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THE EFFECT OF ANTI-ARRHYTHMIC AGENTS ON THE ACTIONS OF OUABAIN IN THE ISOLATED PERFUSED GUINEA PIG HEART.

The Ohio State University, Ph.D., 1971
Pharmacology

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THE EFFECT OF ANTI-ARRHYTHMIC AGENTS ON THE
ACTIOnS OF OUABAIN IN THE
ISOLATED PERFUSED GUINEA PIG HEART

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Steven Ivan Baskin, Pharm. D.

The Ohio State University
1971

Approved by

Advisor
Department of Pharmacology
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The opportunity for me to be able to attend Graduate School was originally made possible by my wife Eileen and my family, who had the patience to see me through this period of time.

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During the course of my graduate training there are a large number of student friends, staff and faculty who have assisted me in many ways. Thank you.
VITA

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Studies in Biological Oxidations, Drs. G. Brierly, A. J. Merola and J. Rieske

Studies in Enzymology and Protein Biochemistry, Dr. J. Alben

Studies in Protein Synthesis and Cellular Control, Drs. F. A. Kruger and R. McCluer
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"The pharmacologist confronted with the actions of cardiac glycosides is a little like the medieval philosopher confronted with the universe. Many things happen, and some things obviously happen as the result of other things, but identification of a first cause turns out to be rather difficult."

I. M. Glynn, 1969
INTRODUCTION

With the knowledge that ouabain is probably accumulated by the heart by a specific process rather than by simple diffusion, (Dutta et al., 1968a; Dutta and Marks, 1969), this study was undertaken to investigate the pharmacological correlates, if any, of this specific accumulation process. The first facet of this study was to search for a drug that would effectively reduce the ouabain accumulation by the heart and thereby demonstrate the potentiality of combatting cardiac glycoside induced arrhythmia and secondly whether agents that are claimed to prevent cardiac glycoside induced arrhythmia affect the specific accumulation of ouabain by the isolated heart. The second facet of this study was to investigate, in-depth, the action of drugs which had been most effective in lowering the accumulation of ouabain. A protocol which would challenge their ability to prevent the onset of arrhythmia in a time dependent arrhythmogenic situation would be used for studying their ability to reduce ouabain accumulation. The third facet was to investigate any changes in the ionic status of the hearts in the presence of the selected agents and whether there was any relationship between the ability to prevent cardiac glycoside induced arrhythmias by the selected agents and their ability to change the accumulated H³-ouabain, and concomitantly to alter the ionic status of the isolated perfused guinea pig hearts. The ultimate purpose of this study is to develop further understanding of mechanisms of action of cardiac glycosides at the cellular level.
REVIEW OF THE LITERATURE

History of Cardiac Glycosides and Recognition of Arrhythmias Associated with Them.

As early as 1500 B.C., the Ebers Papyrus mentioned the use of digitalis glycosides in the form of squill by the ancient Egyptians (Moe and Farah, 1967). Although old herbalists described the use of digitalis in folk medicine, it was in 1785 that William Withering, with the publication of "An Account of the Foxglove and Some of its Medical Uses," set in motion further investigations which have led to its pre-eminent use in present day therapeutics. Although Withering was more concerned with the value of digitalis as a diuretic, he was aware that digitalis did exert some action on the heart. He stated, "That it has a power over the motion of the heart, to a degree yet unobserved in any other medicine and that this power may be converted to salutary ends." (Withering, 1937). John Ferriar in 1799 presented evidence "for connection of the circulatory and diuretic effect." He was the first to note of the drug "a primary action on the heart and to relegate the diuretic effect to a position of secondary importance" (Moe and Farah, 1967).

Although ouabain was discovered and so named in 1888 by Arnaud (Jacobs and Bigelow, 1932), it was Sir Thomas Fraser who in 1890 discovered its digitalis-like action while studying African arrow poisons made from strophanthus and introduced it into medicine (Moe and Farah, 1967). Ouabain may be obtained from the
seeds of *Strophanthus gratus* (Wall et Hook), Baillon or from the wood of *Acokanthera schimperi* (A. DC.), Schwf. (Claus and Tyler, 1965).

Jacobs and his co-workers (Jacobs and Bigelow, 1932) were credited for establishing the preliminary structure of ouabain (Figure 1).

Ouabain consists of a cardenolide, a steroid nucleus with C-D rings in the cis configuration and a 5-membered unsaturated lactone ring at C₁₇ and the glycone or sugar moiety rhamnose attached at the three position of the aglycone.

The first toxic effects of the digitaloids were noted by Withering when studying the lethality in turkeys of a crude extract prepared from a digitalis plant. He also noted the toxic effect of this extract in a number of the patients under his care.
Withering was also the first to describe that low maintenance doses could be used over long time periods allowing the patient to be relatively free from digitalis intoxication. He also recognized that the toxic symptoms of the drug could be reversed by withholding the drug. Thus the first treatment for digitalis intoxication was evolved (Withering, 1937).

Among the earliest observers of arrhythmias induced experimentally by digitalis, Boehm may be mentioned in particular for reporting in 1872, that in frogs "the diastolic part of the wave was interrupted half-way by the rudiment of a second systole." His explanation was that digitalis increased the contractility to such an extent that two contractions were necessary to complete it. Rothberger and Winterberg (1913) were the first to systematically investigate electrocardiographically the arrhythmias occurring in dogs as a result of strophanthin and digitalis. They found that as a rule, all strophanthin induced ectopic beats originated in the left ventricle. Early studies showed that under certain conditions aberrant systoles produced by ouabain could be mimicked by topical application of barium, but not sodium (Scherf and Schott, 1953). Edens and Huber (1916) found that an increase in serum calcium caused increased extrasystoles. However, the calcium ion has never been shown conclusively to be the cause of all digitalis induced arrhythmias.

In more recent analysis of arrhythmias induced by ouabain in intact dogs, Vassalle, Greenspan and Hoffman showed evidence that ouabain toxicity seems to produce more than one class of arrhythmias. It is not certain at this time whether different classes of ouabain induced arrhythmias as measured by electrocardiographic means are produced by more than one ionic mechanism or an increased change of just one ionic mechanism (Vassalle et al., 1963).
Suggested Ionic Mechanisms for Cardiac Glycoside Induced Arrhythmias

Three molecular mechanisms have been consistently suggested as being related to cardiac glycoside toxicity. Those are (1) changes in the ionic status of calcium, (2) changes in the ionic status of sodium, and (3) changes in the potassium ionic status.

1. Changes in the Ionic Status of Calcium (Ca)

Myocardial calcium levels have been investigated by many authors. Lee et al. (1963) found that cat papillary muscle contained 2.9 mM/kg wet weight Ca, while superfused with 1.1 mM Ca. Gilbert and Fenn (1957) and Shanes and Bianchi (1969) have reported Ca contents of 2.84 mM/kg and 1.36 mM/kg, respectively for the frog sartorius muscle. These values may be low as the Ca concentration in the bathing media were lower (1.8 mM and 1 mM respectively). Grossman and Furchgott (1964) found that the Ca concentration varied directly with the Ca in the bathing media. With a bathing media of 4.50 mM, the total tissue Ca was found to be 3.74 mM/kg. Langer (1968) found 4.21 mM/l Ca in unperfused rabbit ventricle, but 4.25 mM/l in septae perfused with 1.5 mM Ca, and 5.05 mM/l in septae perfused with 2.5 mM Ca. Winegrad and Shanes (1962) found 1.72 mM/kg total Ca in stimulated guinea pig atria after 1 hour incubation in 2.5 mM Ca. Teiger and Farah (1967) recorded 2.01 mM/kg and 2.79 mM/kg Ca after perfusing with 1.8 mM/l and 3.6 mM/l Ca respectively. These results are summarized in Table 1.

From the wide range of Ca values mentioned here, it is difficult to assign any appropriate intracellular Ca concentration which may be related to a cardiac glycoside induced arrhythmia.

A. J. Clark in 1912 postulated that Ca may have an affect on cardiac glycoside action. Loewi (1918), due to the similarity between the positive inotropic effect of cardiac glycosides and Ca ions
Table 1
Reported Muscle Calcium Ion Concentration Values

<table>
<thead>
<tr>
<th>Reference</th>
<th>Preparation</th>
<th>Calcium in bathing media mmole/l</th>
<th>Calcium Concentration mmole/kg wet wt.</th>
</tr>
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<tr>
<td>Lee et al., 1961</td>
<td>Cat papillary</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Gilbert and Fenn, 1957</td>
<td>Frog sartorius</td>
<td>1.8</td>
<td>2.84</td>
</tr>
<tr>
<td>Shanes and Bianchi, 1959</td>
<td>Frog sartorius</td>
<td>1</td>
<td>1.36</td>
</tr>
<tr>
<td>Grossman and Furchgott, 1964</td>
<td>Guinea pig atria</td>
<td>4.50</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.32</td>
<td>2.52</td>
</tr>
<tr>
<td>Langer, 1968</td>
<td>unperfused rabbit ventricle</td>
<td>--</td>
<td>4.21</td>
</tr>
<tr>
<td></td>
<td>perfused rabbit ventricle</td>
<td>1.5</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>5.05</td>
</tr>
<tr>
<td>Winegrad and Shanes, 1962</td>
<td>Guinea pig atria</td>
<td>2.5</td>
<td>1.72</td>
</tr>
<tr>
<td>Teiger and Farah, 1967</td>
<td>Rabbit atria</td>
<td>1.8</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6</td>
<td>2.79</td>
</tr>
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</table>
on the heart muscle, suggested that the cardiac glycosides sensitize the heart to Ca. Wilbrandt and Caviezel (1954) suggested that cardiac glycosides prevent a loss of myocardial Ca probably by depressing Ca outflow. Under therapeutic conditions little actual increase in cellular Ca is seen (Langer, 1970) if at all (Holland and Sabatini-Smith, 1969). Whether the slightly increased flux can account for the inotropy is not confirmed at this time.

Lüttgau and Niedergerke (1958) showed that Ca competes with sodium (Na) in its positive inotropic effect and therefore, there is always an enhancement of positive inotropy in the absence of Na, or when the Na/Ca ratio is reduced. It is possible for an antiarrhythmic agent to exert its effect by altering the Na/Ca ratio.

2. Changes in the Ionic Status of Sodium (Na)

It is well established that the myocardial intracellular concentration of sodium ([Na]_i) is well below that of the outside sodium concentration ([Na]_o) (Langer, 1968). In the calculation of not only the intracellular Na concentration but also the intracellular concentrations of potassium (K) and Ca, an accurate measurement of the extracellular space is needed. The measurement of extracellular space using inulin as a marker has been claimed to give an authentic estimate of extracellular space compared with other existing methods. However for physical and chemical reasons (Phelps, 1965; Falbriar et al., 1969) and for non-uniformity of the inulin molecule (Patterson, 1968) this agent also may not be a true measurement of the extracellular space. A representative sampling of reported extracellular space measurements is seen in Table 2.

The myocardial intracellular Na concentration in fresh tissue is approximately 18-28 meq/l. Perfusion with a physiological salt
Table 2

Reported Muscle Intracellular Na Ion Concentration Values

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Conditions of Experiment</th>
<th>Total H2O/kg wt. ± S.E.</th>
<th>Extracellular H2O/kg wt.</th>
<th>Intracellular H2O/kg wt.</th>
<th>meq/kg wet.</th>
<th>meq in extracellular space per kg wet.</th>
<th>meq in intracellular space per kg wet.</th>
<th>meq/liter</th>
</tr>
</thead>
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<tr>
<td>1 Papillary muscle Dog</td>
<td>Min 3 hr incubation fat-free</td>
<td>791±8</td>
<td>180</td>
<td>611</td>
<td>38.9</td>
<td>26.7</td>
<td>12.2</td>
<td>19.6</td>
</tr>
<tr>
<td>2 Rat Ventricular strip not stimulated, but stimulation does not make any difference</td>
<td>2 hr value 33°C</td>
<td>784±9.6</td>
<td>227</td>
<td>557</td>
<td>64.0</td>
<td>32.9</td>
<td>35.7</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>4 hr value 33°C</td>
<td>780±9.6</td>
<td>238</td>
<td>642</td>
<td>78.8</td>
<td>32.9</td>
<td>45.9</td>
<td>71.5</td>
</tr>
<tr>
<td>in vivo</td>
<td>3 hr body temperature</td>
<td>772±2</td>
<td>206</td>
<td>566</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>27.7</td>
</tr>
<tr>
<td>Rat Ventricle Low sodium</td>
<td>2 hr value 33°C</td>
<td>762±110</td>
<td>227</td>
<td>535</td>
<td>63.1</td>
<td>32.9</td>
<td>30.2</td>
<td>56.5</td>
</tr>
<tr>
<td>3 Papillary muscle Cat</td>
<td>2 hr value 27°C</td>
<td>753±3</td>
<td>240</td>
<td>513</td>
<td>64.86</td>
<td>42.8</td>
<td>22.1</td>
<td>43</td>
</tr>
<tr>
<td>4 Rabbit--left atrium</td>
<td>2 hr value 37°C</td>
<td>780</td>
<td>220</td>
<td>560</td>
<td>64.0</td>
<td>32.8</td>
<td>31.2</td>
<td>55.7</td>
</tr>
</tbody>
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Table 2--Continued

2 Hercus, V. N. et al., 1954.
4 Tuttle et al., 1961.
solution, causes the values to rise after one-two hours of perfusion to 40-60 meq/l (Tuttle et al., 1961). Table 2 shows that species (Vick et al., 1970), temperature (Page and Soloman, 1960), and anoxia (Hercus et al., 1955) tend to influence intracellular Na values. Regional myocardial differences (Barclay et al., 1960) are only but a few of the factors which can change myocardial electrolyte composition.

Sodium exchange has been studied in the isolated dog heart (Conn and Wood, 1959; Langer, 1967), and in the rabbit myometrium (Jones, 1970; Langer, 1970). Particular components of Na exchange have been studied in other tissues as red blood cells (Glynn, 1969), Hela cells (Baker, 1970), frog skeletal muscle (Keynes and Steinhardt, 1968), toad bladder (Crabbe and DeWeer, 1969) and pigeon crop mucosa (Frantz and Rose, 1968). It appears from these studies that the myocardial membrane is relatively impermeable to the Na ion and that at least three Na compartments can be assigned on the basis of kinetic studies.

Cardiac glycosides have been known for a long time to increase the Na ion concentration in cells (Clark, 1912). Although many authors, as Wood and Moe (1942), showed that a toxic dose of many different cardiac glycosides produce an increase of Na in the myocardium, it was Schatzmann (1953) who found that cardiac glycosides inhibit the active movement of Na and K across red cell membrane and that a possible mechanism could be described for the increase in Na found in cells. Schatzmann suggested that cardiac glycosides retained sodium by inhibition of "transport ATPase". Many workers have found the increases in intracellular Na after many different protocols of treatment (Tuttle, 1961; Page, 1964). There seems to be a general consensus that toxic doses of cardiac glycosides increase the intracellular ion concentration.
Cardiac glycosides are also known to inhibit Na-K exchange in red blood cells, squid axons (Baker et al., 1969) and myometrium (Langer, 1970). This action has been attributed to its ability to inhibit a Na-K-ATPase (Bonting et al., 1962). In addition to Na-K inhibition, cardiac glycosides are also thought to inhibit Na-Na exchange (Garrahan and Glynn, 1967). Cardiac glycosides are also thought to increase Na cellular permeability (Holland and Klein, 1959). Most experiments in which the effect of cardiac glycoside on Na transport has been measured, the cells have been preloaded with the radioactive Na and the rate of efflux under control conditions and in the presence of the particular cardiac glycoside. It is generally agreed that cardiac glycosides reduce the turnover of Na in those tissues studied to date.

