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The Ohio State University, Ph.D., 1973
Pharmacology

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IN RELATION TO THE PHARMACOLOGICAL EFFECTS OF OUABAIN
IN DOG: EFFECTS OF DPH AND KCl INFUSION

DISSERTATION

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

By

Hee Min Rhee, B. Sc., M. Sc.

* * * *

The Ohio State University
1973

Approved by

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Advisor
Department of Pharmacology
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Last but not least, my special thanks go to my family for their continued encouragement and understanding, especially to my mother for her persistent dedication to her children and for raising them with uncompromising principle.
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Studies in Biochemical Pharmacology. Professors J. J. O'Neill and D. Couri
Studies in Cardiovascular Pharmacology. Professor S. Dutta
Studies in Cardiovascular Physiology. Professor C. W. Smith
Studies in Electro-Pharmacology. Professor P. B. Hollander
Studies in Muscle Physiology. Professor E. Bozler
Studies in Neuroendocrinology. Professor H. Goldman
Studies in Physical Chemistry. Professor Q. VanWinkle
Studies in Radioisotope Methodology. Professors L. Malspeis and D. R. Feller
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>adenosine triphosphate</td>
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<td>ATPase</td>
<td>adenosine triphosphatase</td>
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<td>a-v</td>
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<td>C_{inulin}</td>
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<td>dp/dt</td>
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<td>dilantin(5,5-diphenyl hydantoin)</td>
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<td>F.A.B.P.</td>
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<td>g</td>
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<td>GFR</td>
<td>glomerular filtration rate</td>
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<td>tritium</td>
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<tr>
<td>H.S.</td>
<td>highly significant</td>
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<td>IIT</td>
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<td>k</td>
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<td>kg</td>
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<td>N.S.</td>
<td>non significant</td>
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<tr>
<td>O.D.</td>
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<td>3b-xhamnosyl-1b, 5, 1la, 14, 19-pentahydroxy-20:22-cardenolide</td>
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<td>P&lt;sub&gt;ouabain&lt;/sub&gt;</td>
<td>plasma concentration of ouabain</td>
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<td>p</td>
<td>pressure or probability</td>
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<td>pH</td>
<td>negative log of hydrogen ion concentration</td>
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<td>Pi</td>
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<td>pM</td>
<td>picomolar</td>
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<tr>
<td>POPOP</td>
<td>1,4-bis-2-(5-phenyl-oxazole) benzene</td>
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<td>PPO</td>
<td>2,5-diphenyloxazole</td>
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r  correlation coefficient
R - C  resistance - capacitance (differentiating circuit)
rpm  rotation per minute
RV  right ventricle or right ventricular
RVct  right ventricular contractile tension
S. A.  specific activity
S. E.  standard error of the mean
sec  second
s/p  supernate to pellet ratio
SR  sarcoplasmic reticulum
SRF  sarcoplasmic reticulum fragment
t  time
T  temperature
T/M  tissue to medium ratio
t₀  zero time or control period just prior to drug treatment
Tris  tris(hydroxymethyl) amino methane or 2-amino-2-hydroxy methyl-1,3-propanediol
U_x  urine concentration of substance, x
Vmax  maximum velocity
I. INTRODUCTION

Ever since William Withering(1785) introduced digitalis in modern medical practice nearly 200 years ago, there has been a great deal of interest in this group of drug. During the first hundred years after the initial remedial use of digitalis in dropsy, one sees a period of controversy over its clinical usefulness and its gradual acceptance. Once its clinical usefulness became clear, another disputation arose as to the vital organ that mediated the therapeutic and toxic effects of digitalis. By the beginning of this century it became increasingly clear because of the works of A. J. Clark(1933) and Otto Loewi(1918) that the heart is the site of the primary effect of digitalis. Thus, it fell upon the researchers of our time to study the characteristic and site of digitalis effect on the heart.

In 1913, Weiszäcker was the first to note a correlation between the action of digitalis and its myocardial activity. With the isolated cat papillary muscle under hypodynamic state, Gold and Cattel(1940) first demonstrated that digitalis increased the force of isometric contraction under a constant rate of stimulation. Subsequent studies (Sonnenblick et al., 1966; Ellis and Dimond, 1966) confirmed the positive inotropic action of digitalis regardless of the functional state of myocardium. However, the molecular mechanism of digitalis for the inotropic action has not been completely elucidated(Marks, 1964; Langer, 1968).

In recent years, in an attempt to understand the mechanism(s) of the positive inotropic action of cardiac glycoside, their effects on the major cellular systems have been subjected to investigation by various workers. The possibility that digitalis may increase the cardiac contractile force by its effect on the energy metabolism was extensively examined(Wollenberger, 1951; Lee et al., 1960; Furchgott and Gubareff, 1958). It is now generally agreed that the metabolic effects of digitalis appear to be secondary to its effects on the movement of cellular electrolyte(Lee and Klaus, 1971).

The effects of cardiac glycosides on the physicochemical or enzymatic properties of the isolated contractile protein indicate that the positive
inotropic action of this agent probably is not mediated by their direct interaction with the contractile protein (Katz, 1966; Luchi and Kritcher, 1967; and Lee and Klaus, 1971). A considerable gravity of experimental evidences suggests that the positive inotropic response to digitalis is due to its effect on the phasic changes of available free calcium concentration of the myocardial cell (Langer, 1965; Ebashi and Endo, 1968; Bailey and Dresel, 1968).

In the early sixties Repke (1963) pioneered the hypothesis that the Transport ATPase (Mg-dependent, Na and K activated Adenosine Triphosphohydrolase, e.c. 3.6.1.3) is the receptor that culminates digitalis effect on the heart. This has attracted a great deal of interest not only because of the good correlations between the susceptibility of cardiac glycoside to the NaK ATPase and their potencies among different species, but also because of specific digitalis binding to this enzyme in parallel with the inhibition of the enzyme in vitro. Recently, Besch et al. (1970) studied the effect of ouabain on three distinct cellular systems--mitochondrial ATPase, calcium accumulating capability of the sarcoplasmic reticulum fragments and the NaK ATPase in the dog heart. The results of their study indicate that the effect of ouabain reflected reproducibly only to the inhibition of NaK ATPase activity, not to any other cellular systems studied. Akera et al. (1970) were also able to demonstrate the causal relationship between the inotropic response and the NaK ATPase inhibition induced by ouabain in dog myocardium.

In spite of these interesting correlations between digitalis and the NaK ATPase activity, there are still many questions that remain to be answered for the wide acceptance of this hypothesis. For example, the positive inotropic effect of cardiac glycosides has been demonstrated without any substantial changes in the intracellular Na⁺ and K⁺ concentrations which would be expected from the inhibition of Na and K pump (Tuttle et al., 1961; Holland et al., 1954). Furthermore, Lee and Klaus (1971) pointed out that one should be cautious to draw any conclusion on the basis of the comparison between an in vitro determination of NaK ATPase activity and an arbitrary inotropic response observed in vivo. Mason et al. (1971) suggested the inhibi-
tion of the NaK ATPase might be better related to the arrhythmia or toxicity induced by cardiac glycosides than to the positive inotropic response. A direct evidence of the dissociation between the positive inotropy and the inhibition of the NaK ATPase activity has been recently demonstrated by Okita (1972).

Since a direct correlation between the inhibition of the NaK ATPase and the positive inotropic effect of ouabain has not been investigated under a proper defined experimental condition, the main theme of this study is to establish the relationship, if any, between the inhibition of the NaK ATPase activity and the positive inotropic effect induced by ouabain. Additionally, an effort has been made to reexamine the prevalent concept of the causal relationship between ouabain induced arrhythmia and the inhibition of the NaK ATPase activity. At the same time, in considering the weight of digitalis induced toxicity and the frequent administration of KCl or DPH for the management of the adverse effect of digitalis, a part of this study was devoted to examine the antiarrhythmic action of DPH or KCl in terms of their effects on the accumulation of ouabain to the major myocardial subcellular fractions and on the activity of the NaK ATPase.
II. REVIEW OF THE LITERATURE

In order to elucidate the site or sites of the positive inotropic action of digitaloids, various attempts have been made particularly during the last two decades to inquire into the interactions of this group of drugs with ill or well defined macromolecules of contractile apparatus in vitro. Thus, the literature of this period reveals that as the mechanism of cardiac muscle contraction has become more involved over the years implicating not only contractile protein but other subcellular organelles associated with the ionic pump, so did the site of interactions for digitalis effect. In early years the site of digitalis effect was limited to the hypothesis involving actin, myosin or actomyosin system because of the observation that these proteins are affected by digitalis in vitro. In recent years a number of studies revealed the role of digitaloids to alter the calcium binding property of the cardiac sarcoplasmic reticulum membrane or to affect ionic pump by altering the sarcolemma bound transport enzyme and their connection to the positive inotropic event. Prior to consideration of the effects of digitalis on these various proposed cardiac cellular sites, it may be pertinent to discuss what is meant by the term myocardial contractility and the basic contractile mechanism underlying an inotropic event.

