin as in previous experiments with loosely bound digoxin. Insulin increased the positive inotropic effect and the binding of digoxin by the microsomal fraction. To obtain the amounts of the digoxin on the calcium-labile fraction,

TABLE 19

The ^{3}H-digoxin uptake by the microsomal fraction and by the calcium-labile fraction in a guinea pig heart perfused with glucose-free Krebs-Henseleit solution for 30 min.

Treatment	Digoxin (ng/mg microsomal protein)	
	Microsomal fraction	Ca-labile fraction
digoxin 2×10^{-7} g/ml	3.25	0.46

microsomal fraction was resuspended in urea solution (see Methods). We have shown previously that calcium-labile fraction comprises approximately 25% of the total microsomal fraction in untreated hearts. The ratio of calcium-labile to urea releasable digoxin was not changed significantly in insulin treated hearts as compared to the untreated hearts (i.e., untreated heart, 0.22; insulin treated heart, .17). Aldosterone decreased the positive inotropic effect but did not decrease the total microsomal digoxin significantly in this series of experiments. However, aldosterone decreased significantly the ratio of calcium-labile to urea releasable digoxin (aldosterone treated heart, .10).

The inotropic effect in these group of hearts were correlated with the digoxin amounts of calcium-labile fraction as shown on the figure 17. There was a highly significant correlation between the positive inotropic effect and digoxin content of the calcium labile fraction in these hearts ($r = .88$, $P < .01$). The coefficient of correlation between the positive inotropic effect and the quantity of digoxin remaining on the microsomes after the calcium-labile digoxin had been removed by resuspension in 5 M urea solution was not significantly different from zero ($r = .45$, $P > .05$). These results are consistent with those in Section B, 2. Loosely bound digoxin obtained by resuspension in Krebs-Henseleit solution was correlated with the positive inotropic effect, but the digoxin which remained bound after that resuspension procedure was not correlated.

c. Binding capacities on the Ca-labile fraction

To investigate the total binding capacities by the calcium-labile fraction, the hearts were perfused with digoxin from threshold inotropic concentration 5×10^{-8} to concentration which caused

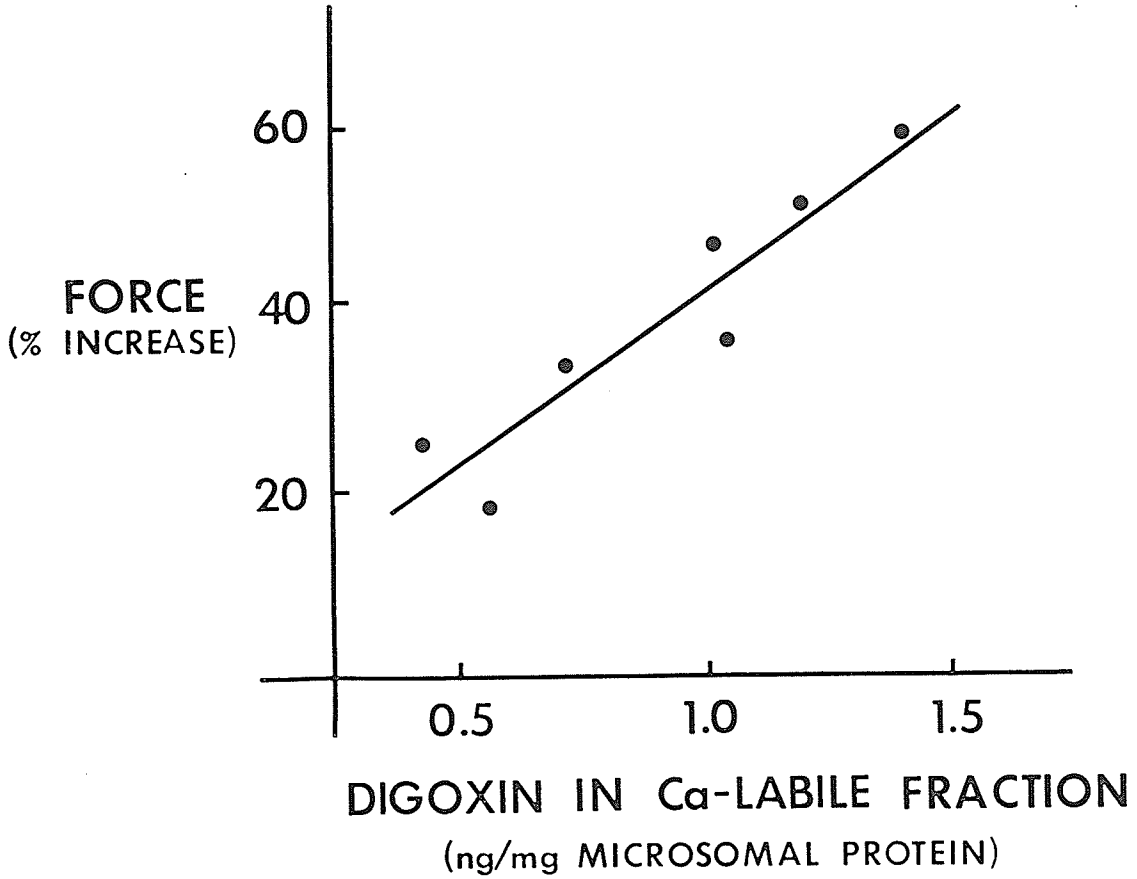


Figure 17.

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 x 10^{-7}g/ml) or insulin (2 mU/ml).

contracture (1×10^{-6} g/ml). The digoxin content in the calcium-labile fraction was plotted against the digoxin concentration in the perfusate (fig. 18). The microsomal digoxin content was also plotted against the digoxin concentration in the perfusate (fig. 18). Figure 18 shows that an increase in the digoxin concentration of the perfusate caused an increase in the quantity of digoxin bound on the calcium-labile fraction, indicating the uptake of digoxin by the calcium-labile fraction was dose-related. Over the range of digoxin concentrations in the perfusate, a saturable component in digoxin uptake became evident, indicated by the fact that the slope fell progressively with higher perfusion concentrations in both total and calcium-labile microsomal fractions (fig. 18). When the digoxin content in the calcium-labile fraction was plotted against the logarithm of digoxin concentration in the perfusate (fig. 19), a sigmoid pattern was observed. To determine if the binding site became saturable, a concentration of digoxin clearly in the toxic range, 1×10^{-5} g/ml was used. However, the amounts of digoxin bound in both fractions, microsomal and calcium-labile fraction, was nearly doubled by increasing the dose from 1×10^{-6} to 1×10^{-5} g/ml, i.e. from 12.94 to 20.70 ng/mg microsomal protein in the total microsomal fraction, and from 2.26 to 4.4 ng/mg microsomal protein in the calcium-labile fraction.

As shown in figure 20A, a total tissue uptake in guinea pig hearts also follows a sigmoid pattern as a function of perfusate digoxin concentrations, suggesting that digoxin uptake by the heart is also a saturable process at higher concentrations of digoxin. When the hearts were exposed to the toxic concentration of digoxin (1×10^{-5} g/ml) for