3. Changes in the Ionic Status of Potassium (K)

Potassium is generally recognized to be the main intracellular monovalent cation. Table 3 illustrates typical intracellular K concentrations reported by the various workers in cardiac muscle (Boyer and Poindexter, 1940; Conn, 1956; Gertler et al., 1956; Grupp and Charles, 1964; Hajdu, 1953; Hagan, 1939; Rayner and Weatherall, 1957; Schreiber, 1956; Tuttle et al., 1961; Vick and Kahn, 1957; Wedd, 1939).

A common range for intracellular K would be 125-175 meq/l. Table 3 is illustrative of the wide range of intracellular values. It would be difficult to assign a particular concentration to intracellular K as the individual authors used different analytical methods when dealing with the subject. Tuttle et al. (1961) observed a gradual lowering of K concentration in 2 hrs with time of perfusion in rabbit myocardium, while Page and Soloman (1960) did not observe any K change after the first hr of perfusion. Decreased extracellular
# Table 3

Reported Muscle Intracellular Potassium Ion Concentration Values

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Conditions of Experiment</th>
<th>Total H₂O/kg wet wt ± S.E.</th>
<th>Extracellular H₂O/kg wet wt</th>
<th>Intracellular H₂O/kg wet wt</th>
<th>meq/kg wet</th>
<th>meq in extracellular space per kg wet wt</th>
<th>meq in intracellular space per kg wet wt</th>
<th>meq/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹Papillary muscle Dog</td>
<td>Min 3 hr incubation fat-free 37°C</td>
<td>791±8</td>
<td>180</td>
<td>611</td>
<td>76.1</td>
<td>0.97</td>
<td>75.13</td>
<td>122.96</td>
</tr>
<tr>
<td>²Rat Ventricular strip non-stimulated</td>
<td>2 hr., 33°C</td>
<td>784±9.6</td>
<td>227</td>
<td>557</td>
<td>98.0</td>
<td>1.34</td>
<td>97.21</td>
<td>173.54</td>
</tr>
<tr>
<td>Rat Ventricular strip stimulated</td>
<td>4 hr., 33°C</td>
<td>780±9.6</td>
<td>238</td>
<td>642</td>
<td>89.7</td>
<td>1.34</td>
<td>88.36</td>
<td>137.63</td>
</tr>
<tr>
<td>in vivo</td>
<td>3 hr body temp</td>
<td>772±2</td>
<td>206</td>
<td>566</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>140.0</td>
</tr>
<tr>
<td>Rat Ventricular strip low sodium</td>
<td>2 hr., 33°C</td>
<td>762±110</td>
<td>227</td>
<td>535</td>
<td>111.25</td>
<td>1.34</td>
<td>109.91</td>
<td>205.44</td>
</tr>
<tr>
<td>³Papillary Muscle Cat</td>
<td>2 hr., 27°C</td>
<td>753±3</td>
<td>240</td>
<td>513</td>
<td>128</td>
<td>83.1</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td>⁴Rabbit left atria</td>
<td>2 hr., 37°C</td>
<td>780</td>
<td>220</td>
<td>560</td>
<td>77.32</td>
<td>0.57</td>
<td>76.73</td>
<td>137.00</td>
</tr>
</tbody>
</table>

¹Vick, et al., 1970.
²Hercus, et al., 1955.
⁴Tuttle et al., 1961.
Na (See Table 3) is in most cases associated with an increase in the intracellular K concentration.

Although with a toxic concentration of cardiac glycosides there appears to be universal agreement that intracellular K is lowered (Hagan, 1939), there is lack of agreement on what effect a "therapeutic" concentration of cardiac glycosides has on the intracellular K content. Hagan (1939), Boyer and Poindexter (1940), Tuttle et al. (1962) and Brown (1962) found that at therapeutic doses intracellular K concentrations increased above control values while at "toxic" doses of digitalis compounds, intracellular K was lost. On the other hand, since Webb (1941) and Sanyal and Saunders (1961) found that intracellular K content was unaltered with "therapeutic" doses and a loss occurred with "toxic" concentrations only, while Blackmon et al. (1960) and Langer (1970) found a loss of K even with "therapeutic" doses.

The Accumulation of Cardiac Glycosides by the Heart

One of the fundamental questions related to pharmacology is the localization of the drug with relation to biological actions (Leake, 1961). Since the accumulation of a drug at a biological site may be related to an ionic mechanism of action, it might prove fruitful to investigate this aspect of cardiac glycosides.

Clark (1937) suggested with reference to cardiac glycosides "There appears to be a simple relation between concentration and time until initiation of biological effects, for the time until systolic arrest, which is the clearest end point there evidently exists a complex time relationship, with a long period of biological lag." When he uses the term "complex time" he is comparing time concentration curves with the passage of dye into one celled algae as simple models.
St. George, Freedman and Ishida, using the embryonic duck heart assay method, found approximately eighty-nine percent of digitoxin in the supernatant fraction of hearts after intravenous administration of the cardiac drug to rats. Approximately three percent was present in the mitochondrial fraction. They made no attempt to investigate the microsomal fraction (St. George et al., 1953).

Harvey and Pieper (1955) found fifty-seven percent of digitoxin radioactivity in the supernatant fraction and forty-three percent in the particulate fraction of isolated guinea pig hearts perfused with C\textsuperscript{14}-digitoxin. For the particulate fractions, the nuclear fraction had twelve percent, the "debris" twenty-six percent and mitochondria six percent. As in the St. George study, the microsomal fraction was not isolated. When the heart was washed by perfusion with non-radioactive digitoxin to clear the vascular space of radioactive drug, the supernate fraction accounted for only twelve percent of the recovered radioactivity whereas the radioactivity on a concentration basis was not appreciably altered in the particulate fraction.

Spratt and Okita (1958) showed that from in vivo studies over ninety percent of unchanged H\textsuperscript{3}-digitoxin was found in the soluble supernate fractions of heart, liver and kidney cells of the rat. For the particulate fractions of the rat heart, 5.4, 1.9, and 1.2 percent were present in the microsomes, mitochondria and nuclei respectively.

The subcellular distribution of H\textsuperscript{3}-digoxin was further studied (Dutta et al., 1968b) in guinea pig and rat hearts. Dutta and his co-workers found that in both rat and guinea pig the subcellular fraction which accumulated the greatest H\textsuperscript{3}-digoxin ng/g of tissue was the microsomal fraction. The concentration of atrial H\textsuperscript{3}-digoxin was approximately two-thirds that of the ventricles in the guinea pig.
heart at all times. They suggest from this study that the microsomal binding may be directly related to the differences seen in the biological activities in the rat and guinea pig. In further investigations into the nature of accumulation of cardiac glycosides, Dutta and co-workers (1968b) provide evidence, based on distinct differences on the effect of K on the uptake of different cardiac glycosides, for the existence of more than one "receptor site" in reticulum fragments.

Further kinetic studies are indicative of more than one accumulation site in cardiac muscle (Kuschinsky et al., 1967; 1968a; Godfraind and Lesne, 1968; Lullman and Van Zweiten, 1969), in smooth muscle (Godfraind and Lesne, 1968) and in non-myelinated nerve (Landowne and Richie, 1970). There is evidence in both cardiac (Godfraind and Lesne, 1968; Lullman and Van Zweiten, 1969) and smooth muscle (Godfraind and Lesne, 1970) that a non-saturable component of the binding may be related to the polarity of the cardiac glycoside and that a saturable component is related to a pharmacological therapeutic activity.

Sodium-Potassium activated ATPase has been implied by Matsui and Schwartz (1968), Besch et al. (1970), Akera and Brody (1970), and Repke and Portius (1963) to be the pharmacological receptor for digitalis. It is not well defined as to whether these authors are suggesting one receptor to cover all actions of cardiac glycosides. To stress their ideas idealized models have been proposed by Repke and Portius (1966), see Figure 2, and Wilson et al. (1970) (Figure 3) among others.

However, at the time of this investigation the data has been lacking to conclusively link Na-K-activated ATPase with an arrhythmogenic site in the myocardium.
The chemical nature of the ouabain binding site has not as yet been thoroughly studied. Most of the studies have been concerned with the binding of ouabain to Na-K-ATPase. Kuschinsky, Lullman and Van Zweiten (1968b) have implicated a "lipoid" related site for the non-saturable site by their aqueous glycerol extraction studies. They also propose that the ouabain related site may not be of a lipoid nature. Abdulla and Turner (1969) give evidence that ouabain action may be related to a lipid site by showing that ouabain may inhibit turbidity changes in non-water shocked myelin fractions. Although Tanaka and Sakamoto (1969) appear to have demonstrated...
that phospholipids are essential for the activation of Na-K ATPase, the site of binding of ouabain to the enzyme in relationship to an arrhythmogenic activity has not been shown at this time.

Rationale for the Objectives of this Investigation

Since there could be pharmacological correlation between the effectiveness of a specific agent to prevent ouabain toxicity and to prevent specific ouabain accumulation by the heart (Dutta and Marks, 1969), agents that may effectively reduce the ouabain accumulation by the heart were investigated. Agents which have been claimed to prevent cardiac glycoside induced arrhythmia were also surveyed for their ability to lower ouabain accumulation by the myocardium.

The agents surveyed were categorized into three broad groups:

1. Agents which possess a structural similarity to cardiac glycosides

2. Agents which may change the ionic status of cells

3. Agents which may prevent cardiac glycoside induced arrhythmia, irrespective of a claimed mechanism of action

1. Agents which possess a structural similarity to cardiac glycosides

Cardiac glycosides structurally consist of three parts: (1) the sugar moiety, (2) the steroid moiety and (3) the lactone moiety. The sugar portion of the cardiac glycoside has been known since the early structure-action relationship studies by K.K. Chen and his co-workers to alter the toxicity of cardiac glycosides in cats (Chen et al., 1951; Chen and Henderson, 1954). A sugar transport system was implicated by Keyl and Dragstedt (1954a; 1954b) in the transport and fixation of cardiac glycosides to a site of action. Bailey and Dresel (1971) present indirect evidence that the interaction of
the positive inotropic response of cardiac glycosides is related to sugar transport and metabolism. Yoda and Hokin (1970) have claimed that the sugar moiety contributes to the reversibility of binding of cardiac glycosides to Na-K stimulated ATPase from beef brain. The exact contribution of sugars in the accumulation is not known, nor have any experiments shown any competition between sugars and digitalis binding.

The accumulation of cardiac glycosides has been shown to be inhibited by other cardiac glycosides (Kuschinsky et al., 1968; Dutta and Marks, 1969). Aldosterone has been claimed by Lefer and Sayers (1965) to antagonize inotropic action of ouabain. Selye et al. (1969) have also presented evidence that steroids may prevent the onset of cardiac glycoside toxicity. However, other investigators (Levy and Richards, 1963; 1964; 1965; Levy, 1969) measuring many parameters find that aldosterone does not alter cardiac glycoside action. Recently Knox and Sen (1971) have found that aldosterone stimulates Na-K ATPase which may or may not be related to aldosterone's ability to reverse cardiac glycoside toxicity. Other steroids, notably estrogens and corticosterone type steroid derivatives have been observed to modify the activity of cardiac glycosides (Chung, 1969).

Giarman (1948), Walton et al. (1950) and Bennett et al. (1958) have investigated lactone-like moieties for cardiac glycoside-like cardiac effects. At best, little effect of the lactone-like moieties themselves can be seen. However, there is indication that their modification may change the pharmacological nature of the cardiac glycosides (Giarman, 1948; Bennett et al., 1958).

Investigation of the effect of a group of lactones and related compounds tested upon cardiac glycoside toxicity showed that tetrahydrofurfuryl amide (THFA) was the most potent compound against
digitoxin-produced premature ventricular contractions and ventricu-
lar tachycardia and ouabain-produced ventricular tachycardia and
atrial fibrillation in dogs. (Cosmides et al., 1956). However,
Shafer and Adicoff (1970) could find the same beneficial effect of
THFA which Cosmides and his co-workers found.

2. Agents which may change the ionic status of cells

Although it is well established that ethacrynic acid (EA) in-
hibits Na-K ATPases prepared from both kidney (Charnock et al.,
1969; Davis, 1970) and heart (Song and Scheuer, 1968; Tobin and
Brody, unpublished), the mode of action for EA exerting a diuretic
or any other pharmacological effect is not well established (Gordon
and Hartog, 1969; Wolf et al., 1969). Lee et al. (1969) presented
evidence that EA does not alter cardiac glycoside induced arrhythmia
in the dog.

Cocaine, an alkaloid from the leaves of Erythroxylon coca
Lamark, has been claimed to prevent sodium influx in nerve tissue.
Whether this inhibition of Na movement is related to the Na dependent
ouabain (Harrison and Wakin, 1968; Dutta and Marks, 1969) accumu-
lation by the myocardium has not as yet been resolved.

Potassium has been shown by many authors to prevent cardiac
glycoside induced arrhythmias (Loewi, 1918; Sampson et al., 1943;
Fisch and Stone, 1969). Potassium has been claimed by Williams et al.
(1966) to inhibit the arrhythmogenic activity of ouabain without
suppressing the increase in contractile force due to ouabain. It is
possible that potassium's ability to inhibit the arrhythmogenic
activity of ouabain is due to its ability to regulate ouabain accumu-
lation by the heart (Dutta and Marks, 1969). However, part of the
K activity may not be directly related to antagonism of ouabain but
to its own pharmacological properties (Fisch, 1969).
3. Agents which may prevent cardiac glycoside induced arrhythmia irrespective of a claimed mechanism of action

Heparin is the most acidic substance which is synthesized in animal systems. Loubatieres and his co-workers (1963) have demonstrated that Na heparin suppressed the arrhythmia and bradycardia of ouabain in dogs while allowing the inotropic action of ouabain to continue. Related to this, it is also known that the process of digitalization is inhibited by prior administration of heparin (Wilson and Gisvold, 1962). At this time it is not known if the accumulation of ouabain is inhibited by heparin.

Quinidine is an alkaloid from the bark of the family of cinchona trees (Claus and Tyler, 1961). Jean Baptiste de Senac in 1749 was the first to use cinchona for atrial fibrillation. It is now commonly used as an antiarrhythmic agent (Moe and Abildskov, 1967). Goodford and Vaughan Williams (1962) have suggested that quinidine may reduce the "availability" of the Na carrier. By reducing the external K concentration, quinidine-induced atrial arrest can be reversed. This has led to proposals that quinidine's action in part may be K-related (Overman, 1959). If quinidine action is ion related or direct, it is not known at this time if it can affect the accumulation of cardiac glycosides by the myocardium.

Taurine, 2-aminoethanesulfonic acid, was first reported in ox bile in 1827 (Jacobson and Smith, 1968). As early as 1936, Sugihara, Nagasawa and Okabe reported that taurine acts as a stimulant in frog, toad and rabbit heart and caused partial recovery in the oxygen deficient or nicotine disturbed heart. The heart is said to contain a higher concentration of taurine than any other tissues (Scharff and Wool, 1964; Jacobson and Smith, 1968). It was found that canine heart tissue was capable of forming taurine from cysteine and of converting taurine to its deaminated analog, isethionic acid.
The rate of this conversion was increased by digoxin, thus demonstrating the presence of ISA in canine ventricle (Read and Welty, 1962). It was suggested that taurine exists as a cyclic uncharged molecule at body pH, that the cell membrane is relatively permeable to taurine when the latter is injected, and that deamination to ISA permits it to function as a strong intracellular anion capable of retarding the decrease in intracellular K associated with cardiac glycoside induced arrhythmias (Read and Welty, 1962; 1963; 1965).