1. MYOCARDIAL CONTRACTILITY AND ITS ASSESSMENT

For the sake of simplicity and conveniency of terminology, the term "contractility" has been used inaccurately by various workers so that variable parameters such as cardiac output, isometric tension, velocity of shortening, isotonic shortening, stroke work etc., have been measured and expressed frequently in the name of contractility. As a matter of fact, in a carefully written chapter on muscle contraction in a physiology textbook (Bayliss, 1959), the term is not used. According to Hamlin and Smith (1968), contractility is defined in terms of ultrastructural and biochemical energetics, as a quality of muscle whereby it responds to stimulation by shortening. Inotropy is a synonym for contractility such that a positive inotropic agent is a substance which increases the force of contraction while a negative inotropic agent is one which decreases it.
Badeer (1967) indicated that contractility could be defined as the property of muscle tissue to shorten or to develop tension, or both, due to the development of intermolecular forces, the exact nature of which is still not clearly defined.

The mechanical output of a muscle in a single isometric twitch does not give a true picture of the degree of activity of the contractile component during the course of contraction because the external manifestation of activity depends upon the stretching of the series elastic component, which process usually takes time. Holland and Sabatini-Smith (1969) indicate the degree of activity of contractile unit may be expressed as a maximal isometric tension that the contractile unit is able to develop or just bear without lengthening at a given moment. Therefore, in order to measure the active state of muscle accurately, the recording must consequently be arranged in such a way that no movement of the series elastic element occurs, and in such a way that the capacity of the muscle to shorten and do work is translated into an actual performance.

In examining effects of a drug on myocardial contractility, Blinks and Kock-Weser (1963) emphasized that investigators have at least some control over four physical variables which can influence cardiac contractility. They are the time interval between contractions, the resistance to shortening, the fiber length at the onset of contraction and the temperature. Since alteration of any of these variables affect the function of heart muscle, efforts have been made to control all of them in the more careful pharmacological studies. Frequently, it has been difficult to evaluate the accurate contractile response to certain drugs, because effort has usually been directed only toward the prevention of physically induced changes that might be confused with the effect of the drug under study. When one is interested in evaluating the inotropic response of any drug, one usually considers the measurement of one of the following: 1) the rise of tension in the early part of systolic period, which is roughly proportional to the onset and the rise of the active state, 2) the peak tension which may give a clue to the maximal intensity of active state, 3) the time required to reach the peak tension and 4) the total duration of contraction, which indicate alterations in the time courses of activation. The justification of the above parameters being
used as an index of cardiac performance is based on the concepts that have been developed, largely through the effort of A. V. Hill and his associates (Hill, 1938, 1952), to account for the mechanical behavior of skeletal muscle. In essence, muscle behaves as if it were composed of three components: 1) an active contractile component which can develop tension or shorten according to a particular force-velocity frequency relationship, 2) a passive elastic component mechanically in series with the contractile component (series elastic element), 3) a passive elastic component mechanically in parallel with the contractile component (parallel elastic element). The length and tension changes in the muscle as a whole depend on the interactions among these three elements.

With the isolated cardiac muscle, chiefly papillary muscle, it has been shown that the interaction behavior of three different elements in contractile mechanics of skeletal muscle must also be considered in the analysis of the contractile mechanics (Hill, 1949). Since most studies on inotropic responses have not been shown to have an appreciable influence on the properties of the passive elastic components (Jewell and Blinks, 1968; Edman, 1965), it may be safe to say that inotropic alterations of the myocardium have to be achieved through an influence on the properties of the contractile element only. Based on this concept and the fact that pressure varies as a function of the integrated heart wall tension, the rate of rise of intraventricular pressure (dP/dt, or dP/dt/P) which is derived from the Hill model (see figure 1) has been extensively employed for the quantitative evaluation of inotropic properties of heart muscle (Reeves et al., 1960; Barnett et al., 1961).

2. BASIC MODEL OF CONTRACTILE MACHINERY

In order to visualize the effect of cardiac glycosides on contractile behavior, it is pertinent to review the molecular nature of the contractile machinery and the sequence of events involved in the excitation-contraction-relaxation process. It was once proposed by Szent-Gyorgyi and his collaborators (Hajdu, 1953; Hajdu and Szent-Gyorgyi, 1952) that myosin molecules are kept apart in resting state chiefly by K. In order to contract the repellant action of K on the myosin is removed by a loss of K from the cell during the repolarization phase of the cardiac action.
According to A. V. Hill (1938, 1949), skeletal as well as cardiac muscles consist of at least three different muscle components and the velocity of shortening of contractile element ($V_{ce}$) is the sum of the velocity of lengthening of the series elastic component ($V_{se}$) and the velocity of shortening of circumferential fiber ($V_{cf}$). That is,

$$V_{ce} = V_{se} + V_{cf}$$

$$= \frac{dq}{dt} - \frac{db}{dt}$$

Here $b$, the midwall radius of left ventricle
$k$, a stiffness constant
(Sonnenblick, 1962)
$q$, stress, the product of pressure and geometry ($q = cp$, $c$ is a constant depending on the geometry of ventricle)

During the isometric contraction, $b$ is a constant and therefore $V_{cf} = 0$.

Thus, $V_{ce} = V_{se}$

$$= \frac{dq}{dt}$$

Substituting $q = cp$,

$$V_{ce} = \frac{dcp}{dt} = \frac{dp}{dt} \frac{1}{k}$$

Figure 1. Justification of $dp/dt$ as an index of myocardial contractility
potential. As the muscle cell loss $K$, the repellent forces are decreased, folding of the myosin molecules occurs and somehow they combine with actin to form actomyosin which is responsible for the generation of active state.

In the intervening years, however, a great deal of knowledge has been acquired on the sequence of excitation-contraction process. As it generally is understood, the change in membrane potential is transmitted via the sarco tubular systems to the sarcoplasmic reticulum where calcium is bound in the resting state. This impulse somehow releases calcium from the SR to the level of high enough to interact between actin and myosin, which allows to generate tension by pulling the contractile protein toward the center of sarcomere. Relaxation phase may be followed by sequestration of calcium into the SR where an active pumping process has been defined (Ebashi and Endo, 1968).

It is now known that troponin which consists of three subunits (Ebashi, 1972; Katz, 1972) has many carboxyl groups that combine the released calcium. Calcium bound troponin modulates the inhibitory property of tropomyosin so that troponin allows to form heavy meromyosin cross bridges between myosin and actin molecules. Little is known about the site of the actin which recognizes the myosin molecule and the exact mechanism to deliver calcium rapidly enough to sarcomere to account for the speed of onset of contraction following the membrane depolarization.

The concept of cardiac muscle contraction as we know today can be best summarized by quoting a paragraph from a recent review of Szent-Gyorgyi (1970) on muscle contraction. "According to our present knowledge, excitation induces a liberation of Ca by the endoplasmic reticulum. The troponin, with its agglomerated COOH groups, has a strong affinity to the Ca. It binds the Ca which neutralizes its negative charge, and neutralizes herewith its repulsion of the meromyosin globule. Thus actomyosin is formed. Once actomyosin is formed the heavy meromyosin has to hydrate and has to shorten, pulling the actin filament towards the middle of the sarcomere. The cycle is completed by the splitting of ATP. By this splitting the meromyosin loses its hydration, while the inorganic phosphate formed binds the calcium as secondary Ca-phosphate,
thus releasing the COOH groups of troponin, which, then, repel myosin and let it go, getting the stage ready for the next contraction. We can call this series of events a "microcycle." The repeated microcycles of the various meromyosins integrate to a muscle contraction."

3. CHARACTERISTICS OF DIGITALIS INDUCED POSITIVE INOTROPIC EFFECT

In spite of the clear cut demonstration of Wigger and Stimson (1927) on the positive inotropic effect of cardiac glycosides, full acceptance of this fact had to wait until Gold and Cattel (1940) showed the effect of ouabain on the electrically driven cat papillary muscle. Unfortunately this study left a deep impression that the positive inotropic effect of cardiac glycosides is dependent upon the functional state of myocardium so that inotropic action can only be manifested on failing heart muscle. Subsequent careful studies (Selzer and Kelly, 1964; Sonnenblick et al., 1966; Ellis and Dimond, 1966; Cotton and Stopp, 1958) showed that cardiac glycosides do produce the inotropic action, regardless of the functional state of the myocardium. Therefore, the mechanism by which digitalis increases the force of contraction is probably not simply a reversal of the process which induced the hypodynamic state of the heart (Lee and Klaus, 1971).