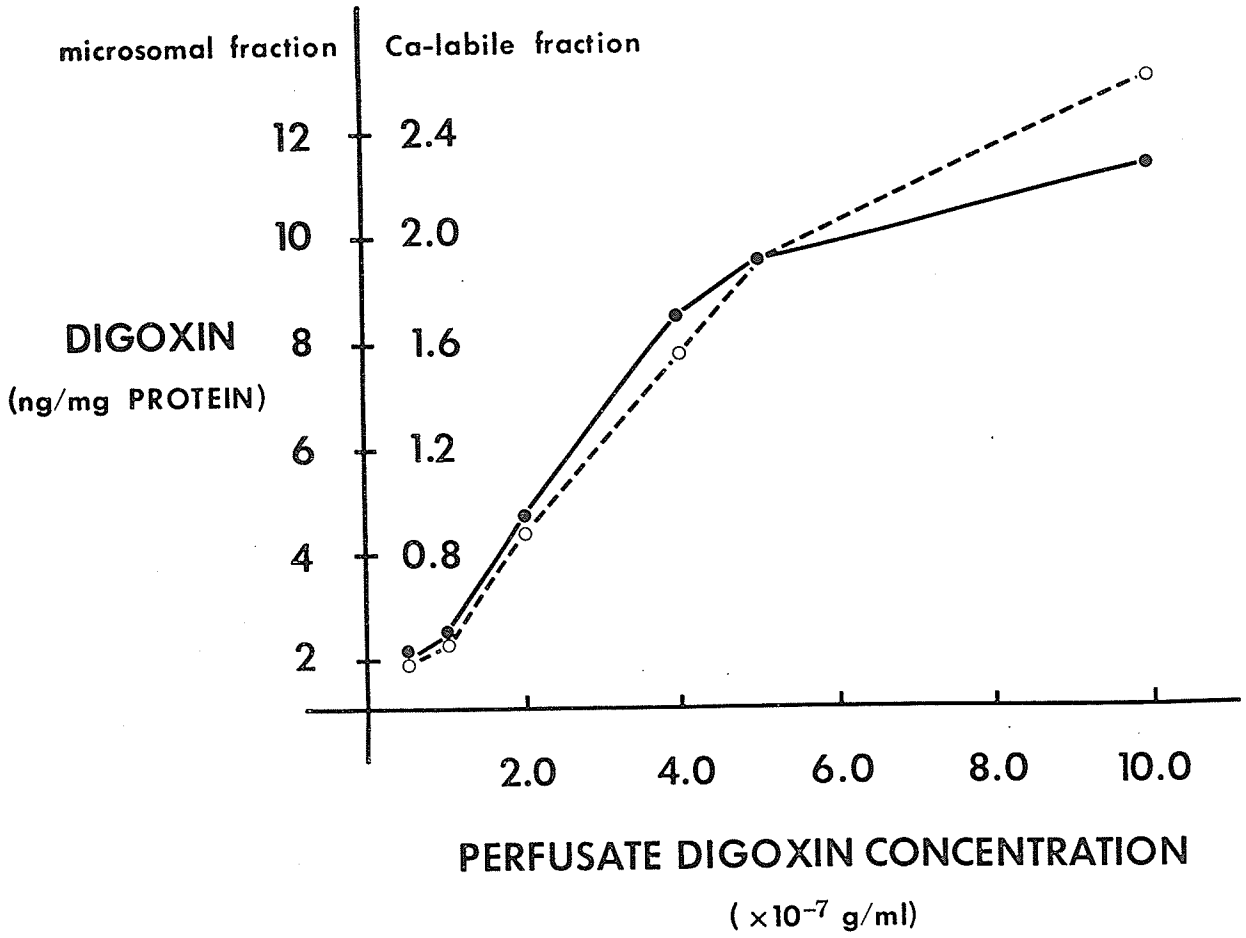


Figure 18.

Digoxin concentration for guinea pig heart microsomal o ---- o and Ca-labile fraction ●-----●, when the hearts were perfused with digoxin at various concentrations. Individual hearts were perfused for 30 min with a single concentration of digoxin, then washed out for 4 min with Krebs-Henseleit solution. Each point represents the mean of at least 2 hearts.

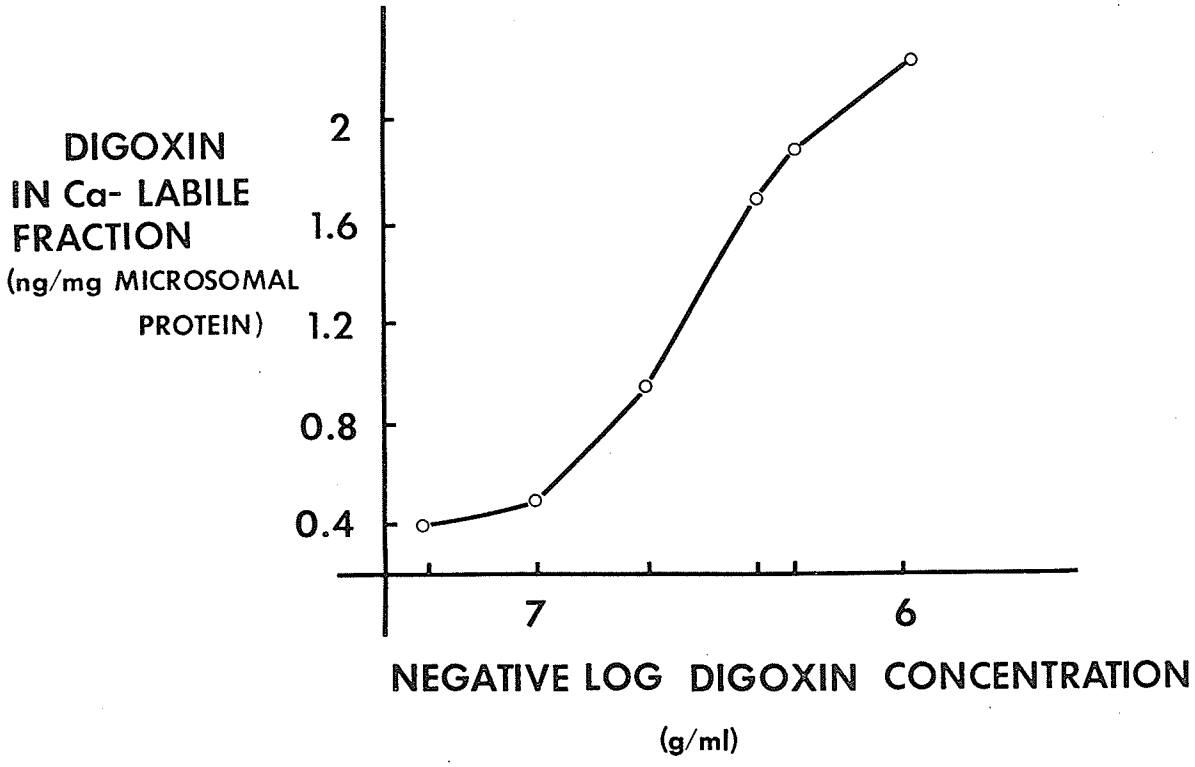


Figure 19.

Digoxin concentration on calcium-labile fraction, when the hearts were perfused with various digoxin concentrations. Each heart was perfused with a single digoxin concentration for 30 min, then subjected to 4 min of wash with drug-free Krebs-Henseleit solution. Each point represents the mean of at least 2 hearts.

30 min, the hearts after brief, initial positive inotropic effect developed contracture. This was followed by a gradual decrease in resting tension to the base line. Total tissue uptake was increased approximately ten times above that measured at 1×10^{-6} g/ml (fig. 20, B). Digoxin uptake by the heart appeared to be related to the concentration of perfusate in the guinea-pig hearts (fig. 20, A). However, it should be noted that digoxin was taken up by the tissue increased by only 4 times, increasing the concentration of perfusate from 1×10^{-7} to 1×10^{-6} g/ml (from 212 to 870 ng/g tissue).

In the range from 4×10^{-7} to 1×10^{-6} g/ml of digoxin in the perfusate a saturable component in digoxin uptake by myocardial tissue became evident, indicated by the fact that uptake by tissue was increased approximately 13%, by increasing the digoxin concentration in the perfusate from 4×10^{-7} to 1×10^{-6} g/ml, respectively (from 768 ng/g tissue to 870 ng/g tissue).

d. The effect of Krebs-Henseleit solution on the release of bound ouabain.

It was shown in previous experiments that resuspension of the microsomal pellet obtained from the hearts perfused with digoxin in Krebs-Henseleit solution released 46.8% of the total microsomal digoxin into the supernatant. These experiments, therefore were designed to determine if ouabain has the same binding properties as digoxin.