Reserpine, an alkaloid present in the dried root of Rauwolfa serpentina (Linne) Bentham ex Kurz (Claus and Tyler, 1961) is known to reduce the biogenic amine content both centrally and peripherally (Shore, 1966). There is conflicting evidence concerning the ability of reserpine to modify the contractile changes produced by cardiac glycosides on isolated heart muscle (Levy and Richards, 1965; Ciofalo et al., 1966). In in vivo experiments, reserpine has been found to modify the arrhythmic activity of cardiac glycosides (Roberts et al., 1963; Boyajy and Nash, 1965; Takagi et al., 1965) in most, but not all, of the recent investigations. However, in in vitro preparations the reverse is more likely to be found (Boyajy and Nash, 1965; Levy and Richards, 1965). Due to differences in types of mechanical and electrical parameters measured in a large variety of different preparations it is difficult to resolve existing differences. Reynolds and Horne (1969) did attempt to show that there were differences in agents which would produce a change in ouabain lethality compared with the production of ectopic rhythm. They also pointed out that tetrabenazine also decreased the mean dose of ouabain to ectopic rhythm to the same degree as reserpine and suggest that reserpine may be acting centrally. Marcus and his co-workers (1968) have found in whole dog preparations a lower myocardial digoxin concentration in those animals pretreated with
reserpine. Later investigations by the same authors found no change in guinea pig heart preparations (Marcus, unpublished). Accumulation of digoxin, digitoxin and digitoxigenin was studied in isolated guinea pig hearts which were pretreated with reserpine (Kuschinsky and Van Zwieten, 1969). It was found that pretreatment of the animals with reserpine did not alter the uptake of digoxin in isolated atria. Also reserpine did not affect the inotropic dose in electrically driven atria. Similar findings were obtained with ouabain in isolated perfused hearts (Baskin and Dutta, 1970).

Although it was in 1908 that Biltz (1908) synthesized diphenylhydantoin (Figure 4), it was not until 1937, on the basis that increased conjugated phenol concentrations in uremia caused decreased motor function, that Merritt and Putnam (1938) described the efficacy of diphenylhydantoin (DPH) in protecting animals from electrically induced convulsive seizures with little sedative effect.

![Figure 4](image)

**Figure 4**

Structure of Sodium Diphenylhydantoin
The following year, Drake and his co-workers (1939) found that DPH caused consistent inhibition of contractile activity in excised rabbit and dog uterine muscle (5.66 x 10^{-5}M). Diphenylhydantoin was found to produce both negative inotropic and chronotropic activity in the presence of atropine or vagotomized in situ dog hearts (Baudouin and Hazard, 1941).

Harris and Kokernot (1950) were the first to show that DPH could be used to prevent ectopic ventricular tachycardia in acute myocardial infarction and suggested that anti-convulsant drugs would be useful in controlling ectopic cardiac arrhythmias because their production may be similar to that of production of focal cerebral impulses that produce seizure. Widespread clinical use of DPH as an antiarrhythmic agent was not seen until the early sixties (Mercer and Osborne, 1967). The use of DPH in the treatment of cardiac glycoside-produced arrhythmias was proposed by Mosey and Tyler (1952, 1964) and Lang et al. (1965).

To this date no single mechanism for the action of DPH in preventing the onset of cardiac glycoside induced arrhythmias has been shown. It has been shown that DPH increases the coronary blood flow in the dog heart (Haft et al., 1967). Gupta and his co-workers (1967) have suggested that this change may be in part related to the antiarrhythmic action of this drug. However, other workers have failed to repeat this work and conclusive evidence in favor of such a mechanism is lacking (Winbury, 1964; Rowe, 1964; Mercer et al., 1967). Both anticholinergic and cholinergic actions have been claimed for DPH by different investigators (Bose et al., 1963; Conn, 1967; Rosati et al., 1967; Gupta et al., 1967) on the basis of effect of DPH on heart rate and on atrioventricular conduction. However, data showing a direct effect of DPH upon acetylcholine to produce a change in heart rate does not exist. Bigger and his associates (1970) felt
that DPH accelerated atrioventricular conduction largely through a
direct action. As yet there is no unequivocal evidence for a signifi-
cant anti-cholinergic or cholinergic effect of DPH. Four mecha-
nisms for the antiarrhythmic action of DPH are proposed involving
changes in the ionic status of the heart: (1) changes in calcium
metabolism, (2) changes solely in potassium metabolism,
(3) changes in passive sodium movements, and (4) changes in
active sodium and potassium movements which could be related to a
Na-K stimulated ATPase.

Little has been published regarding the effects of DPH on Ca
ionic metabolism. Harris and Kokernot (1950) proposed that Ca
may in part be related to the antiarrhythmic action of DPH. Both
Blaustein (1967) and Mule' (1969) have shown that, in vitro, DPH
bound more Ca to phospholipids. Govier and Holland (1964) and
Holland and Sekul (1961) claimed that with ouabain (6 x 10^-5M)
there was increased uptake and exchange of Ca^45, but Grossman and
Furchgott (1954) found that strophanthin-K (1 x 10^-5M) decreased
the apparent Ca exchange in guinea pig auricles, and Thomas (1960)
found only slight increase in uptake of Ca^45 in frog hearts during
contracture. However, Ca^45 uptake may be largely dependent on
the nature of the tissue since Thomas found with increasing larger-
contracture-producing concentrations of ouabain there was slightly
less Ca^45 taken up into the tissues. If there is actually more free
Ca in the tissue treated with ouabain, and if DPH binds this excess
free Ca, it is not known at this time.

The effect of DPH on K^42 uptake and egress were measured by
Cushman and his co-workers (1966). They found that DPH when
compared to controls and ouabain treated samples, did not alter
either the uptake nor egress of K^42 in isolated, noncontractile dog
heart slices. Also the total DPH (2 x 10^-4M) was claimed not to
affect the total K loss in ouabain (5 x 10^-5M) treated slices.
Although Helfant and his co-workers (1968) sought to show that DPH manifests its antiarrhythmic effect in digitalis toxicity by reversing the myocardial $K/A-V$ difference, Miller and Gilmore (1970) could not find a correlation between the antiarrhythmic property of DPH and the inotropic or $K$ losing effects of acetylstrophanthidin. Due to conflicting reports, the exact role that $K$ plays in the action of DPH is unknown at this time.

The third possibility concerning ionic mechanism of action for DPH is that DPH alters Na permeability. Lullman and Weber (1967) interpret their electrophysiological findings to be related to a decreased Na permeability in the myocardial cell. Lullman and Weber did not measure ion fluxes in their studies, but were inferred from changes in either resting potential in guinea pig atria or changes in dog ventricular fiber action potentials. This study does not provide direct evidence concerning Na entry rates into cardiac cells. Sano et al. (1968) and Pincus et al. (1970) have also claimed that DPH decreases Na influx into lobster nerves. However, this data is not entirely consistent or conclusive. At the time of this investigation no one has shown by direct measurement whether DPH will change the Na influx rate into the myocardium.

The fourth proposal regarding an ionic mechanism of action for DPH was originally proposed by Woodbury (1955). He interpreted from his Na turnover and total electrolyte findings which were largely concerned with rat brain electrolyte studies that DPH was involved in the active extrusion of Na from brain cells. He was careful not to categorically apply his findings to the heart in which he did not see significant changes in Na and K concentrations but he did suggest that the findings of Harris and Kokernot (1950) concerning DPH preventing arrhythmia might be related to his findings.
Pincus and Giarman (1967) and later Rawson and Pincus (1968) presented evidence that DPH increased ouabain's action of inhibiting brain ATPase (Na-K assay ratio 3.3:1) in rat, guinea pig and human brain. In examining the effect of DPH on synaptosome Na-K ATPase, Festoff and Appel (1968) found that DPH produced significant stimulation of enzymatic activity under conditions of a high Na-K assay ratio (25-50:1). At assay ratios of 5-10:1, DPH produced little or no effect, and at low Na-K ratios (less than 5:1) DPH was inhibitory. J.W. Woodbury (1963) has claimed that the increased plateau seen in action potentials in the frog ventricle with a very high dose of DPH are due to increased activity in Na-K ATPase. Diphenylhydantoin has been also said to increase the transport of Na across the dog kidney (Koch et al., 1962) and frog skin (Watson, 1970).

Several authors have also found that DPH does not affect Na-K ATPase. Using a Na-K ATPase assay with a Na-K assay ratio of 10:1, Gibson and Harris found that DPH had no effect on human myocardial Na-K ATPase. In 8-16 day embryonic chick cells, Sperelakis and Henn (1970) likewise found that DPH had no effect on Na-K ATPase. However, they used a 5:1 Na-K ratio in their assay procedure. Formby (1969) measuring both the in vitro and in vivo effect of DPH on Na-K ATPase and K activated phosphohydrolase in particulate membrane fractions from rat brain, found that in vivo conditions DPH failed to alter either enzymatic activity, whereas in in vitro conditions DPH under specified conditions (for example in the Na-K ATPase assay, the Na:K ratio was 5:1), the enzymatic activity was inhibited. He could not justify the differences seen. As can be inferred from the preceding discussion, the ionic mechanism regarding DPH is in dispute. Hopefully this study will untangle the quagmire which has developed in this area.
MATERIALS AND METHODS

Animals

Male guinea pigs (Cavia porcellus) weighing 250-450g, Hartley strain, were purchased from the Glenn Carr Farms, Columbus, Ohio through the Ohio State University Vivarium, and were used for all experiments. Animals were maintained on food and water ad libitum prior to sacrifice.

Experimental Methods

Removal of heart and mounting it on the cannula of the modified Langendorff apparatus was previously described by Lindower (1968). The isolated perfused heart apparatus (Figures 5-6) was maintained at constant temperature by a Lauda K-2/R Temperature circulator (Brinkmann Instruments, Westbury, New York). Figures 5 and 6 illustrate schematically and photographically the isolated Langendorff apparatus. The temperature of the perfusate was constantly monitored by placing a thermistor probe (model 44TA Thermistor, Yellow Springs Instrument Co., Yellow Springs, Ohio) at a site close to the heart. The temperature was kept at 28°C in all experiments. The times for all the experiments were monitored by Precision timer model 69235 (Precision Scientific, Chicago, Ill.). The experiment time was recorded by either model SC polygraph (Grass Co., Quincy, Mass.) or the Type R-dynograph (Beckman, Inc., Offner Division, Schiller Park, Ill.) which also recorded all
Figure 5

Photograph of the Physical Arrangement of the Instrumentation Used in the Isolated Perfused Guinea Pig Heart Experiments

1. Precision timer, model 69235
2. thermistor, model 44TA
3. stimulator, model 5D5
4. Holter roller pump, model RL 175
5. temperature circulator, model K-2/R
6. arterial pressure transducer, model P-23 AC
7. displacement muscle transducer, model 33-03-981
Figure 6
Schematic Representation of the Isolated Perfused Heart Apparatus with Constant Temperature (C.T.) Control

1 - Thermistor, 2 and 3 - Electrodes
4 and 5 - Mechanical and Pressure Transducers. T - Traps O - Stopcocks
electrical and mechanical parameters. Three-way disposable stopcocks and i.v. tubes were changed often. The perfusion was maintained at a rate of four millimeters per minute using a Holter roller pump, model RL 175 (Extracorporeal Med. Specialties, Mt. Laurel Township, New Jersey).

The perfusion medium used was a modified Krebs-Hensleit (K-H) solution (Table 4) which was constantly oxygenated and maintained at pH 7.4 by being gassed with 95% O₂ - 5% CO₂ mixture (Winograd and Shanes, 1961). The pH was measured by Model 76A Expandometric pH meter (Beckman Inc., Fullerton, Calif.).

All chemicals used were at least reagent grade. The DPH was a gift of Dr. A. Glaasko, Parke-Davis Co., Ann Arbor, Michigan.

**Experimental Procedure**

Two basic procedures were used and they are illustrated in Figure 7.

```
Rx +

A ← (K-H) 1 x 10⁻⁷M ouabain
  64 minutes  64 minutes

B ← (K-H) 5 x 10⁻⁶M ouabain
  64 minutes  15 minutes
```

*Figure 7*

Schematic Diagram of the Two Basic Experimental Procedures Which were Used
<table>
<thead>
<tr>
<th>Solute</th>
<th>Final Concentration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/l</td>
<td>mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.285</td>
<td>27.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.897</td>
<td>118.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.358</td>
<td>4.8</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.136</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.296</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O</td>
<td>0.368</td>
<td>2.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0</td>
<td>11.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ion</th>
<th>meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>5.8</td>
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<tr>
<td>Na⁺</td>
<td>145.2</td>
</tr>
<tr>
<td>Ca ++</td>
<td>5.0</td>
</tr>
<tr>
<td>Mg ++</td>
<td>2.4</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>27.2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>127.8</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>1.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>2.4</td>
</tr>
</tbody>
</table>
In procedure A an inotropic concentration \(1 \times 10^{-7} \text{M}\) of ouabain was used (Lindower, 1968). A PE 240 catheter (Clay Adams Co., Baltimore, Maryland) was inserted into the left ventricle with the pulmonary vein tied in place by a ligature and attached to a P-23 AC arterial pressure transducer (Statham Inc., Hato Rey, Puerto Rico). The first derivative of the ventricular pressure was recorded on the type R dynograph incorporating a R-C differentiating circuit. The differentiating circuit consisted of a 33K resistor and a 0.1 microfarad capacitor interposed between the voltage input and output. In the majority of the procedure A experiments, aortic pressure was also monitored with the same type of pressure transducer (Figure 5). During the course of the procedure A experiments, the hearts were allowed to beat spontaneously.

Prior to measuring contractile response, the transducers were calibrated with a manometer in millimeters of mercury. The thermometer was calibrated internally. In procedure A and in procedure B, there was no washout time which differed from the experiments of Dutta et al. (1968a, b).

In procedure B, concentration of ouabain \(5 \times 10^{-6} \text{M}\) which would produce both inotropy and arrhythmia was used (Figure 7). The period of perfusion with \(5 \times 10^{-6} \text{M}\) ouabain was set at 15 min for by that time period 100% of the hearts demonstrated abnormalities of rhythm. This total time period of 79 minutes (64 minutes equilibration and 15 minutes with \(5 \times 10^{-6} \text{M}\) ouabain) was convenient.

As in procedure A, the hearts were removed from the beaker containing K-H to which ten U.S.P. units of Sodium Heparin (Organon, W. Orange, N.J.) was added and tied to the Langendorff apparatus. The atria were removed and the AV activity was removed by excising the atrial-ventricular bundle as in the manner of Benforado (1958). The remaining cardiac muscle was kept in contact with a
stimulating electrode and was stimulated by a model SD 5 stimulator, (Grass Instrument Co., Quincy, Mass.) at 75 beats/min, for 5 millisecond duration, and a voltage 10% above the threshold. For twelve experiments, a simple passive type differentiator was used with the Grass polygraph, whose design (Figure 8) was given to us by Mr. Edmund Johnson of the Grass Instrument Company.

![Diagram of Differentiating Circuit](image)

Muscle response in the procedure B experiments was measured with a displacement isotonic muscle transducer model 33-03-981, (Clevite Inc., Brush Instruments Division, Cleveland, Ohio).
A linear deflection (muscle contraction) is minimal with the rotary transducer. The tangent error at full scale is 1.08°. A balsa stick 10 inches long is attached to the plastic hub on the transducer which is balanced with reference to gravity. A stainless steel hook at the end of one end of the balsa stick is attached to an inferior section of the ventricular wall directly in a vertical position with the stimulator. The transducer is rotated around the plastic hub so as to be at the center of the angular displacement of the stick before each rotation.