While studying the mechanism of digitalis-induced inotropic action, Sanyal and Saunders (1958) and Moran (1963, 1967) noted that the positive inotropic effect of ouabain was found to be dependent on and related to the number of contractions after exposure of the heart to ouabain and not to the time of exposure in different preparations. Therefore, Moran proposed a hypothesis which explains this phenomenon, i.e., a reaction between the glycoside and the myocardial cells occurs with each contraction and that each reaction leads to progressive increase in contractility. It may be appropriate to point out that Glynn (1969) indicated each beat of the heart leads to an entry of sodium, so that cardiotonic effect develops only after a certain number of beats. Govier and Holland (1965) also showed that the Ca exchange in isolated rabbit atria is contraction dependent and ouabain augments the increase in calcium influx induced by contraction.

a. Role of Myocardial Energy Metabolism for the Inotropic Action of Cardiac Glycosides

Studies on the action of cardiac glycosides with different cardiac
preparations indicated, in general, digitalis increases cardiac mechanical performance to a great extent (Sarnoff et al., 1964; Jacobson, 1968; Goksel et al., 1963; Bing et al., 1950) without concomitant increase of oxygen consumption, that is, digitalis increases a mechanical efficiency which may not be able to observe all the time in normal heart (Bing et al., 1950). Wollenberger (1947), Finkelstein and Bodansky (1948) and Crevasse and Wheat (1962) have shown that this increase in oxygen consumption caused by cardiac glycosides is most pronounced in media containing glucose and calcium (Langemann et al., 1953), and is not observed when pyruvate, malate, or succinate is substrate (Hermann et al., 1954).

Although considerable efforts have been directed to correlate the inotropic effect of cardiac glycoside and the cellular concentration of intermediary metabolites (Flaut and Gertler, 1959; Casten and Marsh, 1953; Ellis, 1953), the difficulties in the measurements of steady state level of these metabolites and the possibilities of the change of turnover rate of metabolites which may likely occur during the course of experiment raises question as to the significance of these studies. Since high energy phosphate compounds, chiefly ATP and creatine phosphate are vital for the contractile event, extensive studies have been made on the effect of digitalis on the level of these compounds in various biological preparations.

It has been found to be true that the effect of cardiac glycoside on the concentrations of high energy phosphate compounds is not consistent depending upon the techniques and the preparations employed (Staub, 1959; Rebar et al., 1957; Greiner, 1952; Furchgott and Lee, 1961) so that Wollenberger (1953) once considered that these compounds are probably not changed by therapeutic concentration of digitalis. It appears that studies of Furchgott and Cubareff (1958), Lee et al (1960), Lee et al (1961) and the most recent investigation of Rhee et al (1972) would agree with this observation, though toxic concentration of cardiac glycosides decreases the myocardial content of ATP, CP with an increase of the content of ADP, AMP and inorganic phosphate as shown by Wollenberger (1951), Greiner (1952) and Wollenberger (1949).

The effect of toxic dose of cardiac glycoside led to the examination
of the effect of digitalis on oxidative phosphorylation (Grisolia, 1955 a, b), in isolated mitochondria and showed that digitalis did decrease mitochondrial synthesis of ATP and CP. But later results indicate that it may not be the case as reported by the various other workers (Lee and McElboy, 1955; Flaut et al., 1957; Rothlin and Schoelly, 1950).

b. Role of Calcium for the Inotropic Action of Cardiac Glycosides

The similarity of cardiac glycosides and calcium toward myocardial contractile response suggested the idea that the action of digitalis is mediated by calcium; however, there are many dissimilarities in their effect such as the speed of action (Wilbrandt, 1958), K fluxes in red blood cell (Schatzmann, 1953) and the rate of decay of post-stimulation potentiation (Tuttle and Farah, 1962). In 1883, Ringer found that in a Ca free medium hearts failed to contract and Clark (1912) showed that digitoxin effects on frog heart were not seen in the absence of Ca in perfusion fluid. The synergistic effect of Ca and digitalis has been observed repeatedly by many investigators (Berliner, 1936; Bower and Mengle, 1936) and it is well known that digitalis-induced arrhythmia can be prevented or abolished by producing a hypocalcemic state by the administration of either EDTA or citrate (Eliot and Blount, 1961; Bernstein et al., 1959; Brothers and Kabakow, 1957; Soffer et al., 1961). From these observations, Otto Loewi (1918) concluded that digitalis sensitized the effect of calcium on the myocardium. The possible site of action of Ca inside myoplasm is beyond doubt from the work of Heilbrunn and Wiercinski (1947), who had demonstrated the shortening upon a direct microinjection of CaCl₂ into frog skeletal muscle cells.

In 1954 Wilbrandt and Caviezel suggested that digitalis prevents a loss of myocardial Ca, probably by depressing Ca efflux. Although Harvey and Daniel (1952) failed to observe this effect of digitalis on Ca⁴⁵ exchange in perfused guinea pig heart, Thomas et al (1958) noted an increased uptake of Ca accompanying the ouabain induced contracture in the frog heart. A similar observation was made by Holland and Sekul (1959) with isolated rabbit atria. Later it was shown that concentrations of digitalis producing contracture in addition to causing an increased uptake of Ca⁴⁵ also caused a rise of tissue Ca (Grossman and Proughgott,
1964; Lullman and Holland, 1962). However, with therapeutic doses of
digitalis, Ca exchangeability was increased, without or with little
change of the total tissue calcium content.

It has been also known that there are several specific Ca pools
or compartments in cardiac muscle, which may be responsible for the
determination of the extent of contraction. With isolated cat myocardial
tissue Bailey and Dresel (1968) found at least three calcium pools:
calcium pool 1, which half life is about 5 seconds and represents
extracellular-space; pool 2, a labile calcium pool with a half life
of 43 seconds or so; the other pool represents intracellular Ca which
is relatively nonexchangeable. However, Langer and his associates were
able to determine four compartments of Ca with a washout technique
after the load of Ca$^{45}$ in the frog heart (Sopsis and Langer, 1970)
and rabbit ventricle (Langer and Brady, 1968). In an effort to correlate
the inotropic action of digitalis and Ca pool affected by digitalis,
Bailey and Harvey (1969) found that ouabain increased Ca influx into
the labile pool, which may be comparable to the compartment described
by Langer (Sanborn and Langer, 1970) as lanthanum-sensitive site.

According to Holland and Sabatini-Smith (1969) contractile activity
occurs when the concentration of calcium in the medium is as low
as 0.2 to 0.3 micromolar, which means, in resting state, the myoplasmic
Ca concentration must be below this level. Since Weber and Herz (1963)
and Hasselbach (1964) showed that isolated sarcoplasmic reticulum can
reduce Ca up to 0.1 micromolar or even lesser than this and almost all
Ca is sequestered in the reticulum in resting fiber, Glynn (1969) concluded
that in excitation-contraction coupling the signal in the transverse
tubules causes a release of Ca from a bound state and that this liberated
Ca causes contraction by acting directly on the contractile filament of
the myofibril.

c. Role of the NaK ATPase For the Inotropic Action of Cardiac Glycosides

In 1931, Calhoun and Harrison observed a marked reduction of
myocardial K content after administration of toxic or lethal dose of
digitalis in dogs. A considerable body of evidence (Bay and Bay, 1956;
Blackman et al., 1960; Harris et al., 1955; Lee, 1963; Holland, 1960)
with different preparations and with various species supports this
observation, though the effect of low or therapeutic doses of digitalis on the level of $K$ is still a matter of controversy. Effect of cardiac glycosides on the coronary arteriovenous difference in $K$ concentration indicated that venous $K$ was raised following intravenous injection of cardiac glycoside (Wood and Noe, 1940). This was confirmed later by Hajdu and Leonard (1959), Klaus (1964) and Vick and Kahn (1957). Grupp and Charles (1964) could not find a relationship between coronary $A-V$ $K$ difference and cardiac contractility because low dose of cardiac glycoside produces either no increase or only a temporary increase in coronary venous potassium concentration. Furthermore, Brown et al (1962) found that in the digitalized heart the initial negative balance of $K$ was followed by a positive balance of $K$. Farah (1969) explains this phenomenon as a matter of method and speed of administration of cardiac glycoside because, when digitalis is given intravenously, the local digitalis concentration may be toxic especially in cardiac fibers close to the capillaries, which may reflect a loss of intracellular $K$ and increase the $K$ in the coronary venous blood. Once redistribution of digitalis has achieved, some of the initial potassium loss may be restored and produced a biphasic $K$ balance.

However, a number of investigators have shown that a positive inotropic effect of cardiac glycoside was frequently associated with an increased intracellular $K$ concentration (Carslake and Weatherall, 1962; Hagen, 1939; Holland et al., 1954; Tuttle et al., 1961). For instance, Tuttle et al (1961) and Witt and Tuttle (1960) have reported a reduction of the spontaneous $K$ loss from isolated rabbit atria with considerable inotropic manifestation at low doses of ouabain. In some of these studies, the intracellular sodium concentration was reduced instead of an increase when low concentrations of digitalis were used (Klaus et al., 1962; Tuttle et al., 1961). These observations may suggest that therapeutic dose of cardiac glycosides may have actually stimulated the sodium pump in cardiac cells, and thus increased the rate of exchange of Na or $K$. However, these results are difficult to accept because the determination of intracellular sodium concentration is rather inaccurate as generally agreed.

Schatzmann (1953) noted that in red blood cell cardiac glycoside
inhibited the active pump of Na and K without much affecting respiration or glycolysis. This observation was reexamined by Whittam and Ager (1965), who demonstrated that the inhibition of the pump by ouabain was associated with a reduction of glycolysis in such a way that three sodium ions were pumped out for each molecule of ATP consumed. Glynn (1957) showed that in red blood cells as well as squid axon the inhibition of K transport by digitalis requires the presence of the extracellular Na. Also cardiac glycosides inhibit Na exchange in red blood cells and the backward pumping mechanism which is accompanied by the formation of ATP when the concentration gradient of Na and K is steep (Glynn, 1969).