A single heart was perfused with ^{3}H-ouabain for 30 min and fractionated to obtain the microsomal fraction by the usual method. The microsomal fraction was resuspended in Krebs-Henseleit solution for 5 min and centrifuged. ^{3}H-ouabain content released by resuspension of the microsomes was approximately 60% of the total microsomal ouabain. This

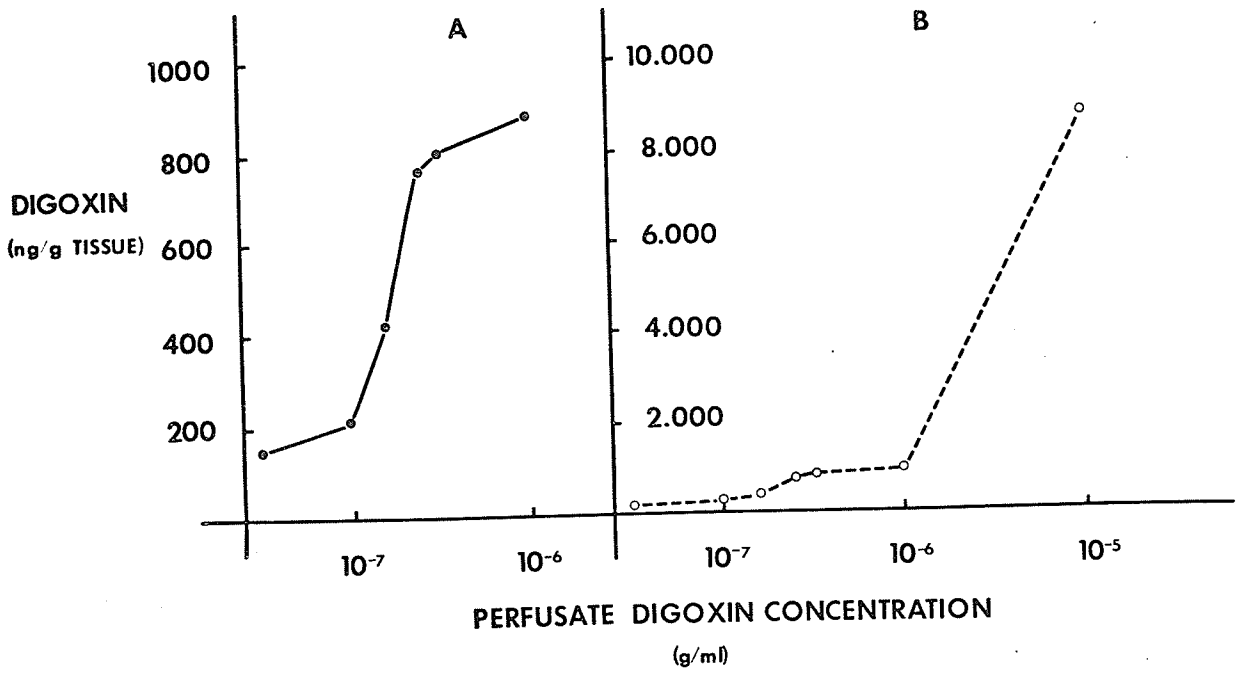


Figure 20.

Myocardial content of digoxin as a function of dose. Each point on curve represents mean of 2-3 animals. A, solid line, represents expanded curve of dotted line B. The curve in the concentration range of 10^{-6} to 10^{-5} g/ml was not shown in solid line.

suggests that ouabain was probably bound to microsomal fraction more specifically than digoxin, and is probably more sensitive to calcium ions. (see Section A, 2, e for the ouabain uptake by the microsomal and total tissue). Therefore, it may be possible that considerable amounts of bound ouabain by the microsomal fraction may be released during washout with Krebs-Henseleit solution containing calcium for 4 min. Isolated microsomal fraction may not be affected by digoxin in the extracellular space, since it was shown by Dutta and Marks (1968a) that added digoxin to the homogenized cardiac tissue in sucrose solution did not bind to total prticulate fraction.

6. Gel filtration

The 3rd supernatant obtained from resuspension of microsomal pellet was used to determine whether the calcium-labile digoxin removed from the particulate by resuspension was free or remained associated with a protein component. The microsomal pellet was resuspended in the Krebs-Henseleit solution and centrifuged. The 3rd supernatant was layered on the tope of a G-15 Sephadex column. The elution pattern obtained from one such experiment is shown in figure 21. The elution pattern from Sephadex G-15 indicated that retention of the radio-activity was the same as for an authentic sample of free ^{3}H-digoxin. The protein peak corresponds to the elution volume of serum albumin. Resuspension in sucrose solution has been tried and it is clear that the 3rd supernatant contained free ^{3}H-digoxin.

7. Dog heart

a. The effect of resuspension media on the release of bound digoxin.

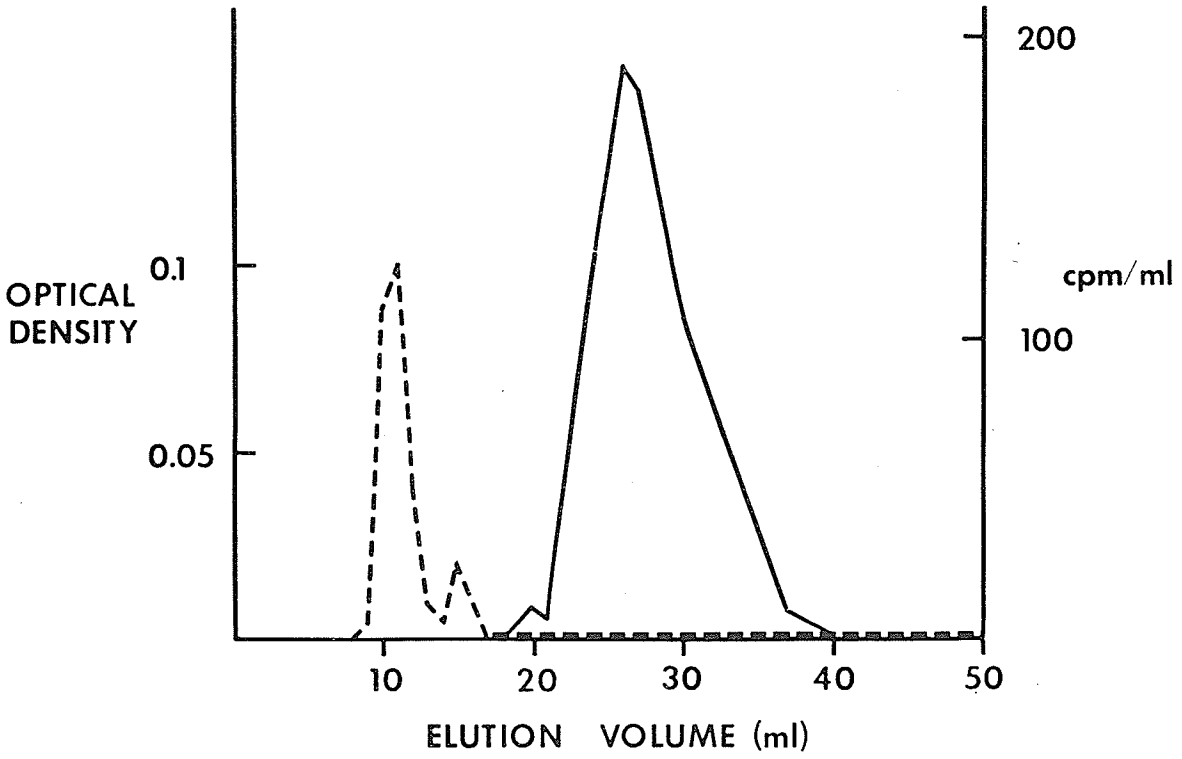


Figure 21.

Elution diagram of 3rd supernate with 0.9% sodium chloride solution from 1.5 by 22 cm Sephadex G-15 column. The right ordinate indicates the radio-activity of the eluate; the left the optical density at 280 mu; and the abscissa indicates the elution volume in ml. The dotted line indicates the protein content of the eluate (Optical Density). Solic line indicates the ^{3}H-digoxin content of the eluate (CPM/ml).

It was shown that approximately 25% of the digoxin bound by the microsomal fraction was calcium-labile and was directly related to the positive inotropic effect. To investigate whether calcium-labile fraction exists in the dog hearts, the microsomal fraction obtained from dog hearts were exposed to digoxin in vitro for 30 min and then centrifuged. The microsomal pellet obtained was resuspended in the same media used for the guinea pig hearts. The effect of calcium on the release of bound digoxin from the microsomal fraction are given in table 20. Calcium ion released bound digoxin from the microsomal pellet in dog heart.

b. Fractionation of calcium-labile fraction by density gradient in dog heart.