In the majority of procedure B experiments, the ventricular pressure was measured using an arterial pressure transducer P 23 AC by inserting a PE 190 catheter via the pulmonary artery into the right ventricle. In both procedures A and B, electrogram recordings were maintained and recorded by surface electrodes attached to the aortic cannula and to an exploring silver wire electrode located near the apex of the heart. In the procedure B experiments, the stimulus rate was monitored by attaching leads from the output of the stimulator to the input of the preamplifier of the 4th channel of the polygraph.

\( ^{3} \text{H}-\text{Ouabain Analysis} \)

\( ^{3} \text{H}-\text{Ouabain was obtained from the New England Nuclear Corporation (Boston, Massachusetts) (11.7 curies/mM). It was prepared by catalytic exchange with tritiated water and purified by absorption chromatography. Radiochemical purity was established by paper and thin layer chromatographic methods by New England Nuclear Corporation. This highly labeled }^{3} \text{H}-\text{ouabain was routinely diluted 1-20 with purified non-labeled ouabain before use.} \)

Ouabain uptake studies were made by measurement of the glycoside content of the heart homogenate and its centrifugation fractions.
After perfusion for the specified time period in either procedure, the hearts were removed from the perfusion apparatus and the ventricles were diced into small cubes and weighed after blotting. The tissue was homogenized with 40 passes of a Potter-Elvehjem homogenizer in an ice bath to make a 10% suspension. The suspending solvent was sucrose-EDTA (0.33M-0.001M) in procedure A and sucrose-EGTA (0.33M-0.001M) buffered to pH 7.4 with Tris-Cl (0.01M) in procedure B. The homogenate was centrifuged at 166,000 x g for one hour to separate the particulate fraction from the soluble fraction (Figure 9).

The pellet was resuspended in the sucrose-chelator solution, was centrifuged at 450 x g for 15 minutes for sedimentation of a nuclear fraction. Then the resultant supernatant was centrifuged at 12,000 x g for 15 minutes to sediment the mitochondrial fraction, and finally at 166,000 x g for one hour to separate the microsomal fraction from the final supernatant fraction.

In the experiments in which subcellular fractions were investigated the subcellular fractions were resuspended in a precise volume of distilled water using a model 8223 vortex mixer (Scientific Products, Evanston, Ill). Aliquots of each particulate fraction and of the supernatant fraction were mixed with Bray's scintillation cocktail (Bray, 1960) (Table 4) and counted in a model 4321 Packard Tricarb Scintillation Spectrometer (Packard Instruments, Downers Grove, Illinois). Complete extraction was reported by Dutta et al. (1968a) using similar methods.

The radioactivity of the homogenate samples were converted to disintegrations per minute (dpm) with the aid of a quench curve made by plotting percent efficiency of a series of standards against the external standard counts. The homogenate samples were subsequently
### HEART TISSUE

10% suspension in sucrose-EDTA solution
homogenize 40 passes (approximately 3 min)

### HEART HOMOGENATE (aliquot)

Centrifuge 166,000 x g (60 min)

### PARTICULATE

10% suspension in sucrose-EDTA
homogenize 15 passes (approximately 1 min)
Centrifuge 480 x g (15 min)

### SUPERNATE

Centrifuge 12,000 x g (15 min)

### NUCLEAR FRACTION

---

### INITIAL SUPERNATE

---

### MITOCHONDRIAL FRACTION

---

### FINAL SUPERNATE

---

### MICROSONAL FRACTION

---

**Figure 9**

Illustration of Heart-Fractionation Flow Pattern

*Gornall's method was modified by the addition of five percent sodium deoxycholate to each sample to facilitate solubilization of the protein (Dutta et al., 1968b).

**When appropriate, corrections were applied for the ratio-decay of tritium (half-life: 12.27 years).
## Table 5
Composition of Bray's Scintillation Cocktail

<table>
<thead>
<tr>
<th>Agents</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napthalene</td>
<td>60 grams</td>
</tr>
<tr>
<td>1,4 bis (5-phenyl-o-oxazolyl) benzene (POPOP)</td>
<td>200 milligrams</td>
</tr>
<tr>
<td>2, 5-diphenyloxazole (PPO)</td>
<td>4 grams</td>
</tr>
<tr>
<td>Methanol, anhydrous</td>
<td>100 milliliters</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>20 milliliters</td>
</tr>
<tr>
<td>p-Dioxane, to make a total volume of</td>
<td>1 liter</td>
</tr>
</tbody>
</table>
expressed as picomole/gram wet weight. Protein was analyzed on each fraction (Figure 9) by the biuret method (Gornall et al., 1949).

Analysis of Sodium and Potassium

An aliquot (2 ml) of the 10% tissue homogenate was placed in plastic tubes containing an equal volume of 14 N nitric acid containing 2% lanthanum, lanthanum oxide, AAS grade (Alfa Inorganic Inc., Beverly, Massachusetts). After twenty-four hours the digest was heated slowly to 100°C in a temp-block module heater (Scientific Products, Evanston, Illinois) and maintained for one hour and allowed to cool to room temperature and was reconstituted to the original volume with distilled water modified after a manner described by Tuttle et al. (1961). Both homogenate and subcellular fractions were analyzed for Na and K and were diluted to appropriate concentrations for analysis. Early analysis was carried out at (Na-5890 Å) and (K-7665 Å) by a model 303 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, Connecticut). Later most of the Na and K analyses were carried out on a flame emission spectrophotometer, model 143 (Instrumentation Laboratory Inc., Lexington, Massachusetts) using a lithium internal standard.

Analysis of Interstitial Space

In order to obtain values for intracellular ions, the volume of the interstitial space was determined by measuring the inulin content in the heart which was perfused with one percent (w/v) inulin in K-H solution for periods of 32, 64 and 128 minutes. At the end of each incubation period, the heart was removed from the perfusion apparatus and blotted lightly on filter paper. The tissue was weighed and placed for 24 hours in cold 10% trichloroacetic acid. They were then homogenized, centrifuged and an aliquot was taken for inulin
analysis. Hearts were also perfused with inulin free K-H to serve as controls and analyzed. Inulin was determined by the alcoholic resorcinol method of Schreiner (1950).

The water content was determined in all different series of the procedure B experiments by measuring wet and dry weight of the tissue samples. The samples were dried at 150°C in glazed crucibles (Thermal American Fused Quartz Co., Montville, New Jersey) until a constant weight was achieved.

Analysis of Calcium

From the same digest in which Na and K was analyzed, Ca was also measured at 4227 A by a model 303 Atomic Absorption spectrophotometer (Perkin-Elmer, Norwalk, Connecticut). In all absorption studies, values were measured as percent absorption and converted to absorbance. The (sucrose-EGTA)-nitric acid 1:1 solution was used as blank values.

Intracellular ion concentrations were derived as follows, using K for illustrative purposes.

\[ [K]_i = \frac{(K_t - V_{in} [K_0])}{(V - V_{in})} \]

in which:

- \([K]_i\) = the intracellular K concentration
- \([K]_0\) = the K concentration of the medium
- \(K_t\) = the total K content of the muscle
- \(V_{in}\) = the volume of the inulin space
- \(V\) = the volume of the total muscle water, assuming specific gravity of 1.00

No assumptions were made as to the physical state of the intracellular Na, K or Ca.
Accumulation of Na\textsuperscript{22}

Na\textsuperscript{22} was obtained carrier free from International Chemical and Nuclear Inc. (Irvine, California). Aliquots of tissue homogenate, perfusate and subcellular samples were pipetted using Oxford automatic pipettes and analyzed in the model 5000 auto-gamma spectrometer (Packard, Downers Grove, Illinois). The Na\textsuperscript{22} spectrum was calibrated and the settings with the highest figure of merit (Efficiency\textsuperscript{2}/Background = E\textsuperscript{2}/B) was used in counting the samples.

Accumulation of Ca\textsuperscript{45}

Ca\textsuperscript{45} (13.6 microcurie/mg Ca) was purchased from International Chemical and Nuclear Co. (Irvine, California). Aliquots of the ten percent homogenate, perfusate and subcellular fractions were pipetted with the aid of oxford automatic pipettes into Bray's scintillation cocktail and counted in the scintillation spectrometer.

All Ca\textsuperscript{45} (half life: 163 days) were corrected for radioactive decay by the following formula:

\[ \ln C_0 = \ln C_t - kt \]

where

- \( C_0 \): original radioactivity
- \( C_t \): radioactivity at time \( t \)
- \( k \): decay rate for particular isotope
- \( t \): time of analysis of radioactivity

Uptake or accumulation of Na\textsuperscript{22} or Ca\textsuperscript{45} was defined as the total quantity of ion per kilogram taken up (exchange and net gain) by the tissue from the external medium over a given time period. It is calculated from the tissue concentration of radioactive ion and the specific activity of the bathing solution by the following formula:
\[ \frac{\text{cpm/kg tissue}}{\text{cpm/meq ion in K-H}} = \frac{\text{Ion uptake in meq/kg tissue}}{\text{wet weight}} \]

Conversion to intracellular ion concentrations was as described earlier.

All statistical analyses were carried out with the aid of the Programma 101, Olivetti Underwood (New York, New York) using the student "t" test (Snedecor, 1955). All t values which gave P greater than 0.05 were arbitrarily decided to be not significant in this study.
RESULTS

Influence of Agents Which are Structurally Related to Ouabain Upon
Ouabain Accumulation by Guinea Pig Hearts

It was proposed that agents which bear a specific structural
similarity to some part of the ouabain molecule might alter the accu-
mulation of ouabain by the myocardium. Table 6 shows a summary of
these results obtained in experiments designed to test this hypothesis.
These experiments were performed by methods referred to as pro-
cedure A. None of the sugars tried had any influence on the accumu-
lation of ouabain under conditions in which 2 g/l of glucose was present
as a substrate. Possibly there might have been alteration of the accu-
mulation of ouabain under conditions in which another sugar was sub-
stituted for glucose (Bailey and Dresel, 1971), but this was not done
because of the desire to keep as close as possible to physiological con-
ditions. Digitonin was included in the survey because of its steroid
nature and because of its proclivity to combine with cholesterol. If
like digitonin, ouabain in some way interacts with cellular cholesterol,
then it would be expected that digitonin would compete with ouabain
and reduce its accumulation. Table 6 shows that digitonin did not in-
terfere with the accumulation of ouabain. It was shown by Cosmides,
Miya and Carr (1956) that THFA had the most effect of a large group
of lactone derivatives tested for protection against digitoxin toxicity.
In experiments which were carried out in Krebs-Henseleit (K-H)
solution, however, significant effects of THFA in altering ouabain
accumulation were not observed.
Table 6

Influence of Agents Which are Structurally Related to Ouabain upon Ouabain Accumulation by Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Ouabain Accumulation (pmole/g) ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain 1 x 10^{-7} M</td>
<td>(7) c 182.5 ± 7.5</td>
</tr>
<tr>
<td>+Rhamnose 1 x 10^{-2} M</td>
<td>(3) 217.7 ± 19.4 NS</td>
</tr>
<tr>
<td>+Raffinose 1 x 10^{-2} M</td>
<td>(2) 169.9 ± 21.9 NS</td>
</tr>
<tr>
<td>+3-O-Methyl-D-Glucose 1 x 10^{-2} M</td>
<td>(2) 198.1 ± 23.6 NS</td>
</tr>
<tr>
<td>+Tetrahydrofuraneryl alcohol 1 x 10^{-5} M</td>
<td>(3) 216.2 ± 35.8 NS</td>
</tr>
<tr>
<td>+Digitonin 1 x 10^{-7} M</td>
<td>(2) 197.2 ± 21.6 NS</td>
</tr>
</tbody>
</table>

hearts were equilibrated for 64 minutes with K-H medium, then perfused for 64 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C.

NS = not significant

Number of experiments
Influence of Agents Which May Affect the Ionic Status of the Cardiac Cell Upon Ouabain Accumulation by Guinea Pig Hearts

The second broad group of agents which were examined for their influence upon ouabain accumulation in isolated guinea pig hearts were compounds which alter the ionic status of cells in some way. Table 7 shows the compounds in this group which were tested and their effect on the accumulation of ouabain. Cocaine is believed to exert its anesthetic action by inhibiting sodium influx. It is conceivable that the retardation of Na ion movement might influence the accumulation of ouabain. We found that there was a slight but insignificant reduction of ouabain accumulation by the myocardium produced by cocaine in the dose used. In Table 7 sodium ethacrynate (1 x 10^{-3} M) was shown to cause only a slight, but insignificant reduction in ouabain binding. Valinomycin has the ability to make the K ion more lipophilic, and thereby it increases the intramitochondrial K (Wenner et al., 1967). Table 7 shows that valinomycin does not significantly influence the accumulation of ouabain. This suggests an increase of the intramitochondrial K ion concentration does not appear to alter ouabain accumulation. Doubling the extracellular K concentration does significantly reduce the ouabain accumulation by the myocardium. The increase in extracellular K ion concentration has a profound influence on decreasing ouabain accumulation. This confirms the well-known observation that potassium interferes with ouabain accumulation processes (Dutta and Marks, 1969).

Influence of Antiarrhythmic Agents Upon Ouabain Accumulation by Guinea Pig Hearts

The third broad group of agents examined for their effect on altering the accumulation of ouabain were chosen on the basis that they have been reported to be antagonistic to the development of
Table 7
Influence of Agents Which May Affect Ionic Status of the Cardiac Cell upon Accumulation by Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Agents</th>
<th>Ouabain Accumulation (pmole/g)±S.E.</th>
<th>(7)(^c)</th>
<th>182.5 ± 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain 1 x 10(^{-7}) M</td>
<td></td>
<td>(3)</td>
<td>167.8 ± 10.3</td>
</tr>
<tr>
<td>+Cocaine 1 x 10(^{-5}) M</td>
<td></td>
<td>(4)</td>
<td>150.3 ± 11.9</td>
</tr>
<tr>
<td>+Sodium Ethacrynate 1 x 10(^{-3}) M</td>
<td></td>
<td>(3)</td>
<td>189.6 ± 9.4</td>
</tr>
<tr>
<td>+Valinomycin 1 x 10(^{-6}) M</td>
<td></td>
<td>(3)</td>
<td>89.6 ± 11.0</td>
</tr>
<tr>
<td>+Potassium ions 2 x normal</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 64 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C.

\(^b\) NS = not significant

\(^c\) Number of experiments
arrhythmias due to toxic concentrations of ouabain. From Table 8 it can be seen that quinidinе SO₄ (1 x 10⁻⁶ M) does not significantly reduce the accumulation of ouabain. The mechanism by which quinidinе exerts its antiarrhythmic action, therefore, does not appear to be related to ouabain accumulation.

Administration of 3 mg/kg reserpine to the guinea pigs 18 hours before sacrifice did not alter the accumulation of ouabain as seen in Table 8. This dose has been shown to reduce the cardiac pools of biogenic amines (Shore, 1966). There have been many conflicting reports regarding the use of reserpine in the prevention of cardiac arrhythmia and the decrease of the lethality of cardiac glycosides (Roberts et al., 1963; Ciofalo et al., 1967). Horne and Reynolds (1967) among others, present evidence which indicates that reserpine's effect is on the central nervous system rather than on the heart per se. This would be in accordance with the present finding in which no effect on ouabain accumulation by the myocardium was found.

Taurine has been claimed to prevent digoxin induced arrhythmias by acting as a precursor to isethionic acid which regulates the intracellular K ion concentration (Read and Welty, 1965). Mechanisms by which taurine prevents digoxin toxicity appear to be unrelated to the accumulation of ouabain, as can be seen from Table 8.