Considerable experimental evidence supports the idea that Na and K transport across the membrane of animal cells is carried out by transport-ATPase, which is specifically inhibited by active cardiac glycosides, though the exact mechanism by which the translocation of Na and K is accomplished is still beyond our complete understanding. Skou (1957) discovered this enzyme from the homogenate of peripheral nerve of the crab and subsequently identified its activity in a variety of tissues of different species (Bonting et al., 1961). Other investigators contributed to our understanding on the chemistry of the enzyme, the mode and site of the inhibitory action of cardiac glycosides on the enzyme and the possible models of translocation of Na and K (Skou, 1960; Dunham and Glynn, 1961; Gibbs et al., 1965; Uesugi et al., 1971; Skou, 1972; Sen et al., 1969; Albers et al., 1968; Opit and Charnock, 1965). Figure 2 shows one of the possible models of the NaK ATPase system, which exists in a series of allosteric transition states coupled to cyclic phosphorylation and dephosphorylation. This model also reflects a possible picture how translocation of Na and K is accomplished in this system. All these are based on the experimental evidences for the allosteric nature of this enzyme, which is supported by the conformational studies with various techniques (Yoda and Hokin, 1970b; Lindenmayer and Schwartz, 1970; Nagai et al., 1970; Ponzetti, Jr. and Titus, 1972; Allen et al., 1970b; Stryer, 1968).

Repke (1963, 1964, 1965) considered that the stimulation of NaK ATPase by cardiac glycoside might be responsible for the inotropic
Fig. 2. Hypothetical mechanism relating NaATPase to active transport of Na⁺ and K⁺

The circle denotes a plasma membrane of which the enzyme is a component. The phosphorylation site is on the cytoplasmic surface of the membrane. The cation effector sites of the enzyme are considered identical with the active transport carrier. The enzyme is denoted as "cis" or "trans" when the carriers are adjacent or opposite the phosphorylation site.

Reaction 1 is the Na⁺-dependent ATP-ADP exchange (4-6). In the native enzyme, the phosphorylated cis enzyme is rapidly converted to the trans form (reaction 2). In the presence of K⁺, reaction 3 activates the hydrolytic step (reaction 4). The trans enzyme becomes less stable as a result of the dephosphorylation and reverts to the cis form (reaction 5). The cycle is completed when Na⁺ displaces K⁺ from the cis enzyme (reaction 6). See the reference (Albers et al., 1968) for the detailed discussion.
effect of digitalis, which hypothesis was abandoned by himself later due to failure of reproducible activation of the enzyme with digitalis. Wilbrandt and Weiss (1960) noted the very low doses of strophanthoside sometimes stimulates Na transport across frog skin. Stimulatory effect of cardiac glycosides was also reported on cardiac microsome, on chick kidney and brain ATPase (Palmer et al., 1966; Palmer and Nechay, 1964; Lee and Yu, 1963). Thus Repke (1963) argued that the inotropic effect of digitalis may be related to the stimulation of the NaK ATPase and the toxic effects are related to the inhibition. But the difficulty is that demonstration of the stimulatory effect of NaK ATPase by cardiac glycosides is not easy while positive inotropic effect is readily reproducible. Also inotropic effect of cardiac glycosides may be associated with either a slight fall in K and an increase of Na or no change of any of these ions, as indicated by Tuttle et al. (1961).

However, Repke (1963) concluded that NaK ATPase is the receptor of cardiac glycosides on the basis of following observations. 1) NaK ATPase is not inhibited by inactive cardiac glycosides but by active glycosides, whose I_{50} produced a toxic effect on heart muscle. 2) Na and K were required for the action of digitalis on both NaK ATPase activity and cardiac muscle inotropic response. 3) Correlation was possible between the sensitivity of cardiac glycosides to NaK ATPase and that to toxic effects among different species. 4) The action of cardiac glycosides on cardiac muscle and on enzyme preparation was both slow and dose dependent.

In spite of this striking good correlation, there are still many problems to accept this hypothesis, that is, positive inotropic effect of cardiac glycosides has been demonstrated without any substantial changes in intracellular Na and K concentrations expected from the inhibition of NaK ATPase. Many reports (Boyer and Poindexter, 1940; Carslake and Weatherall, 1962; Holland et al., 1954; Tuttle et al., 1961) have documented an increase of K concentration during inotropic action of cardiac glycosides. Moreover, Ca is known to inhibit NaK ATPase while Ca is absolutely necessary to produce contraction and actually increases the speed of action of cardiac glycoside on cardiac muscle (Berliner, 1936; Bower and Mengele, 1936).
Recently Besch et al (1970) and Akera et al (1970) reported that a certain fraction of NaK ATPase was inhibited in myocardial tissue of dog at the time of inotropic effect of ouabain was manifested. However, it requires caution to draw any conclusion on the basis of comparison between an effect in vitro artificially created situation and an effect in vivo. Since most enzyme operates only with a fraction of the full maximal capacity in vivo (Lee and Klaus, 1971) and in vitro experimentally constructed condition, the enzyme activity is usually at its maximum, it is unknown how much inhibition of NaK ATPase activity in vitro is required to conclude that interference with the Na and K exchange has actually taken place in vivo. Therefore, Lee and Klaus (1971) concluded there is no known basis to justify a comparison between data in vitro and in vivo, in the case of NaK ATPase inhibition.

Moreover, Okita and his associates (Roth-Schechter et al., 1970; Okita, 1969 and 1972) have shown that there is no quantitative correlation between the inhibition of NaK ATPase in vitro and the inotropic effect in vivo because, in electrically driven isolated rabbit atria, inotropic effect of strophanthidol 3-bromoacetate could be washed out but the NaK ATPase inhibition persisted, suggesting the dissociation of the inhibition of NaK ATPase and the positive inotropic effect of cardiac glycosides.

However, Glynn believes that the positive inotropic effect is probably due to the inhibition of Na pump which increases the intracellular Na concentration and thus in turn displaces Ca from the carrier and allows more free calcium to exert its action on the contractile elements (Glynn, 1969). Also Schatzmann (1963) has demonstrated that the inhibition of NaK ATPase preparation from the pig heart with ouabain concentration as low as $4 \times 10^{-10} \text{M}$, which may indicate at the therapeutic concentration of cardiac glycoside inhibition of NaK ATPase.

In consideration of the role of calcium in excitation and contraction coupling, it may be reasonable to suppose that an increase in the intracellular concentration of ionized calcium will likely produce an increase of contractile force. Gersmeyer and Holland (1963) and Grossman and Furchgott (1964) demonstrated that an increase of Ca$^{45}$ uptake was
associated with an increase of contractile performance. Also Palmer and Posey (1967) observed that in cardiac sarcoplasmic vesicle, increase of internal sodium concentration releases Ca. Farah (1969) indicated the positive inotropic effect of cardiac glycosides is associated with increased Ca exchange. He concluded that the positive inotropic effect of digitalis is apparently not causally related to the decrease in intracellular K concentration but dependent upon the ratio of Na to K and on the ratio of Na to Ca, because abnormally high Ca, Na, K or abnormally low Ca interrupts the positive inotropic effect of cardiac glycosides.

4. SITE OF DIGITALIS ACCUMULATION BY THE MYOCARDIUM

From the foregoing review it is noted that information concerning the site of action of digitalis was obtained basically from the various biochemical and biophysical responses to these agents in vitro. This is particularly true of proposals regarding the effect of digitaloids on the Na-K ATPase system or calcium binding property of the sarcoplasmic reticulum membraneous fraction. With the continued development of interest in the site of digitalis action it became imperative to investigate whether heart in general or certain subcellular fraction of the heart in particular bind digitalis preferentially. Furthermore, as the response to a drug is due to a consequence of its binding to its receptor, it is logical that as soon as supportive techniques to measure intracellular concentration of digitaloid became available various workers wanted to investigate whether digitaloids undergo interaction with any particular component of the cardiac cell.

Earlier work indicates the liver accumulates about three times as much digitoxin and ten times as much digitoxin metabolites as does the heart (Okita et al., 1955). This confirmed earlier findings of Fischer et al. (1952) who showed the heart possesses no greater affinity for cardiac glycosides. In a later study Dutta and Marks (1966) showed a similar result with 16 organs of both guinea pig and rat, though there are species differences. Despite the lack of specific affinity of heart muscle for digitalis, it is the heart which is chiefly benefited by digitalis. Thus, question remains where in the heart does it act.
Although Harvey and Pieper (1955) found only 57 per cent of digitoxin radioactivity in the supernatant fraction in isolated guinea pig heart, after intravenous administration of digitoxin to the rat, St. George et al (1953) identified about 89 per cent of it in supernatant fraction of heart, measured by the sensitive bioassay method of embryonic duck heart. Spratt and Okita (1958) obtained similar results using radioactive digitoxin in rats. With perfused bullfrog hearts and dog's heart-lung preparations, Fozzard and Smith (1965) could identify 60 per cent of tritiated digoxin over the A bands of the sarcomere, similar to observation made by Tubbs et al (1964). Sonnenblick et al (1964) could identify the uptake of the tritiated digoxin in the transverse tubular system of cat myocardium. Conrad and Baxter (1964) attempted to correlate the change of myocardial contractility and the intracellular distribution of radiolabeled digoxin in the rat and dog heart. But, due to diffusion and redistribution of drugs during fixation and dehydration procedures, the significance of these studies is questionable.