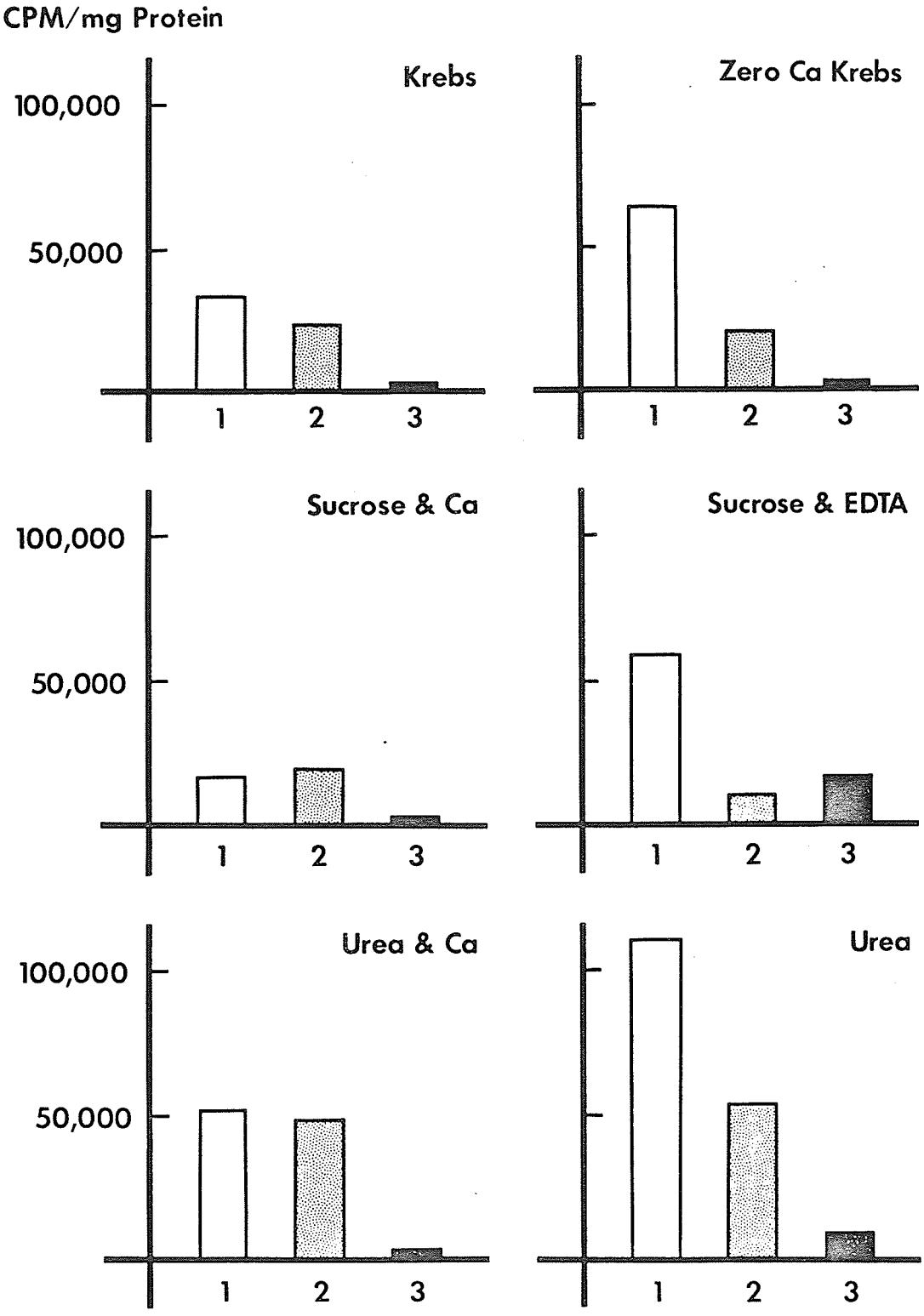
It was shown that digoxin contained in the calcium-labile site may be responsible for the positive inotropic effect. Therefore, we attempted to isolate the digoxin bound to the calcium-labile fraction of the microsomes by recentrifugation of the microsomal fraction on the discontinuous sucrose density gradient ranging in concentration from 35 to 60% of sucrose.

The resuspension media was 11% sucrose solution containing EDTA, zero calcium Krebs-Henseleit solution or 5 M urea solution. The top of the liquid column after centrifugation was assumed not to contain any particulate matter. Three layers appeared below the top. Bound ^{3}H-digoxin was recovered with the highest specific activity at the interface between the sample layer and 35% sucrose (1st) in either of zero calcium Krebs-Henseleit solution or sucrose solution containing EDTA or 5 M urea resuspension. There was lower specific activity between the 35 and 45% interface (2nd), 45 and 60% interface (3rd) (fig. 22).

TABLE 20

The effect of various ion on the release of bound digoxin from the microsomal fraction of dog

Medium	% of total microsomal digoxin (per mg protein) removed by resuspension procedure in dog heart
Sucrose plus EDTA	67.84
Sucrose plus 2.5 mM Ca	79.82
5 M Urea	78.63
5 M Urea plus 2.5 mM Ca	92.01


CPM/mg Protein
Krebs
Zero Ca Krebs
Sucrose & Ca
Sucrose & EDTA
Urea & Ca
Urea
100,000
50,000
1
2
3

Addition of 2.5 mM calcium to each of the resuspension media decreased significantly the specific activity of material at the 1st interface in the Krebs-Henseleit solution, the sucrose solution or 5 M urea (fig. 22). There was no significant change of specific activity in the 2nd and 3rd interface of the sucrose gradient in each resuspension, (fig. 22). The results suggest that the calcium-labile fraction was bound to the light microsomal fraction.

When the radioactivity on each interface was expressed as a percent of total microsomal digoxin, the digoxin concentration in the non-particulate layer (see above) was greater than the subfractions obtained at the interfaces between the gradients (fig. 23). It is interesting to note that digoxin content in the 1st interface was decreased in all resuspension media when calcium was added, but increased in the 2nd interface, that is, the total digoxin content were consistently but not significantly greater in these layers when the microsomal fraction was resuspended in the presence of calcium. Calcium caused no change in total digoxin content of the 3rd interface.

In figure 24, the protein contents in each layers are shown. Calcium ion did not affect the distribution of protein in each layer, but the distribution of protein was remarkably dependent upon the resuspension media. We have used similar quantities of microsomal fraction for the resuspension in each media, but total quantities recovered from each tube of sucrose gradient was different, especially in sucrose resuspended media. Results suggest that urea may solubilize the protein of microsomal fraction, thus, increases the protein amounts in sample layer, whereas Krebs-Henseleit solution may aggregate the protein of the microsomal fraction, thus sediment into heavy layers (fig. 24).

Figure 23.

Dog microsomal pellet was resuspended in various media for 5 min and layered on the discontinuous sucrose gradient and centrifuged. Numerals S, 1, 2 and 3 on the ordinate represent the sample layers; the sample: 35% sucrose interface; the 35:45% sucrose interface and the 45:60% interface, respectively.