Loubatieres and co-workers (1963) have presented evidence that heparin will prevent the toxicity of cardiac glycosides without altering the positive inotropic effect due to ouabain. With 1.0 unit/ml of Sodium Heparin a small but significant reduction of ouabain accumulation is seen (Table 8). Thus, it is possible that the antagonism of heparin is due to the reduced accumulation of ouabain by the myocardium.
Table 8
Influence of Antiarrhythmic Agents Upon Ouabain Accumulation by Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Ouabain Accumulation (pmole/g) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain 1 x 10^{-7} M</td>
<td>(7)c 182.5 ± 7.5</td>
</tr>
<tr>
<td>+Quinidine Sulfate 1 x 10^{-6} M</td>
<td>(4) 185.7 ± 25.6 NS</td>
</tr>
<tr>
<td>+Reserpine pretreatment 3 mg/kg</td>
<td>(4) 167.7 ± 13.0 NS</td>
</tr>
<tr>
<td>+Taurine 2 x 10^{-4} M</td>
<td>(2) 158.9 ± 11.7 NS</td>
</tr>
<tr>
<td>+Sodium Heparin 0.8 m unit/ml</td>
<td>(2) 167.4 ± 25.7 NS</td>
</tr>
<tr>
<td>l.0 unit/ml</td>
<td>(3) 151.4 ± 3.4 P&lt;0.01</td>
</tr>
<tr>
<td>+Diphenylhydantoin 1 x 10^{-4} M</td>
<td>(4) 82.7 ± 2.0 P&lt;0.001</td>
</tr>
</tbody>
</table>

Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 64 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C.

NS = not significant

Number of experiments
The agent in this class of compounds which exerted the greatest effect in reducing the accumulation of ouabain was diphenylhydantoin (DPH). Under conditions used in procedure A, there was a reduction from 182.5 picomole/g to 82.7 picomole/g.

Distribution of Ouabain in Subcellular Fractions of Hearts Perfused With Antiarrhythmic Drugs

Subcellular distribution of the H\textsuperscript{3}-ouabain which was accumulated by the perfused hearts was studied by methods previously outlined by Dutta et al. (1968a). As illustrated in Table 9, those agents, such as quinidine, which did not alter the homogenate content of ouabain did not significantly affect any subcellular fractions. Diphenylhydantoin reduced the accumulation of ouabain in the subcellular fractions proportionally to the reduction in homogenate content. There was no fraction which was affected more than the others and, in general, the same pattern of ouabain accumulation is seen either with ouabain alone or in the presence of the other drugs studied.

Volume of the Space Accessible to Inulin in Guinea Pig Ventricles

Figure 10 shows the extracellular space as determined by inulin at different time intervals in guinea pig ventricle. The method of Tuttle et al. (1961) was used to give a value of 29.75 percent at zero time. Unlike experiments using procedure A or B, there was no equilibrium with K-H solution alone. In order to estimate inulin space, a 1 percent inulin in K-H solution was perfused and inulin concentrations were determined in hearts at desired time intervals.

Table 10 shows the effect of agents on water concentration in the guinea pig hearts. As can be seen, none of the agents used singly or in combination had any influence on the water concentration of the perfused guinea pig hearts.
### Table 9

Distribution of Ouabain in Subcellular Fractions of Hearts Perfused with Anti-arrhythmic Drugs

<table>
<thead>
<tr>
<th>Agents</th>
<th>Ouabain-H$^3$(picomole/mg protein)</th>
<th>S. E.</th>
<th>S/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear</td>
<td>Mitochondrial</td>
<td>Microsomal</td>
</tr>
<tr>
<td>Ouabain 1 x 10^{-7}M</td>
<td>0.75±0.06</td>
<td>0.80±0.09</td>
<td>1.51±0.18</td>
</tr>
<tr>
<td>(7)$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+2 x potassium</td>
<td>0.23±0.04</td>
<td>0.39±0.21</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>(3)</td>
<td><strong>P &lt; .001</strong></td>
<td>NS$^b$</td>
<td><strong>P &lt; .001</strong></td>
</tr>
<tr>
<td>+Diphenylhydantoin 1 x 10^{-4}M</td>
<td>0.31±0.08</td>
<td>0.23±0.03</td>
<td>0.53±0.17</td>
</tr>
<tr>
<td>(4)</td>
<td><strong>P &lt; 0.005</strong></td>
<td><strong>P &lt; 0.001</strong></td>
<td><strong>P &lt; 0.005</strong></td>
</tr>
<tr>
<td>+Quinidine 1 x 10^{-6}M</td>
<td>0.87±0.08</td>
<td>0.86±0.05</td>
<td>1.71±0.04</td>
</tr>
<tr>
<td>(4)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^a$hearts were equilibrated for 64 minutes with K-H medium, then perfused for 64 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C

$^b$NS = not significant

$^c$Number of experiments
Figure 10
The Extracellular Space as Determined by Inulin at Different Time Intervals

The percent of inulin solution in ventricular tissue is on the ordinate. Time of incubation in inulin-KH solution is on abscissa. Standard errors are represented by vertical lines through each point. Each point comprises at least three values.
Table 10
Effect of Agents on Water Content in the Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Myocardial Water Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.97 ± 5.45</td>
</tr>
<tr>
<td>Ouabain $5 \times 10^{-6}$ M</td>
<td>81.18 ± 1.42, NS</td>
</tr>
<tr>
<td>Diphenylhydantoin $6 \times 10^{-5}$ M</td>
<td>81.24 ± 0.69, NS</td>
</tr>
<tr>
<td>Diphenylhydantoin $1 \times 10^{-4}$ M</td>
<td>80.28 ± 0.47, NS</td>
</tr>
<tr>
<td>1.5x normal potassium</td>
<td>83.80 ± 0.72, NS</td>
</tr>
<tr>
<td>No perfusion</td>
<td>82.97 ± 0.28, NS</td>
</tr>
<tr>
<td>Ouabain $5 \times 10^{-6}$ M</td>
<td></td>
</tr>
<tr>
<td>+ 1.5x normal potassium</td>
<td>82.99 ± 0.53, NS</td>
</tr>
<tr>
<td>+ Diphenylhydantoin $6 \times 10^{-5}$ M</td>
<td>80.43 ± 0.97, NS</td>
</tr>
<tr>
<td>+ Diphenylhydantoin $1 \times 10^{-4}$ M</td>
<td>82.26 ± 0.89, NS</td>
</tr>
</tbody>
</table>

Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C.

NS = not significant

Number of experiments.
Effect of Antiarrhythmic Agents on the Ability of Ouabain to Produce Inotropy and Arrhythmia

Figure 11 illustrates three representative experiments showing the effects of $5 \times 10^{-6}$ M ouabain on the mechanical activity of the paced heart. As shown in the top panel, positive inotropy is seen within 2 - 4 minutes, which gradually reaches its maximum at 9 minutes. Following that period there is an onset of arrhythmia as indicated by the presence of extra beats which continued until the end of the experiment at 15 minutes.

In the middle panel, it is seen that in the presence of $6 \times 10^{-5}$ M DPH, ouabain still is able to exert its positive inotropic effect. However, DPH seems to prevent the production of extra beats as shown in the right-hand side of the middle panel. These findings are comparable with earlier observations made by Helfant and his associates (1965) in the intact dog.

In the bottom panel, similarly in the presence of 1.5x normal K ouabain is able to exert its positive inotropic effect. However, K prevents the onset of extra beats in this time period as was observed with the administration of DPH. These findings are in agreement with those of Williams and his co-workers (1966).

It has been reported by earlier workers (Drake et al., 1939; Wollenberger and Krayer, 1948; Covino and Shannon, 1969) that DPH appears to decrease the contractility of muscle. Figure 12 shows that the increasing concentrations of DPH, caused a concomitant depression of heart contractility as measured by the first derivative of the displacement of isotonic lever ($dD/dt$). As can be seen with time, there is a decrease of inotropic activity with $6 \times 10^{-5}$ M and $1 \times 10^{-4}$ M DPH (Figure 13). Figure 14 illustrates the effect of 1.5x normal K on cardiac contractility as measured by displacement ($dD/dt$) with res-
Figure 11

Effect of Diphenylhydantoin and Potassium on the Ability of Ouabain to Induce Inotropy and Arrhythmia
<table>
<thead>
<tr>
<th>AGENT</th>
<th>PARAMETER</th>
<th>INITIAL TIME</th>
<th>3 MINUTES</th>
<th>15 MINUTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>OUABAIN</td>
<td>DISPLACEMENT (D)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-6} \text{M}$</td>
<td>$dD/dt$ DERIV.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>STIMULUS RATE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OUABAIN</td>
<td>$D$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-6} \text{M}$ +</td>
<td>$dD/dt$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPH $6 \times 10^{-5} \text{M}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>STIMULUS RATE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OUABAIN</td>
<td>$D$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-6} \text{M}$ +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POTASSIUM $1.5 \times \text{NORMAL}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>STIMULUS RATE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12
Diphenylhydantoin Dose-Response Relationships

Percent of dD/dt at zero time. Standard errors are represented by vertical lines intersecting each point. Each bar represents the mean of three values.
 CONTROL

DPH $1 \times 10^{-5}$ M

DPH $3 \times 10^{-5}$ M

DPH $6 \times 10^{-5}$ M $p < 0.05$

DPH $1 \times 10^{-4}$ M $p < 0.05$

0 15 30 45 60 75 90 105 120
Figure 13
Diphenylhydantoin Time-Response Relationships

Percent of $dD/dt$ at zero time on ordinate. Perfusion time is on the abscissa. Standard errors are represented by vertical lines intersecting each point.
Figure 14

1.5x Normal Potassium Time-Response Relationship

Percent of dD/dt at zero time is on ordinate. The perfusion time is on the abscissa. Standard errors are represented by vertical lines through each point.
The graph shows the change in $\frac{dD}{dt}$ over perfusion time (MIN.) for two conditions: CONTROL ± SE and 1.5 x NORMAL K ± SE. The y-axis represents $\frac{dD}{dt}$, ranging from 0 to 100, and the x-axis represents perfusion time from 0 to 18 minutes. The CONTROL group shows a steady decrease in $\frac{dD}{dt}$, while the 1.5 x NORMAL K group shows a more pronounced decrease with a sharper decline after 12 minutes.
pect to time. As can be seen with time, there is a decrease in dD/dt.

Many authors, especially Leonard and Hadju (1959) have stressed
the importance of potassium in the maintenance of cardiac contraction.

Further analysis of time response relationship of ouabain (5 x 10^{-6} M)
alone and in the presence of 5 x 10^{-6} M or 1 x 10^{-4} M DPH are shown in
Figure 15. Figure 16 shows ouabain-1.5x normal K time relationships.
The hatched areas in Figure 15 and 16 refer to the onset and duration
of arrhythmia. The reduced dD/dt seen following 9 minutes perfusion
of an arrhythmogenic concentration of ouabain is because of the toxic
manifestations of this agent.

**Effect of Antiarrhythmic Agents on the Accumulation of an Arrhythmogenic Concentration of Ouabain**

Table 11 shows accumulation of tritiated ouabain alone and in
the presence of 6 x 10^{-5} M and 1 x 10^{-4} DPH respectively and 1.5x
normal K. Diphenylhydantoin reduced the accumulation of ouabain by
the myocardium in a dose dependent fashion as seen in this table.
Potassium also significantly reduced the accumulation of ouabain.
This reduction in accumulation of ouabain is seen at the same concen-
trations at which DPH and K exert their antiarrhythmic effects.

**Effect of Antiarrhythmic Agents on Sodium**

In the same hearts in which the accumulation of ouabain was
studied, intracellular ion concentrations were also examined. In
agreement with many other studies (Glynn, 1964), Table 12 also
shows that an arrhythmic concentration of ouabain increases the intrac-
cellular Na ion concentration. The addition of either DPH or 1.5x
normal K prevents the increase in the intracellular Na ion concen-
tration. It should be noted that in the presence of DPH alone (6 x 10^{-5} M
Figure 15
Ouabain/Diphenylhydantoin Time-Response Relationships

Percent of $dD/dt$ at zero time on ordinate. Perfusion time is on the abscissa. Standard errors are represented by vertical lines intersecting each point. The hatched area represents the presence of cardiac arrhythmia.
PERFUSION TIME (MIN)
Figure 16
Ouabain/1.5x Normal Potassium Time-Response Relationships

Percent of dD/dt at zero time on ordinate. Perfusion time is on the abscissa. Standard errors are represented by vertical lines intersecting each point. The hatched area represents the presence of cardiac arrhythmia.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Accumulation (picomole/g) ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain 5 x 10^{-6} M</td>
<td>2980 ± 59</td>
</tr>
<tr>
<td>+Diphenylhydantoin 6 x 10^{-5}M (3)</td>
<td>2621 ± 116 ( P &lt; 0.05 )</td>
</tr>
<tr>
<td>+Diphenylhydantoin 1 x 10^{-4}M (4)</td>
<td>2298 ± 192 ( P &lt; 0.03 )</td>
</tr>
<tr>
<td>+Potassium 1.5x normal</td>
<td>2708 ± 25 ( P &lt; 0.01 )</td>
</tr>
</tbody>
</table>

\( ^{a} \) hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min., temperature 28°C.  
\( ^{b} \) Number of experiments
Table 12
The Effect of Ouabain, Diphenylhydantoin and Extracellular Potassium on Intracellular Sodium Concentrations in Guinea Pig Hearts (15 min)\(^a\)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Intracellular Na meq/l H(_2)O ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(4)</td>
</tr>
<tr>
<td>Ouabain 5 (\times 10^{-6}) M</td>
<td>(5)</td>
</tr>
<tr>
<td>+Diphenylhydantoin 6 (\times 10^{-5}) M (3)</td>
<td>42.03 ± 5.47 (P&lt;0.025^*)</td>
</tr>
<tr>
<td>+1.5x normal potassium</td>
<td>(4)</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 (\times 10^{-4}) M (4)</td>
<td>38.20 ± 7.12 (\text{NS}^b)</td>
</tr>
<tr>
<td>Diphenylhydantoin 6 (\times 10^{-5}) M (3)</td>
<td>41.17 ± 6.03 (\text{NS})</td>
</tr>
</tbody>
</table>

*with reference to ouabain as control.

\(^a\)hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with labeled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28°C.

\(^b\)NS = not significant

\(^c\)Number of experiments
or 1 x 10⁻⁴ M) there was only an insignificant diminution in the intracellular Na ion concentration.

Although DPH alone fails to significantly alter the intracellular Na ion concentration, it does exhibit a dose-dependent effect on the increase in exchange of Na by the myocardium as seen in Table 13. Not only was the accumulation of Na²² by the myocardium analyzed at 15 minutes, but a minute by minute measurement of the perfusate was analyzed. Table 14 and Figure 17 show that with DPH in the medium, more radioactive Na is extracted by the heart as indicated by a decreased Na²² in the perfusate when compared with control. 1.5x normal K also increases the radioactive Na in the guinea pig hearts within a 15 minute period.

Effect of Antiarrhythmic Agents on Potassium

Table 15 shows the effects of the antiarrhythmic concentration of DPH and K on the intracellular K ion concentration. An arrhythmic concentration of ouabain decreases intracellular K concentration while the addition of either DPH or 1.5x normal K prevents the reduction in intracellular K ion. In the absence of ouabain, DPH appears to exert little effect on the intracellular K ion concentration.