In the isolated guinea pig hearts Dutta et al (1968 a; 1968 b) showed the highest digitalis concentrations in the microsomal fractions. They proposed that a digitalis receptor existed in the light particulates derived chiefly from plasma membrane and sarcoplasmic reticulum. This observation is significant because, recently growing body of experimental evidences indicate the NaK ATPase which is prepared from the microsomal fraction has many remarkable characteristics as a possible receptor substance of cardiac glycosides.

In isolated guinea pig hearts, Dutta and Marks (1969) studied the characteristics of digitalis accumulation, including saturability of the ouabain uptake system, dependency of Na and K concentration in perfusion medium and competition between pharmacologically active structurally related compounds. Also it is affected profoundly by chemical agents which affect cellular energy metabolism whether it is glycolysis or electron transport system (Rhee, 1970). More recently tests have been made whether the antiarrhythmic action of diphenylhydantoin is accomplished by a decrease of ouabain uptake in isolated guinea pig heart, which is true with only high dose of DPH (Baskin, 1971).
III. THE OBJECTIVES OF THIS STUDY AND THE RATIONALE FOR THE OBJECTIVES

Despite the numerous negative or contradictory evidences as discussed, the NaK ATPase has a number of striking characteristics as a possible receptor of cardiac glycosides. Careful experiments of Besch et al (1970) and Akera et al (1970) have been considered as proof of the causal relationship between the inotropic action of ouabain and the inhibition of NaK ATPase activity. Excluding the high dose of ouabain employed in these studies, which is close to lethal dose (Farah and Maresh, 1948; Dutta et al., 1972), the inotropic response to ouabain at one moment does not guarantee the true inotropic response to ouabain a minute later because the effect of ouabain is transient in nature. The inotropic action of ouabain may convert to a fatal ventricular fibrillation under such experimental conditions as demonstrated (Dutta et al., 1972).

At this time, even correlation between the inhibition of the NaK ATPase activity and the inotropic response of ouabain is still a matter of controversy because no study has clearly demonstrated the direct relationship between cardiac glycoside-induced inotropism and the inhibition of the NaK ATPase activity under acceptable defined experimental conditions. That is, in principle, it is not justified to conclude any relationship by a simple comparison of the inotropic response to ouabain at one moment and the NaK ATPase inhibition at any arbitrary chosen time without considering the time course interdependency between these two parameters. Therefore, it is imperative to examine the relationship, if any, between ouabain-induced inotropic response and the inhibition of the NaK ATPase activity in such an experimental condition that a steady state inotropic response of ouabain is maintained for sufficient period of time to justify the comparison of ouabain-induced inotropism to any other biochemical parameters.

In considering the gravity of digitalis-induced toxicity in the clinic and the prevalent use of KCl (Zelis et al., 1970; Vassale and Greenspan, 1963) and DPH (Bigger et al., 1968; Helfant et al., 1967a; Jensen and Katzung, 1970) as antiarrhythmic drugs, considerable studies have been made on the mechanism
of their antiarrhythmic action. As suggested (Entman et al., 1972; Mason et al., 1971), if the digitalis-induced toxicity is due to the inhibition of the NaK ATPase, the immediate experimental approach is to test the effect of DPH or KCl on the activity of the NaK ATPase. Akera et al. (1970) studied the effect of exogenous KCl infusion on the NaK ATPase activity in dog. It is of interest that KCl was able to reverse the arrhythmia induced by ouabain to normal sinus rhythm without relieving the inhibition of the NaK ATPase activity induced by ouabain. Spain and Chidsey (1971) could not demonstrate a stimulatory effect of DPH on the NaK ATPase activity prepared from dog myocardium, though the medium of high Na/K (50 mM/0.2 mM) ratio stimulated the NaK ATPase activity significantly. However, Woodbury (1955) and Watson and Woodbury (1972) suggested that the antiarrhythmic property of DPH might be due to the stimulation of the NaK ATPase activity.

Baskin (1971) tested the possibility that the antiarrhythmic effect of DPH and KCl was produced by reducing the accumulation of ouabain in isolated perfused guinea pig hearts. He was able to conclude that the antiarrhythmic effects of KCl and DPH are mediated by the reduction of the accumulation of ouabain because there was a significant decrease of ouabain content in myocardial fractions when the antiarrhythmic effects of KCl and DPH were evident. However, the effective DPH concentration was as high as 10^{-4} M in the perfusion medium.

The present study was undertaken to investigate the following: 1) relationship between pharmacological effects of ouabain and the extent of ouabain content in the major myocardial subcellular fractions including the NaK ATPase, 2) elucidation of the nature of the relationship between ouabain-induced inotropism and the NaK ATPase inhibition under specified conditions, 3) correlation of the NaK ATPase inhibition with other pharmacological effect of ouabain, especially ouabain-induced arrhythmia with reference to ouabain binding to this enzyme and 4) definition of the antiarrhythmic actions of K+ and DPH in terms of the changes in the NaK ATPase activity and ouabain accumulation to the major myocardial subcellular fractions in dog.
IV. MATERIALS AND METHOD

1. ANIMAL PREPARATION AND PHYSIOLOGICAL MEASUREMENTS

All experiments were carried out on female mongrel dogs weighing 9.7 to 23 kg supplied by the Blue Animal Farm (Plain City, Ohio). The dogs were anesthetized with pentobarbital sodium, 30 mg/kg, i.v. without other supporting medications and artificially resired with room air by a Harvard respirator (Harvard Apparatus Co., Dover, Mass. Model 607) via a tracheal intubation. The dogs were ventilated at a rate of 18 strokes per minute with an appropriate tidal volume. Through a midline sternotomy the chest was opened and the following physiological parameters were recorded continuously by either model 5C polygraph (Grass Instrument Co., Quincy, Mass.) or by the Beckman type R-dynograph (Beckman Instruments Inc., Schiller Park, Ill.).

The left intraventricular pressure was monitored by Statham P23AC transducer connected to the chamber by a 15 centimeters long nontoxic radiopaque catheter-needle (Becton, Dickinson & Co., Rutherford, N. J.) which directly punctured the apex dimple of the left ventricle. The right ventricular contractile force was recorded by a Walton-Brodie strain gauge sutured on the appropriate spot of the right ventricle devoid of blood vessels such that the muscle beneath the strain gauge was stretched about 40 to 50 percent of its initial length. The rate of change of the left ventricular pressure (dp/dt) was recorded directly from the ventricular pressure curve by means of a resistance-capacitance (R-C) differentiator.

The end diastolic pressure (EDP) was also measured by increasing the sensitivity of the amplifier of left ventricular pressure so that a few mmHg pressure change can be monitored. Since the EDP was not changed significantly in most of experimental procedures, the dp/dt was employed as a proper estimation of myocardial contractile performance. An "inotropic response" to ouabain was defined as a statistically significant (p<0.05) increase in the maximum LV dp/dt or RVct in comparison to the control values. The maximum dp/dt was measured in millimeters of positive deflection and expressed as percent change from the equilibrium period (t0). Standard lead II electrocardio-
gram, femoral arterial blood pressure (Statham P23AC transducer) and animal's rectal temperature (Model 44TA thermistor probe, Yellow Springs Instrument Co., Yellow Springs, Ohio) were also recorded.

2. OUABAIN DOSE REGIMENS AND THE TREATMENT OF KC1 AND DPH

Animals were divided into two groups—the paced and the intrinsically beating groups. The hearts in the paced dogs were paced at a rate of 10 to 15 per cent above the intrinsic beat by suturing a bipolar electrode on the right ventricle. The heart was stimulated with twice the threshold voltage with 10 milliseconds pulse duration. In the paced dogs 1 ug of ouabain per kg body weight per min was infused through the femoral vein continuously after a loading dose of 3 ug/kg body weight—designated the low dose—with sufficient radioactivity to trace labeled ouabain in the subcellular fractions (the ratio of tritiated to nonlabeled ouabain was at least 1:10). Infusion of ouabain was continued until the manifestation of one of three distinct pharmacological actions of ouabain—initial inotropy, onset of arrhythmia and lethality—was noted. In order to determine the effect of ouabain dose on the accumulation of ouabain in major subcellular fractions and NaK ATPase activity, 2 ug ouabain per kg body weight per minute were infused after a loading dose of 8 ug/kg body weight—designated the high dose—to 4 dogs in the paced preparation.