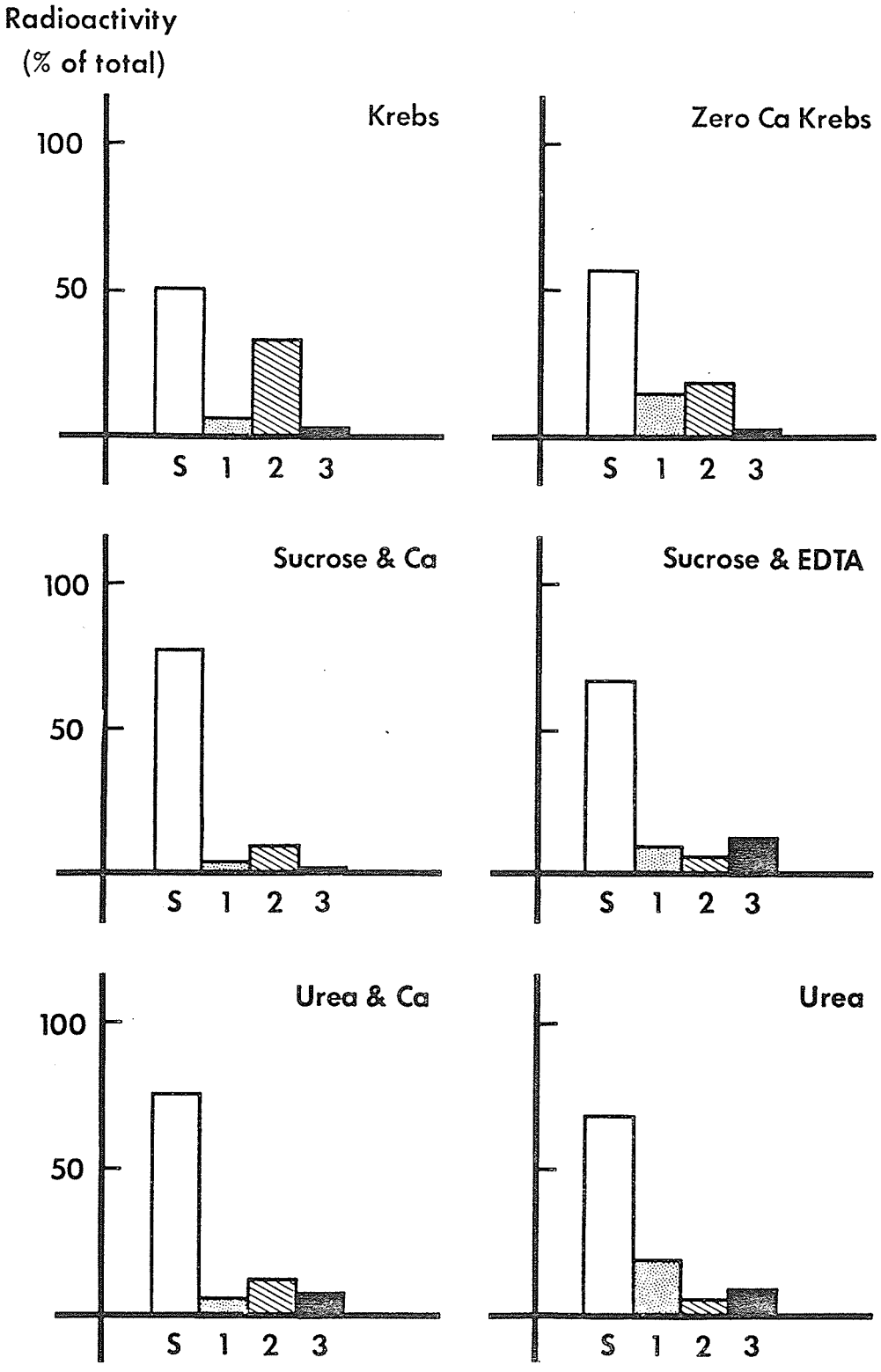
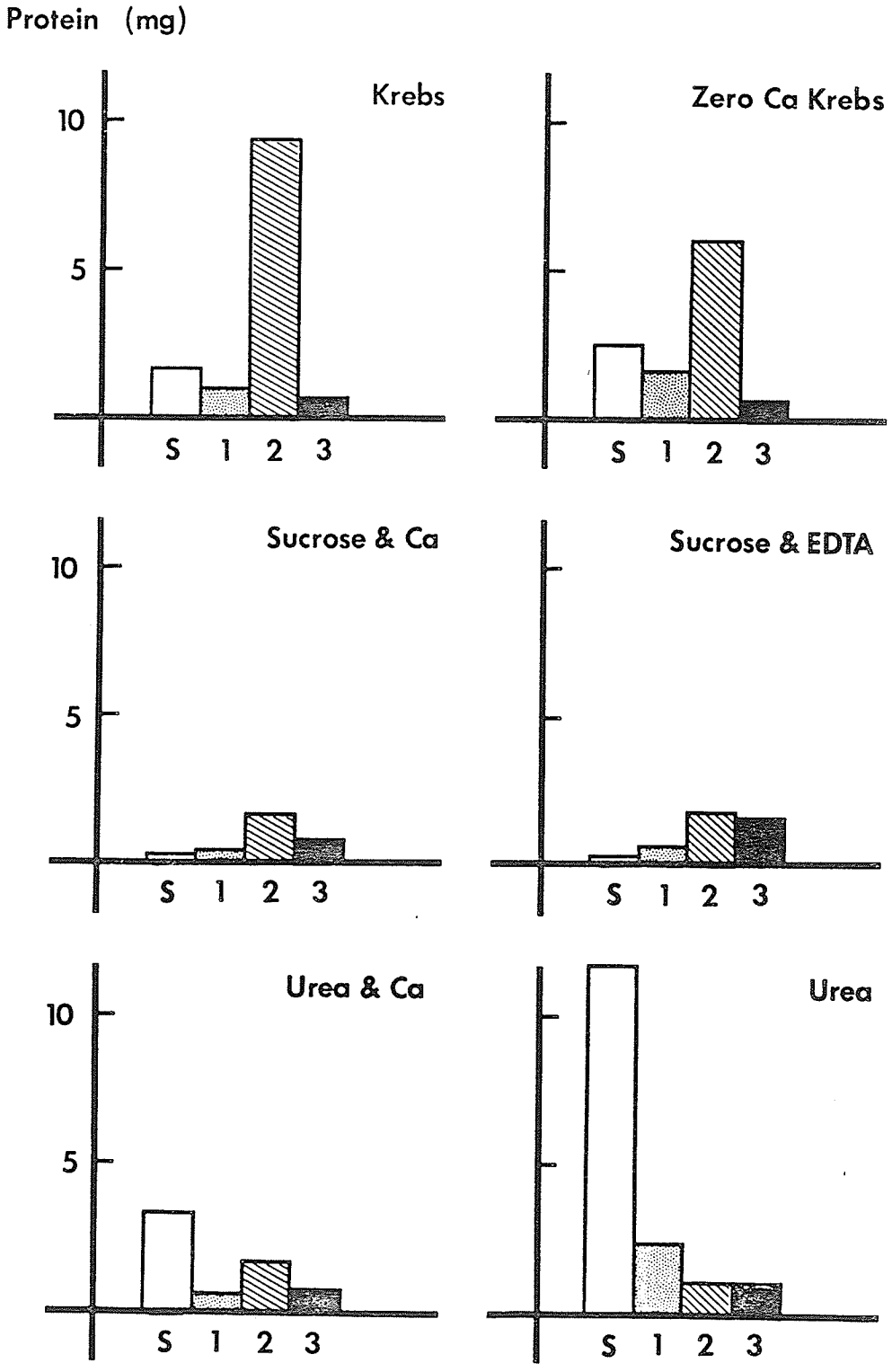

Radioactivity
(% of total)
Krebs
Zero Ca Krebs
Sucrose & Ca
Sucrose & EDTA
Urea & Ca
Urea
100
50
S 1 2 3

Figure 24.

Dog microsomal pellet was resuspended in various media for 5 min and layered on the discontinuous sucrose gradient and centrifuged. Numberals S, 1, 2 and 3 represent the sample layers; the sample: 35% sucrose interface; the 35:45% sucrose interface and the 45:60% sucrose interface, respectively.


Protein (mg)
Krebs
Zero Ca Krebs
Sucrose & Ca
Sucrose & EDTA
Urea & Ca
Urea
10
5
S 1 2 3

c. Electronmicroscopic examination of microsomal fractions

It has been reported that microsomal fraction does not represent a pure preparation of sarcoplasmic reticulum fragment, but contains other subcellular organelles, notably mitochondria (Katz and Repke, 1967). We fractionated the microsomal fraction by the method of Pretorius et al., (1969). Figure 25 is an electronmicroscopic view of cardiac microsomes obtained from dog heart. This view shows vesicles and some of these vesicles have a granular outer surface, ribosome and glycogen granules. Some fibrous material (similar to the actomyocin) was found. Mitochondrial fragments cannot be determined in the electromicroscopic study. However, Pretorius et al., (1969) have reported by using negative staining that the microsomal fraction contained 25 to 30% of mitochondrial contamination and was reduced to 7% after centrifugation on a sucrose gradient. Figure 26 shows a view of the interface between the sample and 35% sucrose (the calcium labile fraction) which was obtained by centrifugation on the sucrose density gradient. This preparation appeared to consist exclusively of vesicles by electronmicroscopic examination. Pretorius and coworkers (1969) have indicated that this microsomal fraction originates from the fragment of sarcoplasmic reticulum because they showed a peculiar elongated appearance when stained with phosphotungstate.