Effect of Antiarrhythmic Agents on Calcium

The effect of an antiarrhythmic concentration of DPH and 1.5x normal K on the intracellular Ca ion concentration is shown in Table 16. It can be seen from this table that within the 15 minute time period of experiment there are no significant changes in the intracellular Ca concentration except for DPH alone. Using Ca⁴⁵, the increased Ca⁴⁵ accumulation in the presence of DPH is further substantiated in Table 17. However, only with 1 x 10⁻⁴ M DPH is a significant change seen.
### Table 13

The Effect of Diphenylhydantoin and 1.5x Normal K on Na\textsuperscript{22} Accumulation\textsuperscript{a}

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Experiments</th>
<th>Intracellular Na meq/l H\textsubscript{2}O ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(5)\textsuperscript{c}</td>
<td>19.31 ± 1.68</td>
</tr>
<tr>
<td>Diphenylhydantoin 3 x 10\textsuperscript{-5}M</td>
<td>(3)</td>
<td>28.94 ± 0.11 P &lt; 0.005</td>
</tr>
<tr>
<td>Diphenylhydantoin 6 x 10\textsuperscript{-5}M</td>
<td>(3)</td>
<td>33.72 ± 4.71 P &lt; 0.025</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 x 10\textsuperscript{-4}M</td>
<td>(5)</td>
<td>38.73 ± 12.01 NS\textsuperscript{b}</td>
</tr>
<tr>
<td>1.5x normal potassium</td>
<td>(5)</td>
<td>36.6 ± 4.7 P &lt; 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a} hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with the various substances indicated. Flow rate 4 ml/min, temperature 28°C.

\textsuperscript{b} NS = not significant

\textsuperscript{c} Number of experiments
Table 14

Effect of Diphenylhydantoin on Time Related Accumulation of Na\textsuperscript{22} \textsuperscript{a}

<table>
<thead>
<tr>
<th>Time</th>
<th>Control ± S. E. \textsuperscript{(3)} \textsuperscript{b}</th>
<th>6x10\textsuperscript{-5}M DPH ± S. E. \textsuperscript{(3)}</th>
<th>1x10\textsuperscript{-4}M DPH ± S. E. \textsuperscript{(5)}</th>
<th>1.5x normal K ± S. E. \textsuperscript{(5)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 min</td>
<td>9670 ± 183</td>
<td>-----</td>
<td>8820 ± 179</td>
<td>8790 ± 124</td>
</tr>
<tr>
<td>1 min</td>
<td>9540 ± 388</td>
<td>9320 ± 56</td>
<td>9390 ± 101</td>
<td>9380 ± 135</td>
</tr>
<tr>
<td>2 min</td>
<td>9740 ± 135</td>
<td>9760 ± 69</td>
<td>8140 ± 987</td>
<td>9550 ± 164</td>
</tr>
<tr>
<td>3 min</td>
<td>9960 ± 218</td>
<td>9830 ± 64</td>
<td>9690 ± 56</td>
<td>9720 ± 131</td>
</tr>
<tr>
<td>4 min</td>
<td>9910 ± 140</td>
<td>9780 ± 72</td>
<td>9850 ± 89</td>
<td>9750 ± 143</td>
</tr>
<tr>
<td>5 min</td>
<td>9930 ± 127</td>
<td>9940 ± 63</td>
<td>9750 ± 176</td>
<td>9680 ± 243</td>
</tr>
<tr>
<td>6 min</td>
<td>9780 ± 112</td>
<td>9930 ± 65</td>
<td>9770 ± 80</td>
<td>9770 ± 131</td>
</tr>
<tr>
<td>8 min</td>
<td>10040 ± 65</td>
<td>10150 ± 30</td>
<td>9970 ± 73</td>
<td>9860 ± 177</td>
</tr>
<tr>
<td>10 min</td>
<td>9810 ± 95</td>
<td>9990 ± 62</td>
<td>-----</td>
<td>9770 ± 129</td>
</tr>
<tr>
<td>12 min</td>
<td>9980 ± 164</td>
<td>9950 ± 74</td>
<td>9990 ± 93</td>
<td>9780 ± 81</td>
</tr>
</tbody>
</table>

\textsuperscript{a}hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with the various substances indicated. Flow rate 4 mg/min, temperature 28°C.

\textsuperscript{b}Number of experiments
Effect of Diphenylhydantoin on Time Related Accumulation of Radioactive Sodium

Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with Na$^{22}$ alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28°C, DPM/ml perfusate of Na$^{22}$ is seen on the ordinate. Perfusion time is seen on the abscissa.
Table 15
The Effect of Ouabain, Diphenylhydantoin and Extracellular Potassium on Intracellular Potassium Concentration\textsuperscript{a}

<table>
<thead>
<tr>
<th>Agent</th>
<th>Intracellular Potassium meq/1 H\textsubscript{2}O ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(4)\textsuperscript{c} 139.2 ± 3.4</td>
</tr>
<tr>
<td>Ouabain 5 \texttimes 10\textsuperscript{-6} M</td>
<td>(5) 120.1 ± 4.5 P&lt;0.025</td>
</tr>
<tr>
<td>+Diphenylhydantoin 6 \texttimes 10\textsuperscript{-5} M (3)</td>
<td>138.7 ± 4.7 P&lt;0.05</td>
</tr>
<tr>
<td>+1.5x normal potassium</td>
<td>(4) 132.7 ± 2.7 P&lt;0.05</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 \texttimes 10\textsuperscript{-4} M (4)</td>
<td>134.0 ± 7.0 NS\textsuperscript{b}</td>
</tr>
<tr>
<td>Diphenylhydantoin 6 \texttimes 10\textsuperscript{-5} M (3)</td>
<td>137.6 ± 5.8 NS</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with the various substances indicated. Flow rate 4 m\textperminute, temperature 28°C.

\textsuperscript{b}NS = not significant

\textsuperscript{c}Number of experiments
Table 16

The Effect of Ouabain, Diphenylhydantoin and Extracellular Potassium on Intracellular Calcium Concentration in Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Intracellular Calcium meq/1 H₂O ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(4)c</td>
</tr>
<tr>
<td>Ouabain 5 x 10⁻⁶ M</td>
<td>(5)</td>
</tr>
<tr>
<td>+Diphenylhydantoin 6 x 10⁻⁵ M (3)</td>
<td></td>
</tr>
<tr>
<td>+1.5x normal potassium</td>
<td>(4)</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 x 10⁻⁴ M (4)</td>
<td></td>
</tr>
<tr>
<td>Diphenylhydantoin 6 x 10⁻⁵ M (3)</td>
<td></td>
</tr>
</tbody>
</table>

*a hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with the various substances indicated. Flow rate 4 ml/min, temperature 28°C.*

b NS = not significant

c Number of experiments
Table 17
The Effect of Diphenylhydantoin on Ca\textsuperscript{45} Accumulation in Isolated Perfused Guinea Pig Hearts\textsuperscript{a}

<table>
<thead>
<tr>
<th>Agent</th>
<th>Intracellular Ca\textsuperscript{45} meq/l H\textsubscript{2}O ± S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(3) \textsuperscript{c} 3.56 ± 0.18</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 x 10\textsuperscript{-5} M</td>
<td>(3) 3.74 ± 0.13 NS\textsuperscript{b}</td>
</tr>
<tr>
<td>Diphenylhydantoin 3 x 10\textsuperscript{-5} M</td>
<td>(3) 4.17 ± 0.26 NS</td>
</tr>
<tr>
<td>Diphenylhydantoin 6 x 10\textsuperscript{-5} M</td>
<td>(3) 3.95 ± 0.20 NS</td>
</tr>
<tr>
<td>Diphenylhydantoin 1 x 10\textsuperscript{-4} M</td>
<td>(3) 5.53 ± 0.40 P &lt; 0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with the various substances indicated. Flow rate 4 ml/min, temperature 28°C.

\textsuperscript{b}NS = not significant

\textsuperscript{c}Number of experiments
Figure 18 shows DPH ($1 \times 10^{-4} \text{M}$) decreases the radioactive Ca$^{45}$ in the heart perfusate when compared to control. This would indicate that more Ca$^{45}$ is accumulated by the myocardium per unit of time.

Table 18 shows the effect of DPH on the subcellular distribution of Ca$^{45}$ in the guinea pig myocardium. As can be seen, the supernatant Ca$^{45}$ decreases while the mitochondrial and microsomal Ca$^{45}$ increases under the influence of DPH.
Hearts were equilibrated for 64 minutes with K-H medium, then perfused for 15 minutes with Ca\textsuperscript{45} alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28°C, DPM x 10\textsuperscript{3}/ml perfusate of Ca\textsuperscript{45} is seen on the ordinate. Perfusion time is seen on the abscissa.
DPM x 10^3 / ml PERFUSATE - Ca^45

CONTROL ± SE

DPH (1x10^-4 M) ± SE

PERFUSION TIME (MIN.)
Table 18
The Effect of Diphenylhydantoin on Ca\(^{45}\) Accumulation in Subcellular Fractions
(meq/mg protein)

<table>
<thead>
<tr>
<th>Agent(^a)</th>
<th>1st Supernatant</th>
<th>Nuclear</th>
<th>Mitochondrial</th>
<th>Microsomal</th>
<th>Final Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1688.5 ± 202.0</td>
<td>6.95 ± 1.62</td>
<td>3.66 ± 0.96</td>
<td>4.48 ± 1.88</td>
<td>741.9 ± 568.4</td>
</tr>
<tr>
<td>DPH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10(^{-5})M</td>
<td>902.2 ± 53.2</td>
<td>4.39 ± 0.53</td>
<td>3.90 ± 1.64</td>
<td>2.47 ± 0.32</td>
<td>297.0 ± 40.7</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.025</td>
<td>NS(^b)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6x10(^{-5})M</td>
<td>1147.6 ± 126.0</td>
<td>4.73 ± 2.06</td>
<td>4.55 ± 2.81</td>
<td>2.55 ± 1.47</td>
<td>316.4 ± 98.4</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3x10(^{-5})M</td>
<td>973.0 ± 29.3</td>
<td>4.33 ± 0.87</td>
<td>4.58 ± 1.70</td>
<td>2.72 ± 0.80</td>
<td>189.5 ± 47.9</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DPH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10(^{-4})M</td>
<td>962.7 ± 100.2</td>
<td>6.04 ± 2.13</td>
<td>15.47 ± 9.13</td>
<td>21.18 ± 18.5</td>
<td>416.6 ± 116.8</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^a\)each value represents 3 experiments
\(^b\)NS = not significant
M ETH O D S O F P R E P A R A T I O N  A N D T H E I R A D E Q U A C Y

Laboratory conditions were kept as constant as possible in preparing the guinea pig for experiment. Selecky et al. (1968) found that there was a seasonal variability of guinea pigs to g-strophanthin but the controls and their corresponding experimental studies in every case were performed during the same seasonal time period so that the seasonal effect could be minimized. Particularly during the months of January and February, increased incidence in pneumonic animals were seen. These animals, if conditions warranted, were not used. Samochewiec (1968) has found that the hearts of rats which were fed atherogenic diets exhibited higher sensitivity to strophanthrin, convallo-toxin and digitoxin when compared with the control hearts. Conceivably this could influence the responses between experiments. Although in all of the animals used in our studies only one animal feed was used, possibly differences could have arisen from different suppliers.

In procedure B, unlike procedure A in which the hearts were allowed to beat spontaneously, the isolated perfused hearts were paced following the method of Benforado (1958). This method consists of excising nodal tissue. Ligation and the use of cauterization were attempted, but did not prove to be as satisfactory. Ligation did not provide consistent suppression of A-V nodal activity during the course of the entire experiment. Cauterization, on the other hand, appeared to be excessively traumatic to the hearts. In these paced perfused hearts
there is a slight but insignificant reduction in mechanical activity which takes place over the period of 79 minutes.

The procedures for the measurement of H\textsuperscript{3}-ouabain were essentially the same as those described by Dutta et al. (1968a, b). The counting efficiencies of the samples were determined individually. This may not have been necessary as there was not more than two percent change of efficiency among the samples. A common efficiency factor could possibly be used in calculating disintegrations per minute.

Samples were homogenized with a teflon Potter-Elvehjem homogenizer except for the inulin studies in which all ground glass homogenizer was used. Preliminary observations suggested that there was incomplete homogenization with the teflon homogenizer. Possibly changing to a ground glass homogenizer may lead to more uniform fractionation. However, further studies involving electron microscopy would be necessary to confirm the belief that glass homogenizers might be superior to teflon homogenizers. The pH of the sucrose-EDTA solution was 5.2 - 5.4, which may alter the consistency of the individual fractions. In procedure B a buffered sucrose-EGTA solution was substituted for an EDTA solution. The sucrose-EGTA solution was buffered at pH 7.4 to attempt to insure consistency from in vivo to the in vitro state. One of the original proposals in this study was to investigate the ion concentrations in the subcellular fraction. EDTA has been claimed to alter the permeability of subcellular fractions to ions more drastically than EGTA (Brierty, personal communication), and therefore, EGTA was used for its milder effect.

Early analysis of Na and K were determined using atomic absorption spectrophotometric methods. However, due to the increased interferences encountered with the atomic absorption method (Baker and
Gar ton, 1961) and the increased stability of a lithium internal standard flame emission method, the later method was used for all values recorded in this thesis. Procedures for the flame emission analysis of Na and K differed from the standard plasma and urine measurements of these ions in that a 20:1 dilution of the samples already existed so the automatic dilutor was not employed and manual dilution techniques were employed to make a final dilution of 400:1 containing 15 meq Li/l as required for proper operation.

Calcium analysis was carried out using lanthanum to prevent interference by phosphate ions. Murdock and Heaton (1970) reported enhancement of calcium by Na and K under high flame temperatures. All Ca standards which were used contained equal Na concentration. However, as Murdock and Heaton indicated, these interferences are apparent at higher flame temperatures than were used in this study. Sodium and K caused no significant change in Ca with the lower temperature air-acetylene flame, which was employed in these experiments.

Ion Accumulation Analysis.

Radioactive calcium was analyzed under conditions which provided the best efficiency²/Background ratio. Due to the higher energy state of the beta particle emitted by the radioactive Ca there was no significant quenching effect under experimental conditions.

Before the radioactive Na was analyzed, a spectral profile for Na²² was recorded and a narrow spectral region was found in which Na²² emitted a large portion of its radiation. However, this small window was not used as it was found that a rather large window gave the best E²/B ratio.
Inulin and Total Water Analysis

The accurate measurement of the extracellular space of a tissue has been desired for many years, so that appropriate intracellular concentrations of ions could be calculated. There has been a growing concern that measurement of the extracellular space using inulin as a marker substance may not be reflecting an accurate inulin space. Phelps (1965) has analyzed physical properties of inulin such as viscosity and dispersion characteristics and has concluded that inulin may not reflect a true extracellular space. Falbraird and co-workers (1969) have claimed that the instability of the chemical assay for inulin makes its use less than desirable. These workers also claim that inulin can produce ultrastructural alterations, although no concrete evidence is offered in their publication. On the other hand, Page (1962) and Bozler (personal communication) express that of all the extracellular markers available, inulin as an extracellular marker would be preferable to others.

Inulin space, in this study, was measured by the alcoholic resorcinol method of Schreiner (1950). The chemical method is influenced by pH, temperature, and slight changes in chemical composition which control reaction conditions and difficulties commonly inherent to a spectrophotometric assay. With all these difficulties, it would appear that a radioactive method would probably be more accurate, but there also appears to be discrepancies using radioactive inulin space method as described by Patterson (1968).

Table 2 shows that there has been a wide degree of values for extracellular space. The value of 29.75% as found in this investigation agrees well with the literature values. Increased accuracy of the methodology for the determination of the extracellular space will have to wait until there are further improvements in techniques.
No changes in the intracellular water content could be seen in this investigation. However, it should be stated that only intracellular water content was determined in experiments involving procedure B. Perhaps this is due to the relatively short time period (15 min) of exposure of the agents to the hearts. Ouabain is known to increase the water concentration in myocardium, but only after longer time periods than used in these experiments (Page et al., 1964; Page, 1964).