The experiments with the intrinsic beating group were designed to determine whether 1) the positive inotropic response induced by the inotropic dose of ouabain is causally related to the inhibition of NaK ATPase; 2) arrhythmia induced by the arrhythmic dose of ouabain is related to the inhibition of the NaK ATPase activity and 3) antiarrhythmic drugs, KC1 and DPH which are known to antagonize the arrhythmia induced by digitalis, alter the effect of ouabain on the NaK ATPase and the accumulation of ouabain in the major myocardial subcellular fractions. In order to maintain the steady state inotropic response to ouabain with respect to time without leading to toxic symptoms for 300 minute experimental period, it was necessary to maintain a constant therapeutic level of plasma ouabain by the manipulation of the ouabain dose regimen. Based on the data from a preliminary experiment on ouabain plasma half life (figure 3) and its renal clearance (tables 1 and 2), an infusion of 0.036 ug ouabain per
Figure 3. Composite graph of the plasma concentration of ouabain. Line 1 represents the plasma ouabain concentration in pm/ml. Line 2 is drawn by extrapolating the solid dots to zero time after a plateau is reached. Line 3 is a second exponential function obtained by subtracting 2 from 1(open dots). See text for explanation.

$t_{1/2} = 16\ min \ k_2 = 0.043\ min^{-1}$

$t_{3/4} = 260\ min \ k_3 = 0.00267\ min^{-1}$
Table 1. The renal clearance of ouabain and plasma half life after its single injection in dog

<table>
<thead>
<tr>
<th>Plasma sampling time (min)</th>
<th>Plasma ouabain pm/ml</th>
<th>Urine collection interval</th>
<th>Urine volume ml/min</th>
<th>Urine ouabain pm/ml</th>
<th>Oubain (GFR) ml/min</th>
<th>P inulin mg/ml</th>
<th>U inulin mg/ml</th>
<th>G inulin (GFR) ml/min</th>
<th>G ouabain / C inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1721</td>
<td>0 - 20</td>
<td>0.20</td>
<td>0</td>
<td>0.131</td>
<td>6.55</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>630</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>344</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>126</td>
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</tr>
<tr>
<td>10</td>
<td>54</td>
<td>0 - 20</td>
<td>0.20</td>
<td>0</td>
<td>0.131</td>
<td>6.55</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>29</td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>30</td>
<td>21</td>
<td>20 - 40</td>
<td>0.19</td>
<td>15808</td>
<td>143.0*</td>
<td>0.154</td>
<td>10.68</td>
<td>13.2</td>
<td>10.8*</td>
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<tr>
<td>50</td>
<td>17</td>
<td>40 - 60</td>
<td>0.25</td>
<td>2052</td>
<td>30.2</td>
<td>0.152</td>
<td>12.06</td>
<td>19.8</td>
<td>1.5</td>
</tr>
<tr>
<td>60</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>11</td>
<td>60 - 80</td>
<td>0.13</td>
<td>1667</td>
<td>19.7</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>90</td>
<td>8.4</td>
<td>80 - 100</td>
<td>0.14</td>
<td>1664</td>
<td>26.1</td>
<td>0.156</td>
<td>18.14</td>
<td>16.3</td>
<td>1.6</td>
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<tr>
<td>110</td>
<td>7.6</td>
<td>100 - 120</td>
<td>0.18</td>
<td>1082</td>
<td>25.6</td>
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<tr>
<td>130</td>
<td>7.0</td>
<td>120 - 140</td>
<td>0.12</td>
<td>1242</td>
<td>21.3</td>
<td></td>
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<tr>
<td>150</td>
<td>6.7</td>
<td>140 - 160</td>
<td>0.13</td>
<td>1007</td>
<td>19.5</td>
<td>0.162</td>
<td>17.96</td>
<td>14.4</td>
<td>1.3</td>
</tr>
<tr>
<td>170</td>
<td>6.2</td>
<td>160 - 180</td>
<td>0.14</td>
<td>926</td>
<td>20.9</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>190</td>
<td>6.2</td>
<td>180 - 200</td>
<td>0.11</td>
<td>1077</td>
<td>19.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>5.8</td>
<td>200 - 220</td>
<td>0.14</td>
<td>800</td>
<td>19.3</td>
<td>0.207</td>
<td>18.90</td>
<td>12.8</td>
<td>1.5</td>
</tr>
<tr>
<td>240</td>
<td>5.4</td>
<td>220 - 260</td>
<td>0.15</td>
<td>709</td>
<td>19.7</td>
<td>0.206</td>
<td>19.42</td>
<td>14.1</td>
<td>1.4</td>
</tr>
<tr>
<td>270</td>
<td>5.0</td>
<td>260 - 280</td>
<td>0.18</td>
<td>519</td>
<td>18.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290</td>
<td>4.6</td>
<td>280 - 300</td>
<td>0.19</td>
<td>489</td>
<td>20.2</td>
<td>0.232</td>
<td>20.58</td>
<td>16.9</td>
<td>1.2</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
<td>260.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEANS</td>
<td></td>
<td></td>
<td></td>
<td>21.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEANS expressed on body weight basis</td>
<td>21.7 ml/min/kg</td>
<td>14.7</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These values were not used in computing the means since they were obtained before equilibrium.
In order to get some idea of the proper dose regimen of ouabain for this study, on the basis of several approximations or assumptions, the following calculations have been made. The assumption was that the main process of ouabain elimination is renal clearance. And the approximation was that the amount of ouabain bound tightly to body tissue which is not subjected to the renal clearance is 40 pm per g of tissue solid at a constant plasma concentration of ouabain, 30pm/ml. This approximation is based on the distribution of digoxin in 15 organs of dog(Doherty and Perkins, 1966). Since the distribution volume(Vd) of ouabain can be calculated from the data of half study (see figure 3) utilizing the following relationship,

\[ V_d = \frac{\text{ouabain dose}}{(A + B)} \]

where, A and B are the plasma ouabain concentrations obtained by extrapolating the ouabain elimination and tissue distribution.

\[ V_d = \frac{30 \text{ ug/kg}}{(10+50) \text{pm/ml}} = \frac{51369 \text{ pm/kg}}{60 \text{ pm/ml}} = 856 \text{ ml/kg} \]

Therefore, to make 30 mp/ml plasma ouabain concentration for 1 kg dog, the amount of ouabain needed(M) can be expressed,

\[ M = 30 \text{ pm/ml} \times V_d + C \]

here C indicates the approximated amount of ouabain (40 pm/g of tissue solid) which is not in equilibrium with the plasma concentration of ouabain. Since muscle has 200g of tissue solid per kg of body weight, C = 40 pm/g x 200g/kg, which is 4.7 ug/kg.

Thus, M = 30 pm/ml x 856 ml/kg + 4.7 ug/kg

\[ = 19.7 \text{ ug/kg} \]

To determine the infusion dose of ouabain, the amount of ouabain cleared per minute has to be calculated. With 1 dog the renal clearance of ouabain was obtained experimentally as 2.1 ml/min/kg (see table 1). This value is quite close to the one obtained from data of ouabain distribution volume and the plasma half life of ouabain. That is, \( t_{1/2} \) final of plasma ouabain concentration was found to be 260 minutes with k of 0.00267 min⁻¹ in one dog experiment. Thus, clearance of ouabain, \( C_{\text{ouabain}} = 856\text{ml/kg} \times 0.00267/\text{min} \]

\[ = 2.28 \text{ ml/min/kg} \]

Therefore, the amount of ouabain(x) eliminated per minute is,

\[ \frac{dx}{dt} = P_x \times C_x \]

where, \( P_x, \) plasma ouabain concentration

\[ = 30\text{pm/ml} \times 2.1\text{ml/min} \times C_x, \] renal clearance of ouabain

\[ = 63 \text{ pm/min/kg} \]

\[ = 0.036 \text{ ug/kg/min} \]

Table 2. Some calculations and estimation for the infusion dose of ouabain
kg body weight per minute, started 10 minutes after a loading dose (20ug/kg) of ouabain injection—designated the inotropic dose—over 1 minute period, produced a relatively constant plasma ouabain concentration (10pm/ml) within 30 minutes after the onset of ouabain infusion.

An infusion of 0.072 ug/kg per minute which was started 10 minutes after a loading dose of ouabain (40ug/kg)—designated the arrhythmic dose—produced about 30 pm/ml or 3x10^-8 M plasma concentration of ouabain. A mild but persistent arrhythmia usually occurred within 60 minutes after the ouabain infusion. The arrhythmia continued for the entire experimental period (300 min) without a fatality. After the induction of the arrhythmia with the arrhythmic dose of ouabain infusion schedule, the persistency of arrhythmia was confirmed up to 3 hours when KCl or DPH administration was initiated to counteract the ouabain-induced arrhythmia. KCl was infused continuously at the rate of 1.2 meq/kg/hr for two more hours along with the ouabain infusion up to the termination of experiment (total 5 hours).