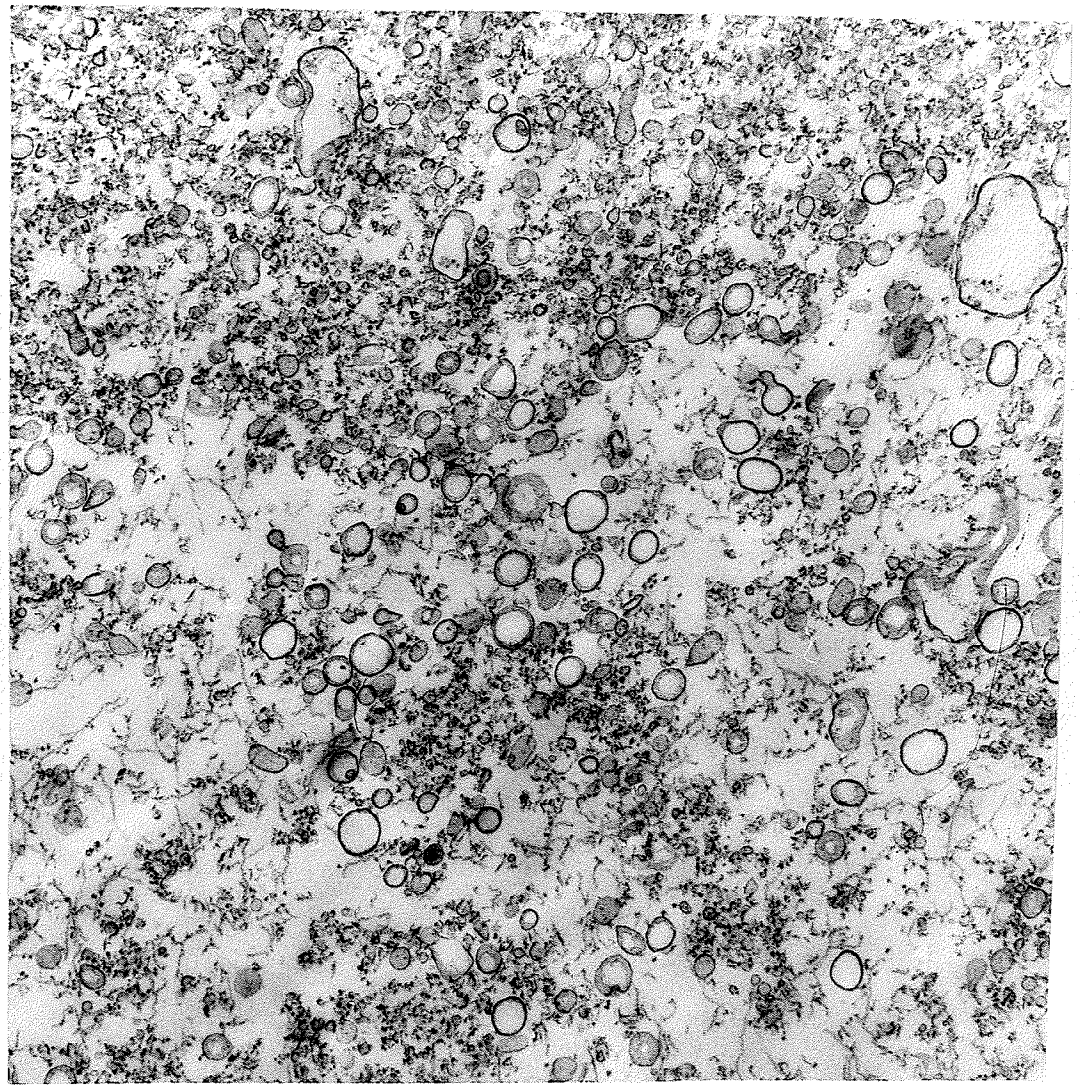


Figure 25.

Electron microscopic view of the microsomal fraction of dog heart. Magnification; 9,500 x 2.7.

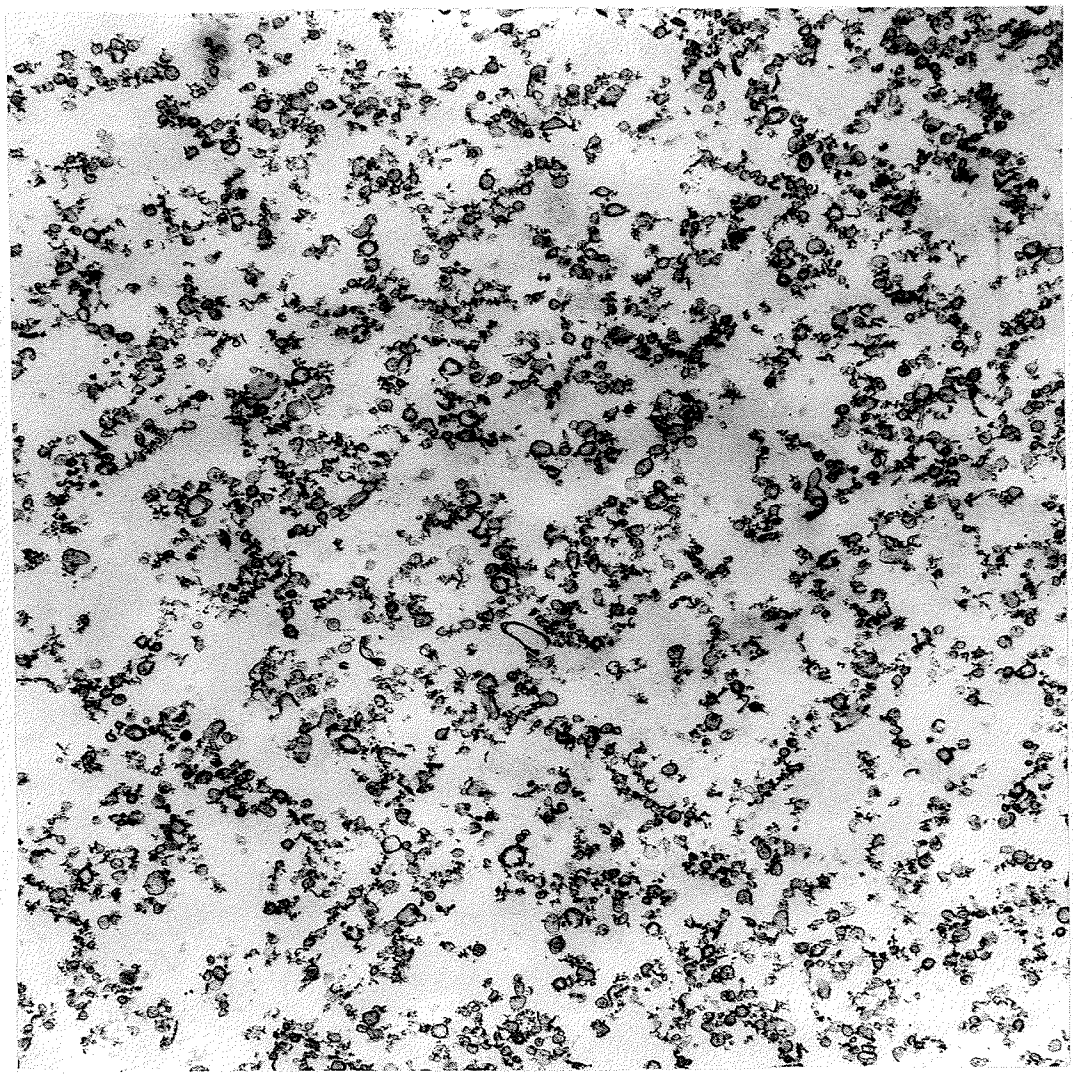


Figure 26.