Ouabain Accumulation and Its Implications

The first objective of this investigation was to carry out a survey of agents which could have the potentiality of reducing the specific accumulation of ouabain (Dutta and Marks, 1969) and might be useful for combating ouabain-induced arrhythmias. Agents which have been claimed to prevent cardiac glycoside induced arrhythmias were also surveyed for their ability to reduce accumulation of ouabain by the myocardium.

Influence of Agents Which Possess a Structural Similarity to Cardiac Glycosides upon Ouabain Accumulation

In all studies done to date, we found that under conditions which were designed to be as physiological as possible, sugars were unable to affect the accumulation of ouabain as seen in Table 6. It has been proposed that the sugar portion of the cardiac glycoside is directly related to the binding of the cardiac glycoside to a transport receptor (Bailey and Dresel, 1971). The results here do not seem to confirm such a proposal. It is felt that if there was any direct interaction of the sugar moiety on ouabain accumulation, a 10^5:1 ratio of rhamnose to ouabain might be reflected in a reduction of H^3-ouabain accumulation by the myocardium. 3-O-methyl-D-glucose, an agent which is known
to compete with glucose across intestinal membranes (Albers, 1967) was also not shown to exert any effect on ouabain accumulation.

Craig and Jacobs (1943) were the first to suggest that the cardiac action of the digitaloids were due to specific stereochemical configuration of the steroid nucleus itself. Although aldosterone has been claimed to prevent the positive inotropy due to ouabain (Lefer and Sayers, 1965), this paper has been refuted by Levy and Richards (1963, 1964). The effect of aldosterone, if true, is not yet known. There are no reports to indicate that aldosterone may be acting by a direct competitive mechanism with cardiac glycosides; perhaps aldosterone is mediating its action through changes in ionic permeability. However, Selye et al. (1969) have suggested that the ability of spironolactone and norbolethone to increase the lethal dose of digitoxin in rats may be directly related.

Digitonin, a steroidal compound, was tested for its ability to reduce ouabain accumulation with the knowledge that this compound has the ability to form complexes with cholesterol (Claus and Tyler, 1965). Since it is well known that cholesterol is an integral part of the plasma membrane (Albers, 1967), it was necessary to ascertain whether ouabain forms any complex at the cholesterol site of the membrane. As digitonin did not affect ouabain accumulation, these results probably suggest that cell membrane cholesterol is not directly involved in the ouabain accumulation process.

It has been claimed by Cosmides and his co-workers (1956) that tetrahydrofurfuryl alcohol (THFA), a lactone-like compound, was capable of preventing cardiac glycoside induced arrhythmias. The implication was that there was a possibility of competition at a locus of toxicological action. However, Shafer and Adicoff (1970) were unable to see the digitalis antagonism by THFA in the intact dog. Moore
(1961) has suggested that antagonism to digitalis may be due to lysis of red blood cells by THFA and subsequent potassium release which could account for the observed effect. The fact that in this study THFA does not reduce the accumulation of $^3$H-ouabain by the myocardium would tend to support Moore's suggestion of indirect mechanism of action.

Although our study did not show that THFA, a lactone-like compound, to have any effect on ouabain accumulation, Katzung and co-workers (1970) did show that modification of the lactone portion of the digitalis compound would modify the inotropic effect due to digitalis without concomitantly changing Na-K ATPase inhibition. Katzung's study as well as Wilson's model, as seen in Figure 3, suggest that the lactone-like moiety does exert an effect on certain sites of action for ouabain.

Influence of Agents Which Affect Ionic Concentrations Upon Ouabain Accumulation.

This investigation showed that $1 \times 10^{-5}$M cocaine did not significantly influence the accumulation of ouabain by the myocardium. However, there appears to be a slight reduction in accumulation, but not the type of changes as are observed with the actual physical reduction of the Na ion (Dutta and Marks, 1969). One could speculate that the presence of the sodium in the system rather than an ability to transport the sodium ion may be important for ouabain accumulation. It would be interesting to know how lidocaine would affect ouabain accumulation. Moore (1970) however, reported that both cocaine and lidocaine enhanced the cardiac uptake of ouabain in non-contracting and paced guinea pig hearts. Since this is reported in a brief abstract it is difficult to judge its merits. Although sodium ethacrynate has been shown to be an inhibitor of Na-K ATPase in the heart (Song and Scheuer, 1958), its cardiac pharmacology has not been fully established. Ethacrynic acid has been claimed to exert some
positive inotropic effect (From, personal communication) but the claim needs further documentation in order to be accepted. Lee and co-workers (Lee et al., 1969) have found that in the dog, there was no effect of ethacrynic acid on the toxicity of cardiac glycosides. It is difficult to ascertain what antagonistic effect ethacrynic acid can have when there is still continuing controversy regarding its direct mechanism of action (Wolf et al., 1969), and possible actions on different energy stores (Gordon and Hartog, 1969). If ethacrynic acid does inhibit sulfhydryl groups in general, caution should be used when attempting to assign its action with a particular biological function.

Valinomycin has been used as a tool to increase the intra-mitochondrial K (Pressman, 1969). In the belief that valinomycin would increase intracellular K in the myocardial cell, studies were undertaken to investigate the effect of valinomycin upon ouabain accumulation. If it is correct to assume that valinomycin does indeed increase the K concentration in myocardial cytoplasm, the negative effect observed by valinomycin on the ouabain accumulation can be interpreted that unlike an increase in the extracellular K, an increase in the intracellular K does not affect ouabain accumulation.

Potassium ions have been recognized for over fifty years to antagonize the effect of cardiac glycosides (Clark, 1912). However, that which is directly related to glycosides and that which is inherently part of the unique pharmacological makeup of K has not always been easy to detect (Logic et al., 1968). It can be observed from Table 7 that twice the normal concentration of potassium has a profound reduction on the accumulation of ouabain. This highly significant effect of K upon the ouabain accumulation was carried on as part of the central theme to attempt to glean more information concerning the
arrhythmic properties of ouabain and was discussed in this context later.

Influence of Agents Which Elicit Their Action Relative to Their Antiarrhythmic Properties.

Quinidine has been traditionally used as one of the mainstays in the clinical management of arrhythmias. Since quinidine is used to prevent ouabain induced arrhythmias, it was investigated for its possible effect on ouabain accumulation. In the preliminary survey a dose was chosen which would not greatly depress the electromechanical properties of the isolated heart. This dose does not seem to affect ouabain accumulation. However, care should be taken to interpret these findings because only a single concentration of this agent was tested in this brief survey. Perhaps a higher concentration would have reduced ouabain accumulation.

Although there is no uniform agreement as to the effect of reserpine in reducing the toxicity or lethality of cardiac glycosides (Ciofalo et al., 1966; Roberts et al., 1963; Levy and Richards, 1965), it is accepted that areas in the central nervous system can be a site of origin for eliciting cardiac arrhythmias (Reynolds and Horne, 1969; Standaert et al., 1969). From the studies of Reynolds and Horne particularly, there is a strong likelihood that locus of action for reserpine in affecting cardiac arrhythmias is the central nervous system and not a direct effect on the myocardium.

Most of the studies which have attempted to correlate the property of catecholamine depletion by reserpine with the antiarrhythmic property of reserpine have not concurrently measured myocardial concentration of any biogenic amine at the time of their study. For this reason it is difficult to judge if the antiarrhythmic effect was due to a particular biogenic amine or some other property of reserpine.
Although Marcus and his co-workers have found reduced ouabain uptake in the dog after pretreatment with reserpine (Marcus et al., 1965) he did not see this phenomenon in the guinea pig (Marcus, personal communication). A species difference most probably does exist regarding reserpine's effect on ouabain accumulation. If this difference is relevant to the decrease of the lethality of ouabain by reserpine, it is not known at this time.

The study of Kuschinsky and Van Zwieten (1969) showed that reserpine did not influence the positive inotropic response due to either digoxin, digitoxin or digitoxigenin in isolated guinea pig atria. Pretreatment with reserpine did not affect the accumulation of the more polar cardiac glycoside, digoxin, by isolated atria. However, after pretreatment with reserpine approximately 20% more digitoxin and digitoxigenin were accumulated. From this present investigation, pretreatment with reserpine did not significantly influence the accumulation of ouabain by the isolated perfused guinea pig heart. This finding would be in agreement with those Kuschinsky and Van Zwieten reported, that polar cardiac glycosides were not influenced by pretreatment with reserpine.

Although Scharff and Wool (1969) found a high concentration of taurine in rat myocardium (39.8 millimole/l), few studies have sought to establish a function for the presence of the large concentrations in the myocardium. In a series of papers, Read and Welty (1962, 1963, 1965) attempt to implicate taurine as a regulator of cardiac intracellular potassium. To the date of this investigation, no other workers have verified their claims.

Read and Welty (1963) postulated that taurine antagonizes digoxin induced arrhythmias by preventing the potassium depletion from the myocardial cell. Like valinomycin, no significant reduction
in ouabain accumulation was observed under the influence of taurine. As with valinomycin, intracellular potassium is postulated to be affected by this agent. The use of these agents, therefore, suggests that an increase of intracellular potassium rather than intracellular concentration of potassium is able to antagonize ouabain accumulation. It is possible that an alteration of intracellular concentration of potassium as affected by these agents may not have caused any effect on ouabain accumulation because of a plasmalemmal asymmetrical affinity for this ion.

At the time of this investigation the role of taurine in the myocardial cell can only be speculated upon. The hydroxyl analog of taurine has been postulated to be an intracellular anion in squid axoplasm which is involved in conductance changes (Mullins, 1959). It would be interesting to know if taurine could serve in the same capacity in the myocardium as Mullins proposes in squid axon.

Mosey and co-workers (1952) were the first to demonstrate that diphenylhydantoin was able to alleviate ventricular arrhythmias resulting from digitalis toxicity. As DPH is believed especially to be useful in the treatment of arrhythmias resulting from an overdose of cardiac glycosides (Mercer and Osborne, 1967), DPH was included as one of the agents in the survey. Of all the agents tried in this preliminary survey, DPH has been most effective in reducing ouabain accumulation. These observations stimulated further investigations and more information regarding its antiarrhythmic properties will be discussed in this context later.

Distribution of Ouabain in Subcellular Fractions as Affected by the Agents Used in the Survey.

There seems to be little if any difference between the homogenerate and subcellular accumulation of $H^3$-ouabain which is influenced by
agents surveyed in this preliminary study. An agent such as DPH which reduced ouabain homogenate accumulation reduced ouabain accumulation in all particulate fractions and no one particulate fraction is exclusively reduced. From this evidence it would not be possible to predict that one subcellular fraction might play more of a contribution in as an arrhythmic locus of action. However, it is interesting to note that Firemark et al. (1963) found that the central nervous system subcellular distribution from DPH is similar to the ouabain subcellular distribution in the myocardium in that DPH and ouabain were found to have the highest subcellular concentration in the microsomal subcellular fraction.

It is noticed that the supernatant/pellet ratio (S/P) (Dutta et al., 1968a) is increased in the presence of DPH and 2x normal potassium. This implies that there is less binding of ouabain or more dissociation of H\textsuperscript{3}-ouabain during fractionation in the presence of these two agents. Quinidine, on the other hand, does not influence homogenate or subcellular ouabain accumulation, nor is there any changes in the S/P ratio affected by quinidine.

**Effect of Antiarrhythmic Agents on the Accumulation of an Arrhythmogenic Concentration of Ouabain**

The second phase of this study was to investigate in-depth the relationship between the prevention of ouabain induced arrhythmia and agents which had been most effective in reducing ouabain accumulation. On this basis DPH and K were selected and further studies using a procedure which would challenge their ability to prevent the arrhythmia induced by an arrhythmogenic concentration of ouabain (5 \times 10^{-6} M) within 15 min in the isolated guinea pig heart. Under the identical conditions in which both DPH and 1.5x normal potassium
prevent ouabain toxicity, these agents significantly reduce the accumulation of ouabain.

The concentrations of DPH and K used in these isolated perfused hearts correspond well with the doses used in vivo by Helfant et al. (1967, 1968) and Scherlag et al. (1968) for DPH and Williams et al. (1966) for K. These results suggest that those agents which are able to effectively reduce the accumulation of ouabain by the guinea pig heart may have the capability to selectively prevent ouabain induced arrhythmias. The clinical application of this type of information will hopefully lead to more judicious and specific therapy for cardiac glycoside induced arrhythmias.

Effect of Antiarrhythmic Agents Alone and in the Presence of Ouabain on the Myocardial Activity.

Early workers as Drake et al. (1939) and Baudouin and Hazard (1941) showed that DPH depressed muscle contractility. However, many of the studies which have described myocardial depression in animals in situ (Baudouin and Hazard, 1941; Boyd and Williams, 1969; Gupta et al., 1967) may be due to rapid systemic injection which causes peripheral hypotension. The transient negative inotropic effect (Conn et al., 1967) may be due to a nervous reflex and is not seen in a denervated animal (Mixter et al., 1966). Although many reports (Mercer and Osborne, 1967) have claimed myocardial depression from DPH in the intact animal, most of these reports could be discounted due to inadequate measurement of myocardial activity, inappropriately rapid rate of injection, or inappropriately large concentrations of DPH administered (Schaff et al., 1969).

In the denervated dog (Schaff et al., 1969) and in isolated preparations (Drake et al., 1939; Covino and Shannon, 1969) there is
decreased contractile activity. The decrease in activity in the de-nervated preparation might reflect the loss of a nervous reflex which is present in the intact animal. This study would agree with the observations made by Covino and Shannon (1969). Figure 12 shows that under the influence of DPH there is depression of myocardial contractility. In Figure 13, it can be seen that there is a time-dependent decrease in myocardial depression under the influence of DPH. Thus, it does appear that in the isolated guinea pig heart, DPH does depress myocardial contractility.

The results of this investigation agree with the well-known findings that increased extracellular K causes negative inotropy in the myocardium (Sampson et al., 1943). The negative inotropy seen with increased K resembles the effect which is observed with the administration of DPH, although it is not known at this time if the commonality seen between the effect of these two agents is related to the similar site.

A minute to minute analysis of ouabain effect on myocardial contractility (isotonic displacement) reveals that at early time periods there appears to be a distinct inhibition of inotropy of ouabain by DPH and K. However, at later time periods, the magnitude of inotropy in the presence of DPH is greater than that of ouabain alone. The results from this study would suggest that the effect of DPH and K on the positive inotropy of ouabain should be examined under conditions in which only positive inotropy and not arrhythmia is present. Apparent increases in ouabain inotropy due to DPH and K measured near the time of ouabain toxicity may be misleading if the enhancement in inotropy seen in the presence of the antiarrhythmic agents is thought to occur at all time periods of drug interaction.
Careful analysis of the literature agree with these findings. Those authors who investigated the effect of acetylstrophanthidin on rapidly produced negative inotropy of K did in fact find that cardiac glycosides did not alter K's negative inotropic effect (Logic et al., 1968). On the other hand, those authors who investigated the effect of K to alter the maximal positive inotropy of cardiac glycosides just before arrhythmia found that K would increase the cardiac glycosides inotropic effect (Williams et al., 1966). Quite similar findings were reported regarding DPH by Scherlag (1965) and Boyd and Williams (1969) who observed that DPH increased the inotropic effect prior to digitalis intoxication.