In the case of DPH experiments, multiple slow injections of DPH (1 mg/kg) into the femoral vein every 10 minutes were given after a loading dose of 8mg per kg i.v. injection. During either the DPH or KCl infusion, the arrhythmic dose of ouabain was infused continuously so that the same amount of ouabain was given to each dog for a comparable period of time. See table 3 for the detailed ouabain dose regimen and the classification of experiments. In all experiments, the control recording was made at least for 30 minutes so that each dog served as its own control before challenging with ouabain or ouabain along with KCl or DPH. During the infusion of ouabain, serial arterial blood samples were taken to monitor the plasma concentration of ouabain and the changes in plasma concentrations of sodium and potassium ions. The plasma concentrations of K^+ and Na^+ were measured by a flame emission photometer (Instrumentation Laboratory Inc., Lexington, Mass., Model II-143) using a 15 meq/l lithium as an internal standard.

In this study nonlabeled ouabain was purchased from Nutritional Biochemicals Corp. or Sigma Chemical Co. and tritiated ouabain (S.A. 13 curies/mM) was obtained from New England Nuclear Corporation. This randomly labeled ouabain was prepared by catalytic exchange with tritiated water and purified by adsorp-
Table 3. Summary of various dose regimens of ouabain used in this study

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>Classification of treatment</th>
<th>Loading dose (ug/kg)</th>
<th>Infusion dose (ug/kg/min)</th>
<th>Duration of expt. (min)</th>
<th>Total dose of ouabain (ug/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The paced heart preparation</td>
<td>Low dose</td>
<td>3</td>
<td>1</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>84±6.8</td>
<td>87±6.7</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>8</td>
<td>2</td>
<td>46±4.5</td>
<td>100±9.0</td>
</tr>
<tr>
<td>The spontaneously beating heart preparation</td>
<td>Inotropic dose</td>
<td>20</td>
<td>0.036</td>
<td>120</td>
<td>24.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.036</td>
<td>300</td>
<td>30.80</td>
</tr>
<tr>
<td></td>
<td>Arrhythmic dose (KCl)</td>
<td>40</td>
<td>0.072</td>
<td>300</td>
<td>61.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>0.072</td>
<td>300</td>
<td>61.60</td>
</tr>
<tr>
<td></td>
<td>Arrhythmic dose (DPH)</td>
<td>40</td>
<td>0.072</td>
<td>300</td>
<td>61.60</td>
</tr>
</tbody>
</table>
tion chromatography. The radiochemical purity of labeled ouabain was checked by the manufacturer routinely by paper and thin layer chromatography and isotope dilution analysis techniques. Before administration of DPH 250mg package of DPH in Steri-Vial (Parke, Davis and Co., Detroit) was dissolved in a solution contained 40% propylene glycol and 10% alcohol in water for injection. In the case of KCl, nonpyrogenic potassium chloride injection, u.s.p.(Travenol Lab. Inc., Morton Grove, Ill.) was used.

3. ANALYSIS OF OUABAIN BINDING AND NaK ATPase PREPARATION

Ouabain binding studies were conducted by estimating the ouabain content of different myocardial fractions. Upon the termination of experiments the heart was rapidly removed and briefly washed in ice-chilled saline solution. Avoiding the apex region where the catheter was inserted, 30 grams of left ventricular muscle were weighed and placed in 120 ml of cold homogenizing solution A which contained deoxycholate(table 4). Subsequently it was homogenized by a Polytron homogenizer(Type Pt. 20, Brinkmann Instruments, N. Y.) at peak speed for 45 seconds to make a 20% homogenate of cardiac muscle. The muscle homogenate was centrifuged at 12,000g for 30 min in a refrigerated ultracentrifuge(Beatman Instruments, Inc., Spinco Division, Model L). The supernate was saved for the preparation of the NaK ATPase and the pellet served to prepare the nuclear and mitochondrial fractions as shown in figure 4. The pellet was resuspended in 4 volumes of sucrose-EDTA(1:1000) solution and centrifuged for 10 minutes at 600g, whose pellet is a nuclear fraction. The supernate was again spun down at 12,000g for 15 minutes to obtained a mitochondrial fraction.

The original supernate was centrifuged for 50 min at 100,000g to produce a supernate and a pellet. This pellet is a microsomal fraction, which was resuspended in 18ml of solution A and further centrifuged for 50 min at the same centrifugal force. The subsequent pellet was again suspended in a mixture of 9 ml of solution B(see table 4) and 9 ml of 2.0M LiBr and stirred for 60 min at 4°C. After centrifuged it again, the pellet was washed out by suspending 8.5 ml of solution B and finally centrifuged at 100,000g for 50 min. This final pellet which was usually 10 to 15 mg of protein was homogenized with 2 to 3 ml of solution B in a Potter-Elvehjem homogenizer by a few strokes of the pestle and stored at -17°C until use or utilized right away after making an
Table 4. Chemical composition of homogenizing solution A and B used for the preparation of the NaK ATPase from the dog myocardium.

<table>
<thead>
<tr>
<th>Chemical agents</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.00 mM</td>
</tr>
<tr>
<td>Na2 EDTA</td>
<td>5.00 mM</td>
</tr>
<tr>
<td>Sodium Deoxycholate</td>
<td>0.15 % (v/v)</td>
</tr>
<tr>
<td>pH adjusted to 6.8 with Tris-base</td>
<td></td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.00 mM</td>
</tr>
<tr>
<td>Tris-EDTA</td>
<td>1.00 mM</td>
</tr>
<tr>
<td>pH adjusted to 7.4</td>
<td></td>
</tr>
</tbody>
</table>
PREPARATION OF NaK ATPase FROM DOG HEART

Left ventricle (30g)
- make 20% homogenate with 120ml of soln A
- homogenize (Polytron) for 45 sec at peak speed
- 20% homogenate (150ml)
- centrifuge for 30 min at 12,000gr

Pellet
- add 0.25M sucrose-EDTA (4x)
- homogenize briefly

Paste
- centrifuge for 10 min at 600gr

Supernate
- centrifuge for 15 min at 14,500gr
- resuspend in sucrose-EDTA (2x)

Pellet
- Nuclear fraction
- resuspend in sucrose-EDTA (2x)

Mitochondrial fraction

Supernate
- centrifuge for 50 min at 100,000gr

Pellet
- suspend in 15ml of soln A
- centrifuge for 50 min at 100,000gr

Supernate
- suspend in a mixture of 9ml of soln B and 9ml of 2M LiBr
- stir for 60 min at 4°C and centrifuge for 50 min at 100,000gr

Supernate
- resuspend in 9ml of soln B
- centrifuge for 50 min at 100,000gr

Pellet
- homogenize in 2-3ml of soln B with a few passes of a Potter-Elvehjem homogenizer

NaK ATPase
- Protein determination a
- Radioactivity counting for ouabain binding b
- NaK ATPase activity determination c

Figure 4. The flow-sheet for the preparation of major myocardial subcellular fractions and the NaK ATPase from the dog myocardium
appropriate dilution for the estimation of NaK ATPase activity.

In order to measure the extent of ouabain accumulation in each different fraction, each fraction was reconstituted with distilled water and appropriate aliquots of each reconstituted particulate fraction or the supernatant fractions were extracted with 15 ml of Bray's scintillation solution (Bray, 1960; see table 5). Subsequently the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 4321, Packard Instruments Co., Inc., Downers Grove, Ill.). Sufficient net counts were taken to assure counting error within permissible range (Loevinger and Berman, 1951). All counts were corrected for quenching by means of a quenching chart prepared by a series of quenched standard samples against the external standard counts (see table 6 and figure 5). By knowing the specific activity of ouabain used, the DPM was converted into picomoles of ouabain in that fraction and expressed per g of heart or milligram of protein measured by modified Biuret method (Gornall et al., 1949). Since there is no known metabolite of ouabain in this species, the radioactivity in each fraction was assumed to be due to intact tritiated ouabain.

4. DETERMINATION OF NaK ATPase ACTIVITY

After determining protein concentration of partially purified NaK ATPase fraction, NaK ATPase was diluted with appropriate volume of solution B so that the final concentration of enzyme is 20 ug/0.1 ml. The final incubation mixture consisted of 4.0 mM ATP, 100 mM NaCl, 10 mM KCl, 5 mM MgCl and 50 mM Tris-HCl (pH 7.4). All these chemicals used were reagent grade or better. Reaction was started either by the addition of ATP or 20 ug of enzyme in final total volume of 0.5 ml of incubation medium. After 10 minutes preincubation period at 37°C omitting either ATP or the enzyme, incubation was continued for another 10 minutes after the reaction started. Mg-dependent ATPase activity was obtained either by ATPase activity assayed in the absence of Na and K or in the presence of 10^{-6}M nonlabeled ouabain. NaK ATPase activity was calculated by subtracting the Mg-dependent ATPase activity from the total ATPase activity which was assayed in complete incubation medium. The liberated inorganic phosphate was measured by the method described by Yoda and Hokin (1970a). 0.5 ml of incubation medium was added to 1 ml of a mixture of
Table 5. Chemical composition of Bray's scintillation solution

<table>
<thead>
<tr>
<th>Chemical agents</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napthalene</td>
<td>60 g</td>
</tr>
<tr>
<td>1,4 bis (5-phenyl-o-oxazoly1) benzene(POPOP)</td>
<td>200 mg</td>
</tr>
<tr>
<td>2,5 diphenyloxazole(PPO)</td>
<td>4 g</td>
</tr>
<tr>
<td>Anhydrous methanol</td>
<td>100 ml</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>20 ml</td>
</tr>
<tr>
<td>1, 4 dioxane</td>
<td>to make total volume of 1 liter</td>
</tr>
</tbody>
</table>


## Table 6: Calibration of Packard Tri-carb liquid scintillation spectrometer, model 4312, for the calculation of tritium counting efficiency.