Electron microscopic view of the calcium-labile fraction obtained from dog microsomes.
Magnification; 9,500 x 2.7.

IV. DISCUSSION

A. The Positive Inotropic Effect and the Microsomal Uptake of ^{3}H-Digoxin

There are two major differences between the material reported in this thesis and previous work in the field. Previous studies on the relationship of the uptake of digitalis glycosides to their pharmacological effect have suffered from the fact that tissue uptake was not measured in the same experiments in which the pharmacological effects were established. We believe that it is essential, especially when interactions between drugs are studied, that a quantitative relationship be based on simultaneous study of the two variables in the same preparations. The second major difference, which makes it difficult to relate the present study with previous ones, is the fact that our major conclusions are based, not on total tissue uptake, but on the uptake by a portion of the microsomal fraction obtained from the hearts. Despite the early demonstration of the lack of reliability of measurements of total cardiac uptake of the glycosides as indicative or correlated with their pharmacological effect, (Kuschinsky et al., 1968a,b), studies in this field have almost without exception utilized total tissue uptake as the measure. According to Dutta and Marks, total tissue uptake of ^{3}H-digoxin is always directly related to microsomal uptake and this total tissue uptake could be taken as an estimate of microsomal binding. However, insulin, aldosterone, and chlorpromazine, and even substrate-free medium did not change the total tissue uptake of ^{3}H-digoxin in our experiments. We suggest that the study of total tissue uptake is probably not sufficient to establish interactions between drugs or comparisons between glycosides. Thus our results indicate that insulin did not facilitate the transport of

digoxin into the cell, but specifically increased the binding of the drug to a site in a structure sedimenting as a microsome which is correlated with the increase in contractile force. Aldosterone and chlorpromazine did not affect total tissue uptake of digoxin by guinea pig hearts but decreased the binding of the drug to the microsomal fraction which is correlated with the decrease in contractile force. We feel that our results are clear enough, that when one also considers the work of Kuschinsky et al., (1968a,b) and Roth-Schechter et al., (1970), we may conclude that the reliance of Dutta and Marks on total tissue uptake as a measure of microsomal binding is incorrect. This makes it extremely difficult to determine exactly how the work reported in this thesis fits into the general picture of the relationship of drug uptake with pharmacological effects.

We found it impossible to study the relationship of tissue distribution to the inotropic effect in spontaneous beating hearts. There were considerable changes in rate during perfusion with digoxin and the positive inotropic effect reached a maximum at 15 min and declined during the next 15 min of perfusion. The positive inotropic effect measured after 30 min could not be correlated with the amount of digoxin taken up by the total tissue or with the binding to the microsomal fraction isolated from these hearts. This is not surprising in view of the report by Kuschinsky, that the uptake of digoxin by the heart was not affected by a reduction of the rate from 180 to 30/min. However, quiescent atria took up ^{3}H-digoxin considerably more slowly than beating hearts, although the maximal uptake at equilibrium (3 hr) was approximately the same (Kuschinsky et al., 1967). It is known

that there is a complex relationship of the interval between beats and the strength of contraction in the heart (Koch-Weser and Blinks, 1963). However, one of the actions of digitalis is to change this relationship such that the strength of contraction is approximately the same at all frequencies of stimulation (Blinks and Koch-Weser, 1963). The changes in rate due to digoxin in our experiments therefore cannot by themselves be the cause of the variability in the positive inotropic effect.

1. 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 (1969, 1971) have shown that highly purified insulin increases the rate of development of the positive inotropic effect of ouabain and k-strophanthidin in rabbit atria (Bailey and Dresel, 1969, 1971). 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. Insulin increased the magnitude of the change in contractile force after treatment with ^{3}H-digoxin for 30 min, but decreased the response at 15 min.

It is well known that insulin increases in concentration of potassium in muscle (Zierler, 1960) and in heart (Regan et al., 1963). Regan et al., (1963) 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 (Regan et al., 1963). Dutta et al., (1968b) showed that the uptake of tritiated cardiac glycoside by the fragments of sarcoplasmic reticulum was inhibited by the addition of KCl in the incubation mixture. Several other investigators (Marcus et al., 1969; Morgan and Binnion, 1970) also showed similar results, i.e. a decrease in the myocardial uptake of ^{3}H-digoxin in the hyperkalemic dog. Prindle (1971) 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 (1969).

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 (Dutta et al., 1968b). 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 (Dutta et al., 1968b). 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 (1968b) 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 contains no transverse tubular system (Sommer and Johnson, 1969), but is still sensitive to the cardiac glycosides.

Marcus et al., (1971) 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 (Hoffman and Singer, 1964; Gettes and Surawicz, 1968; Müller, 1963). In addition, Dutta and Marks (unpublished observation, 1970) 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 (table 9). Assuming that the decreased contractile force by 15 min after the heart was exposed to digoxin and insulin was due to the increased potassium in the cell, 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., (1963) reported that increased net uptake of potassium after insulin infusion into dogs was decreased by infusion of strophanthidin. This potassium loss occurred from 2 to 8 min after injection. Therefore, it seems possible 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 (Dutta and Marks, 1969a). These results are consistent with the observation by Binnion and Morgan (1970), who documented a more than two-fold increase in the 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 (Sampson et al., 1943). This effect is identical to the effect of potassium concentration on the uptake of the cardiac glycosides (Dutta and Marks, 1969a). Bihler (1968) 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 triphosphatase of cardiac muscle(Matsui and Schwartz, 1968). Secondly, Bailey and Dresel (1971) 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., (1969b) have shown that phloretin in similar concentrations decreased the total tissue uptake of ^{3}H-digitoxin by guinea pig hearts.

Thirdly, Bailey and Dresel (1969) 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 microsomal fraction in perfused guinea pig hearts and that insulin increases the inotropic effect as well as the microsomal binding in this preparation (fig.11, table 9). These suggest that sugar transport is required for the initiation of the positive

inotropic response to the cardiac glycoside (Keyl and Dragstedt, 1954a,b; Travis et al., 1956; Bailey and Dresel, 1971).

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(1968b, 1969b and unpublished results) 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 (Dutta et al., unpublished results) also reported the effect of phloretin on inotropic responses to ouabain 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 (Dutta et al., 1969b). 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 depend on the source of energy in the cell. Wenzel and Nichols (1965) showed that fluoride inhibited the effect of ouabain in normal rat ventricular strips and in cat papillary muscles and Berman et al., (1957) showed the same effect in hypodynamic rat ventricle strips. The findings of Majeski and Berman (1965), and of Bennet and Chenoweth (1952) 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 triphosphate available from this pathway (Bailey and Dresel, 1971). Factors which decrease glucose metabolism, that is, the absence of a utilizable substrate in the medium, the shift in metabolism when alternative substrates 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 present experiments, potentiates the total effect.

2. 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. There is some disagreement concerning this effect (see Levy, 1969) 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 microsomal 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 (Idänpään-Heikkila et al., 1968) decreased the total microsomal uptake of digoxin as much as did aldosterone. 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 ($r = 0.73$ $P < .05$).

B. Loosely Bound Digoxin and the Positive Inotropic Effect

Kuschinsky hypothesized that the cellular uptake at different concentrations 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 (Kuschinsky et al., 1967). The availability of the saturable sites was reduced either in the presence of another glycoside or after incubation with metabolic inhibitors (Godfraind et al., 1970; Dutta et al., unpublished observations). It has also been demonstrated that in the guinea pig heart the microsomal uptake

of digoxin is inhibited by dinitrophenol (Dutta and Marks, 1969b) and that binding is ATP dependent in fragments of sarcoplasmic reticulum of the dog heart (Dutta and Marks, 1968b). 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 (table 14). Resuspension of the microsomal pellet in 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 resuspension procedure and the positive inotropic effect appeared to be better than that of the total microsomal uptake. 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.

The correlations which we have shown are based on the use of drugs which decrease the positive inotropic effect of digoxin. This action is recognized for aldosterone (Lefer, 1965) 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 one 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 (Idänpään-Heikkila et al., 1968), 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 (figs. 11 and 12) 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 threshold 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. These experiments indicate that a short period of washout is essential (cf Methods) and that we may have removed an unknown but probably small amount of microsomal digoxin during 4 min drug-free perfusion.