Effect of Antiarrhythmic Agents alone and In Combination with Ouabain on the Ionic Status

Since it is possible that direct measurement of ions could help to elucidate how prevention of ouabain accumulation by antiarrhythmic agents is related, a portion of this investigation was devoted to analyze the role of the intracellular concentration of Na, K and Ca.

In the same hearts in which the accumulation of ouabain was studied, intracellular ion concentrations were also examined. In agreement with other studies (Glynn, 1964), it has been noted that an arrhythmic concentration of ouabain increases the intracellular Na ion concentration. The addition of either antiarrhythmic agent prevents the increase in the intracellular Na ion concentration. Diphenylhydantoin alone (6 x 10^{-5} M or 1 x 10^{-4} M) failed to significantly alter the intracellular Na concentration. These results suggest that the reduction of ouabain accumulation by DPH may have prevented the Na increase.

Although DPH failed to significantly alter the intracellular Na ion concentration, it does exhibit a dose dependent response on the
increase in the uptake of radioactive Na by the myocardium. Increased radioactive Na with no parallel increase in intracellular Na indicates that there is increased Na pumping in the DPH treated heart. This would suggest as Woodbury (1955) first proposed, that DPH may stimulate Na-K activated ATPase. Other workers have also indicated that DPH stimulates Na-K ATPase in a variety of preparations such as the frog skin (Watson, 1970), dog kidney (Koch et al., 1962; Jean, 1970) rat cerebral cortical synaptosomes (Festoff and Appel, 1968) and human eccrine sweat gland (Sato and Dobson, 1969).

Bigger and his co-workers (1970), using electrophysiological methods, suggest that in the presence of DPH, a small increase in sodium pumping in heart muscle does occur. In addition, Katzung and Jensen (1970) presented evidence that the effect of DPH on electrical and mechanical properties of isolated rabbit and dog atria is Na and K dependent. This would further suggest that Na-K ATPase could be related to the effects seen by DPH.

On the other hand, Sperelakis and Henn (1970) failed to see any Na-K ATPase related effect due to DPH in embryonic cultured chick heart cells. However, the embryonic cell preparation may not be the appropriate preparation for evaluation. This is evidenced by the lack of DPH activity on immature rats and mice (Vernadakis and Woodbury, 1965). This lack of sensitivity of DPH in embryonic chick cells may be due to incomplete maturation of Na-K ATPase (Hoffman, personal communication).
Festoff and Appel (1968) found that the Na:K ratio was critical for determining the effect of DPH on synaptosome Na-K ATPase. Diphenylhydantoin produced significant stimulation of enzyme activity under conditions of a high Na:K ratio (25-50:1). At ratios of 5-10:1, DPH produced little or no effect, and at low Na:K ratios (less than 5:1) DPH was inhibitory. Investigation of the literature corroborates these findings. Those investigators who used an Na:K ratio of 5-10:1, did, in fact, find no effect of DPH on Na-K ATPase (Gibson and Harris, 1970; Formby, 1969). Investigators who used an Na:K ratio less than 5:1 did find that DPH inhibited Na-K ATPase (Pincus and Giorman, 1967). It is most probable that the lack of a number of investigators (Pincus and Giorman, 1967; Formby, 1969; Gibson and Harris, 1970) to find stimulation of Na-K ATPase by DPH could be due to the fact that the Na-K ratio used in the enzyme assay system was not appropriate. It is also possible that Na-K ATPase stimulation by DPH was not found because the assay system was performed under a maximal enzyme rate and no further stimulation was possible.

The proposal of Sano et al. (1968) and Lullmann and Weber (1968) that DPH inhibits Na influx does not agree with the data in this study which indicates that DPH increases Na influx in guinea pig myocardium. In addition, this study shows that an agent such as cocaine, which is thought to inhibit Na influx does not appreciably alter ouabain accumulation.

The increment of extracellular K is known to stimulate Na-K ATPase and induce Na turnover similar to what might be expected with DPH (Woodbury, 1969). The increased radioactive intracellular Na seen with the administration of 1.5x normal potassium would be compatible with Woodbury's thesis that K does stimulate the turnover of Na by the myocardium.
A minute to minute analysis of radioactive Na in the perfusate from isolated hearts shows little difference between the treated and the control samples. As can be seen, there appears to be slightly less radioactivity on a minute to minute basis in the perfusate from the heart which received DPH (1 x 10^{-4} M) and 1.5x normal potassium when compared with the control perfusates. This would suggest that there is more radioactive Na entering the heart on a minute to minute basis. There appears to be little difference in the specific activity between the control and the antiarrhythmic agent treated perfusates. Discernable differences between control and DPH (6 x 10^{-5} M) perfusates cannot be seen. However, there is an increase in radioactive intracellular Na at this concentration of DPH.

This investigation shows that an arrhythmic concentration of ouabain decreases intracellular K concentration while the addition of either antiarrhythmic agent prevents the reduction in intracellular K ion. Diphenylhydantoin (6 x 10^{-5} M and 1 x 10^{-4} M) appears to exert little effect on the intracellular K ion concentration. These findings, as a complement to the Na findings, would suggest that the reduction of ouabain accumulation by DPH and K may have prevented the intracellular K loss. These findings also would be in agreement that DPH could act by stimulation of a Na-K ATPase.

Cushman et al. (1966) found that DPH had no effect on the K influx or egress in either control or ouabain treated non-contracting dog heart slices. Since this paper is an abstract it is difficult to evaluate it. Heart slices, if cut too thick, are prone to inadequate nutrition and oxygenation, and if cut too thin, tend to lose too much of their internal milieu to be valuable as a cellular model. Further investigation should be carried out to verify this particular study.
No significant changes in intracellular calcium ion concentration either under the influence of ouabain (5 x 10^{-6} M) alone or in the presence of the antiarrhythmic agents have been noted in this study. Other investigators have reported that under toxic concentrations of ouabain there are increases in intracellular Ca (Holland and Sabitini-Smith, 1969). However, since under conditions of cell death, there is increased intracellular Ca (Blackburn, personal communication) it is difficult to know if changes in intracellular Ca are the cause of toxicity or result from it.

Diphenylhydantoin (1 x 10^{-4} M) alone significantly increases intracellular Ca. To determine the minimum concentration of DPH which would affect Ca concentration, radioactive Ca accumulation was analyzed under the influence of different concentrations of DPH. As seen in Table 17, the minimum concentration of DPH that significantly increases Ca^{45} accumulation is 1 x 10^{-4} M. Little effect is seen at a lower DPH concentration at which antiarrhythmic effects are seen. This would suggest that the effect of DPH on Ca accumulation may not be related to the antiarrhythmic effect of DPH.

The results of this investigation indicate that on a minute to minute basis, more Ca^{45} is accumulated by the myocardium. The increased intracellular Ca under the influence of DPH (1 x 10^{-4} M) would complement the data showing the Ca^{45} differences which are seen in the perfusate samples.

The subcellular distribution of radioactive Ca was determined in hearts which were treated with DPH. The data suggests that the increased Ca^{45} tends to localize in the mitochondrial and microsomal fractions. Additional information should be obtained before any definitive conclusions can be reached regarding the role of Ca and DPH in the myocardium.
Although no direct evidence can be seen about how DPH may exert its antiarrhythmic function by altering Ca accumulation, it is interesting to speculate on how Ca may be involved in the anti-convulsant effects of DPH.

It has been generally recognized in the literature that low Ca medium makes neurons more excitable. The addition of DPH prevents the hyperexcitability due to the Ca loss (Korey, 1951; Woodbury, 1969). Since this investigation showed that DPH tends to cause increased Ca accumulation this might offer an explanation as to how DPH protected against the hyperexcitability in nerves due to Ca loss. This investigational data concerning Ca would support findings from both Blaustein (1967) and Mule's (1969) laboratories, who found that DPH caused increased binding to phospholipids.

Proposed Model System for Diphenylhydantoin and Potassium

This investigation suggests that the action of DPH and K in preventing ouabain induced arrhythmias is due to the reduction of ouabain accumulation. Because of the reduction of ouabain accumulation caused by these antiarrhythmic agents, the increase in intracellular Na and reduction in intracellular K, which are believed to be associated with the ouabain induced toxicity, does not occur. The question arises as how these agents act to reduce ouabain accumulation and thereby antagonize ouabain induced arrhythmias.

If it is assumed that in the doses used in this study DPH and K are able to stimulate Na-K ATPase, it can be visualized that there will be an increased rate of phosphorylation and dephosphorylation of the enzyme and a parallel increased rate of pumping of Na and K. This is directly in contrast to ouabain which combines with this enzyme and stabilizes it so there is a decrease in the turnover rate for the
enzyme. It is proposed that an agent such as DPH or K which stimulates the cyclic phosphorylation and dephosphorylation of Na-K ATPase as indicated by heavy arrow in Figure 19, should prevent the stabilization of the enzyme by ouabain and thus reduce the accumulation of ouabain by the myocardium. The model of Sen, Tobin and Post (1969) is used to illustrate the proposal as to how DPH and K exert their antiarrhythmic actions (Figure 19).

![Diagram of Na-K ATPase](image)
The proposed model would require that DPH and K stimulate Na-K ATPase and exert their effects at a location on the outer side of the plasmalemma. The survey of the literature indicates that DPH stimulates Na-K ATPase in many biological systems (Woodbury, 1969). Furthermore, this study showed that DPH increased radioactive intracellular Na without increasing total intracellular Na which would indirectly suggest that Na-K ATPase is being stimulated by DPH. Dephosphorylation of Na-K ATPase is known to be catalyzed by K rather than by Na (Sen et al., 1969). The resemblance of the action of DPH to K suggests that DPH also may be influencing this dephosphorylation process which may occur at the outer surface of the plasma membrane. The results of Festoff and Appel (1968) who found that a Na:K ratio of 20-50:1 is needed to show stimulation of Na-K ATPase agree with this proposal. Although both Na and K stimulate Na-K ATPase, only increased K highly depresses ouabain accumulation. Initial ouabain accumulation takes place on the outside surface of the plasmalemma where K probably for the most part is acting (Sjodin, 1971). Sodium, on the other hand, is affecting Na extrusion initially on the inner surface of the plasmalemma (Sjodin, 1971). To inhibit the initiation of ouabain accumulation, agents such as K and DPH which primarily act on the outer surface of the plasmalemma must be employed. Further evidence to support the proposal that DPH acts on the outer surface of the membrane is that there does seem to be a good relationship between the blood level of DPH and the antiarrhythmic effect (Mercer and Osborne, 1967). It also appears that DPH is plasma bound to a high degree (Lunde et al., 1970) which might account for a large reservoir of DPH which might be easily accessible to a locus for antiarrhythmic action.

Although the potentiation of the inotropy due to ouabain by DPH ($1 \times 10^{-4} M$) is unknown at this time, it is proposed that the increase
in intracellular Ca seen in the presence of $1 \times 10^{-4}$ DPH causes the potentiation of the inotropy. With a concentration of DPH ($6 \times 10^{-5}$ M) that did not increase the intracellular Ca there was no concomitant increase in inotropy due to DPH.

The mechanism by which DPH stimulates Na-K ATPase is unknown at this time. This investigation suggests that further study is needed in this area. Three proposals which could account for the mechanism of action of DPH on Na-K ATPase are:

1. DPH could increase the affinity for Na or K for the enzyme.
2. DPH could combine with the enzyme to form an DPH complex which is transported within the cell.
3. DPH may be indirectly stimulating Na-K ATPase as through creatine phosphokinase (CPK), an enzyme which indirectly affects the turnover of ATP.

Although Jean (1970) has proposed that DPH could alter the affinity for Na and K for kidney Na-K ATPase, Festoff and Appel (1969) found that DPH did not alter the Na or K affinity for synaptosomal Na-K ATPase. Lis and Bijan (1970) have some preliminary evidence that hydantoin derivatives can affect CPK, but no work has been carried out using diphenylhydantoin. Lastly, no study to this date has shown that DPH enters the cell by an Na-K ATPase mechanism but the results of Firemark et al. (1963) suggest that DPH accumulation does not take place by a passive process. Future research efforts should be directed toward the search for the mechanism by which DPH can stimulate Na-K ATPase.
In conclusion, the author of this investigation notes a quotation which might summarize this and perhaps all investigations.

"The author believes that the living cell is such a complex system that the number of uncontrolled variables and unknown factors influencing its response are too great to permit proof of any theory regarding the mode of action of drugs being obtained directly from its study."

A. J. Clark, 1937
H³-ouabain accumulation (1 x 10⁻⁷M) was studied in the presence of agents which are known to prevent cardiac glycoside induced arrhythmias on agents which are thought to have the potentiality to reduce ouabain accumulation by the heart and thereby might be able to prevent ouabain induced arrhythmias. The preliminary survey revealed that quinidine, reserpine, taurine and cocaine did not significantly reduce H³-ouabain accumulation, whereas diphenylhydantoin (DPH), potassium and sodium heparin did significantly reduce H³-ouabain accumulation in isolated perfused guinea pig hearts.

Since in the preliminary studies, only one concentration was chosen for the rapid survey of these various compounds upon the H³-ouabain accumulation, caution should be applied in interpreting the negative effect on the ouabain accumulation demonstrated by quinidine and cocaine. It is quite possible that in the presence of higher concentrations of quinidine and cocaine, there may have been an appreciable depression of ouabain accumulation by the isolated heart.

At the end of the survey period an in-depth study was undertaken to investigate the relationship between the prevention of ouabain induced arrhythmia by DPH (6 x 10⁻⁵M) and (1 x 10⁻⁴M) and K (1.5x normal) their effects on ouabain accumulation and electrolyte composition in the isolated guinea pig heart.
the electro-mechanical properties of these hearts were monitored for the entire duration of the experiment. Results show that DPH and K did not interfere with the positive inotropy of ouabain, but did prevent the production of extra beats within the identical time period. A minute to minute analysis of ouabain effect on myocardial contractility (isotonic displacement) reveals that at early time periods there appears to be a distinct inhibition of inotropy of ouabain by DPH and K. However, at later time periods, the magnitude of inotropy in the presence of DPH is greater than that of ouabain alone. Apparent increases in ouabain inotropy due to DPH and K measured near the time of ouabain toxicity may be misleading if the enhancement in inotropy seen in the presence of the antiarrhythmic agents is thought to occur at all time periods of drug interaction.

Diphenylhydantoin and K prevented the increase in intracellular Na produced by ouabain (5 x 10^-6 M) alone. Diphenylhydantoin produced an increase of radioactive Na exchange with no change in intracellular Na. These data suggest that DPH and K are acting to increase active Na transport by the myocardium. Diphenylhydantoin and K both prevent the reduction of intracellular K by ouabain (5 x 10^-6 M). This would also suggest that DPH and K may be preventing ouabain accumulation and thereby preventing the loss of intracellular K.

Ouabain alone and in the presence of DPH and K did not significantly alter the intracellular Ca. Although DPH (1 x 10^-4 M) did produce increased radioactive Ca accumulation, DPH (6 x 10^-5 M), a concentration which does exert antiarrhythmic effect, does not significantly alter radioactive Ca accumulation. It would appear that changes in Ca are unable to account for the antiarrhythmic effect of DPH.

The most significant observation is that whenever there is prevention of arrhythmia, there is concomitant reduction in ouabain
accumulation. In addition, when comparing hearts perfused under the influence of these various agents, the subcellular fractionation pattern of ouabain remained essentially unchanged. Consistently the concentration of H3-ouabain was found to be greater in the microsomal fraction than any other fraction of the heart homogenate.

The antiarrhythmic effect of DPH and K on ouabain induced toxicity thus appears to be related to the ability of DPH and K to reduce ouabain accumulation by the myocardium and thereby prevent the intracellular Na and K changes which lead to the arrhythmic state.
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