C. 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 Krebs-Henseleit solution. We have also shown (table 14) that approximately 50% of total microsomal digoxin was removed during 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 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 Krebs-Henseleit solution might be responsible for the lability of bound digoxin. Approximately one-fourth of the total microsomal digoxin was released when the microsomal pellet was resuspended in 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. Urea solution was used as a resuspension medium. Dutta et al., (1968a) have shown that bound digoxin to the microsomal fraction may be associated with the membrane macromolecule by hydrogen binding forces. Resuspension of the microsomal fraction in 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 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 5 M urea comprising 50% of the bound digoxin, and a final fraction of approximately 25% of the total microsomal 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 (1966) and Carsten (1967) found that both ouabain and strophanthidin inhibited the calcium uptake of isolated cardiac sarcoplasmic reticulum fragments. Klaus and Lee (1969) showed that cardiac glycosides increased the amount of free releasable calcium fraction of sarcoplasmic reticulum fragments of dog heart. On the basis of these results, they suggested that calcium releasing effect of cardiac glycosides may be related to the positive inotropic effects. Pfordte and Foerster (1970) studied the binding of cardiac glycosides to serum albumin protein in vitro and have shown that calcium ions release cardiac glycosides from their protein binding and suggested that calcium ion and cardiac glycoside compete for the same binding site.

In view of these results, we speculated that calcium-labile fraction may be related to the positive inotropic effect and may 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. The digoxin released by urea was not correlated with the positive inotropic effect. Aldosterone significantly 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.

In a dose-response study with the positive inotropic range of digoxin concentrations, the amount of digoxin in the calcium-labile fraction was correlated well with the positive inotropic effect; however, digoxin uptake by the total tissue and the digoxin content in the total microsomal fraction were correlated equally well so that the results of the dose-response study are consistent with, but do not provide strong evidence for our hypothesis concerning the relationship of the inotropic effect and binding to the calcium-labile site.

Although we have shown some evidence for saturability of digoxin binding on the calcium-labile site within the positive inotropic range of digoxin concentration. At toxic concentrations, we observed a 10-fold increase in total tissue uptake, but only a 2-fold increase in total microsomal uptake. Despite the apparent plateau in the calcium-labile fraction at the highest therapeutic concentrations, this also was doubled at toxic levels.

We have shown that there may be considerable binding of digoxin before a positive inotropic effect could be observed, by extrapolation of the regression lines (figs. 11 and 12) to zero effect. We found again that there is a threshold concentration of bound digoxin on calcium-labile fraction below which no pharmacological effect occurs. 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 microsomal fraction. It was found by electronmicroscopy that this interface did not contain mitochondria fraction and consisted of homogeneous membranous vesicles.

D. General Discussion

1. The microsomal fraction

This subcellular fraction contains a great variety of membrane components which have fused during homogenization to form vesicular structures. They include both sarcolemmal and internal membrane structures. In muscle, the plasma membrane forms the innermost part of a complex sheath or sarcolemma, which consists of several layers and includes collagen microfibers and mucoprotein in its composition (Mauro et al., 1961). The plasma membrane is characteristically invaginated in the form of finger-like projections, known as the transverse component of the sarcotubular system, or T-system. A longitudinal component (or L-system) has no connection with the T-system and is regarded as analogous to the endoplasmic reticulum of other cells (Porter and Palade, 1957).

In skeletal muscle, it is generally understood that the T-tubules terminate in a region known as the triad, where they are in apposition with terminals of the L-system. However, in heart muscle, it has recently been shown that T-system is ramified to a far greater extent, much of it running parallel with the L-system, though never continuous with it (Forssman and Girardier, 1970). Consequently, the T-system in myocardium contributes much more to the microsomal fraction than in the case of skeletal muscle.

Cardiac microsomes contain vesicular fragments that bind calcium, and in the presence of oxalate they take up large quantities of this cation to form dense precipitates of calcium oxalate in their interior (Carsten, 1964; Katz and Repke, 1967; Entman et al., 1969). This fraction does not, however, represent a pure preparation of

sarcoplasmic reticulum fragments, but contains significant amounts of fragments derived from other subcellular organelles, notably mitochondria (Fanburg et al., 1965; Katz and Repke, 1967; Hulsmans, 1961; Webster and Williams, 1964; Imai et al., 1966). In addition, an active Na, K-activated ATPase, presumably derived from the plasma membrane, can be prepared from cardiac microsomes (Auditors and Murray, 1962; Schwartz, 1962; Lee and Yu, 1963; Skou, 1965; Matsui and Schwartz, 1966; Tashima et al., 1966; Portius and Repke, 1967). In addition, microsomes prepared from heart (Romeo et al., 1966) and other tissues (Leighton, 1968) contain fragmented lysosomes and peroxisomes. Dutta and coworkers (1968a) have shown by electron microscopy that this fraction from guinea pig hearts, consists of vesicles and membrane fragments and we have confirmed this in dog hearts.

High-speed centrifugation in sucrose density gradients has been used to remove the mitochondrial fraction of both skeletal (Hasselbach and Makinose, 1963; Seraydarian and Mommaerts, 1965) and cardiac (Carsten, 1964; Katz and Repke, 1967; Entman et al., 1969) microsomes and zonal centrifugation of skeletal muscle microsomes in sucrose has proved useful in the preparation of fragmented sarcoplasmic reticulum with a low proportion of mitochondrial fragments (Schuel, 1965). Recently, Katz and coworkers (1970) demonstrated the applicability of zonal centrifugation to the preparation of fragmented cardiac sarcoplasmic reticulum. This method permits the isolation of small amounts of fragmented sarcoplasmic reticulum from the Na, K activated ATPase that is attributable to the plasma membrane.

2. Experimental procedure

Our experiments were designed to test the subcellular distribution and the inotropic effect of cardiac glycosides, we have measured the subcellular distribution of cardiac glycosides as well as their contractility in the same hearts to enable correlation of their relationship. We have used the experimental procedure reported by Dutta et al., (1968a), but their experimental techniques were modified as follows; the perfusion pressure on the hearts was maintained constant instead of maintaining a constant flow rate. The perfusion medium was not recirculated and the hearts were stimulated at the constant rate. For the preparation of subcellular fractions, Dutta et al., (1968a) have homogenized the tissue in sucrose-EDTA solution for 3 min, whereas we homogenized the tissue by 7 gentle strokes within 1 min. The nuclear fraction was separated by spinning 700 g for 10 min. Protein was measured by the method of Lowry on a Technicon Autoanalyzer. Radioactivity was counted in scintillation liquid containing toluene, Triton X-100, PPO and POPOP. The selection of $30^{o}C$ as the perfusion temperature in these experiments was primarily based on our desire to retain constant mechanical activity in the perfused guinea pig hearts. It seems clear that an interaction of the drug with the microsomal fraction occurred in the whole heart, and not as an artifact of re-distribution during the homogenization and fractionation procedures (Dutta et al., 1968a). The hearts were perfused for 4 min with drug-free medium at the end of perfusion with digoxin. Washout for this period of time has been shown in these and other experiments (Dutta et al., 1968a) to remove more than 90% of the drug from the extra-cellular space of the isolated perfused guinea pig hearts. The

clearance of the extracellular space was studied by perfusing the hearts for 30 min with ^{14}C-inulin and then perfusing with isotope-free Krebs-Henseleit solution for a different period of time. Approximately 95% of ^{14}C-inulin was rapidly cleared from the tissue within 4 min (fig. 2). Thus, a 4 min washout with a Krebs-Henseleit solution containing no ^{3}H-digoxin was adequate to clear the extracellular space of digoxin. It has also been demonstrated that binding of digoxin to the microsomal fraction requires specific ionic conditions that were not present during the fractionation procedure. That is, broken cells would contribute potassium in concentrations that would be inconsistent with binding (Dutta and Marks, 1969a). In addition, we have examined the microsomal digoxin binding in the non-washed hearts with drug-free Krebs-Henseleit solution at the end of digoxin perfusion and found that the digoxin concentration in the microsomes was not higher than that of washed hearts. The microsomal digoxin, therefore, was taken as an estimate of the uptake.

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