- **SAMPLE DESCRIPTION**: 15al Bray Soln, 0.25uc, 0.25uc + 0.05ml, 0.25uc + 0.1ml, 0.25uc + 0.2ml, 0.25uc + 0.3ml, 0.25uc + 0.4ml, 0.25uc + 0.5ml, 0.25uc + 0.6ml, 0.25uc + 0.7ml, 0.25uc + 0.8ml.

- **TIME**: 10.000 MIN-SEC.

- **COUNTS**: RED, GREEN, BLUE.

- **COUNTS PER TIME**: RED, GREEN, BLUE.

- **A.E.S. OR CHANNELS RATIOS**: A/G AUTO, G/R Auto, R/B Auto, G/B Auto.

### Notes:
- 20% heart homogenate was used as quenching material.
- Since 0.25uc has 555000 dpm, % efficiency was calculated as follow:

\[
\text{% Efficiency} = \left( \frac{\text{Counts in red channel}}{555,000} \right) \times 100
\]

Table 6: Calibration of Packard Tri-carb liquid scintillation spectrometer, model 4312, for the calculation of tritium counting efficiency.
Figure 5. The counting efficiency chart for tritium in Tri-carb liquid scintillation spectrometer, model 4312.
4 volumes of 4.5% ammonium molybdate and 1 volume of 60% perchloric acid after the completion of incubation. Three ml of n-butyl acetate were added and the mixture was mixed briefly on Vortex mixer (Scientific Ind. Inc., Queens Village, N. Y.). After centrifugation of the mixture, the clear upper organic solvent layer was read its absorbancy at 350 nm on Beckman DU spectrophotometer (see figure 6). The optical density was relatively stable for several hours and a wide range of orthophosphate could be read by adjusting the wavelength between 310 and 400 nm depending upon the concentration of inorganic phosphate.
Figure 6. Absorbancy of inorganic phosphate at 350 nm. Each point represents at least 6 determinations.
V. RESULTS

1. EXPERIMENTS WITH THE LOW AND HIGH DOSES OF OUABAIN IN DOGS WITH PACED HEARTS.

Experiments with paced hearts were designed to produce various stages of ouabain effect by infusing a relatively high dose of ouabain in a short period of time. Effort has been made to minimize the effect of frequency on the positive inotropic response to ouabain by pacing the heart above its intrinsic rate. In pilot experiments, a continuous infusion of ouabain (1 ug/kg/min) after a loading dose (3 ug/kg)—designated as the low dose experiments—produced three distinct pharmacological effects of ouabain within predictable periods of infusion. That is, a statistically significant positive inotropic response to ouabain could be observed about 15 min following the start of ouabain infusion. Under the low dose schedule arrhythmia and lethality occurred 40 and 85 minutes respectively after the start of infusion of ouabain. In order to study the dose dependent pharmacological effect of ouabain, experiments were done with an infusion of ouabain (2 ug/kg/min) after a loading dose of 8 ug/kg—designated as the high dose experiment. This induced a lethal effect about 40 minutes after the beginning of ouabain infusion. See table 3 for the details of ouabain dose regimen and the total experimental duration.

With the low and high doses of ouabain infusion schedule, the plasma concentration of ouabain with respect to time is shown in figure 7. With the low dose infusion of ouabain, it is noted that the plasma ouabain gradually rises to around $1.2 \times 10^{-7}$M (120pm/ml plasma) in 15 to 20 minutes. About this time there is a significant ($p<0.05$) increase in intraventricular pressure and dp/dt as shown in figure 8. With the low dose of ouabain infusion, the plasma ouabain concentration remained at fairly constant level throughout the experimental period. However, the high dose of ouabain infusion raised the plasma concentration of ouabain up to 170pm/ml in 5 minutes after start of ouabain infusion and it continued to increase till the time of lethality, when the plasma ouabain concentration was around $3 \times 10^{-7}$M.

a. The Effect of The Low and High Doses of Ouabain Infusion on The LV dp/dt and RVct.

By pacing the heart 10 to 15 per cent above its intrinsic rate, the EKG
Figure 7. The plasma concentration of ouabain after the low and high doses of ouabain infusion in the paced heart preparation. Vertical bars indicate standard errors. Parentheses indicate number of animals used.
pattern was changed to such an extent that the typical waves (p or t) disappeared. Figure 8 shows a typical example of the effect of the low dose of ouabain infusion in paced heart. In this specific dog the low dose of ouabain raised the systolic and diastolic femoral arterial blood pressure with a significant increase in LV dp/dt. The onset of arrhythmia was noted at 42 minutes after ouabain infusion as shown by the irregular changes in left ventricular pressure. In this dog the magnitude of intraventricular pressure and dp/dt were reduced upon stimulation. This may be due to the force-frequency relationship because the intrinsic heart rate of this dog was high (172 beats per min) and had to stimulate 190 beats per min to suppress the intrinsic rate. This high stimulation rate may reduce the ventricular filling due to a decrease of ejection volume, which may reflect on the decrease of FABP and LV dp/dt.

Figures 9 and 10, and table 7 show the time course of development of positive inotropic effect of the low and high doses of ouabain expressed as the per cent changes in LV dp/dt and RV contractile force. In paced control dogs, both LV dp/dt and RVct decreased about 40 to 60 minutes after an equilibration period. Both the low and high doses of ouabain produced a significant positive inotropism in a short period of time, although in some dogs the arrhythmia was so severe that it was difficult to evaluate quantitatively the positive inotropic effect of ouabain. The high dose of ouabain rapidly induced a significant positive inotropic response than did the low dose of ouabain. It is of interest to see that the quantitative difference in % change in LV dp/dt and RVct, that is, the magnitude of inotropic response expressed in RVct after 60 minutes of ouabain infusion, was a lot greater than that expressed by the change in LV dp/dt. However, with the high dose schedule, per cent change in LV dp/dt decreased after 30 minutes of ouabain infusion and RVct continued to increase till the time of death.

Under the low and high doses of ouabain infusion schedule, the time required to induce various pharmacological effects of ouabain—inotropism, arrhythmia and lethality—is summarized in table 8. Since a constant rate of ouabain infusion either under the low or high dose schedule was applied to each dog after a certain specific loading doses of ouabain, the infusion time
Figure 8. A sample of the effect of the low dose of ouabain on the hemodynamic parameters in the paced dog hearts.
Figure 9. Effect of the low and high doses of ouabain on the right ventricular contractile force in the paced hearts. Vertical bars indicate standard error. Parentheses indicate number of animals used. According to unpaired t-test between ouabain treated group and the control group, a, b, c, d, and e indicate that the p values are smaller than 0.05, 0.025, 0.01, 0.005 and 0.001 respectively.
Figure 10. Effect of the low and high doses of ouabain on the LV dp/dt change in the paced heart preparation. Vertical bars indicate standard error and parentheses indicate number of animals used. See figure 9 for the meanings of the rest of signs.
Table 7. Per cent change in LV dp/dt and RVct after the low and high doses of ouabain infusion in the paced heart preparation.

PER CENT CHANGE IN LV dp/dt AND RV CONTRACTILE TENSION (CT)

<table>
<thead>
<tr>
<th>Dose Schedule</th>
<th>Time in minute after ouabain treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>loading infusion dose</td>
<td>0</td>
</tr>
<tr>
<td>Control dogs</td>
<td>dp/dt 100</td>
</tr>
<tr>
<td></td>
<td>ct 100</td>
</tr>
<tr>
<td>3</td>
<td>dp/dt 100</td>
</tr>
<tr>
<td></td>
<td>ct 100</td>
</tr>
<tr>
<td>8</td>
<td>dp/dt 100</td>
</tr>
<tr>
<td></td>
<td>ct 100</td>
</tr>
</tbody>
</table>

* Six animals were used for control group, see figure 9 for the replication of other experiments.

* % change in LV dp/dt, mean ± standard error

# % change in RV contractile tension, mean ± standard error
Table 8. Time of induction of various effects following administration of the low and high doses of ouabain

<table>
<thead>
<tr>
<th>Dose Schedule</th>
<th>Significant Onset of Induction of inotropism arrhythmia lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading (ug/kg)</td>
<td>Infusion (ug/kg/min)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>n²</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>4</td>
</tr>
</tbody>
</table>

* All values are expressed as mean ± standard error.

© indicates number of animals used.

According to unpaired t-test between the low and high doses of ouabain treated groups, n.s. indicates p values are greater than 0.05 and a, b and d indicate the p values are smaller than 0.05, 0.025 and 0.005 respectively.
of ouabain also allows one to calculate the total amount of ouabain that was infused up to certain period of time. In this table, it is noted that at the time of a significant inotropic response and lethality induced either by the low or high dose of ouabain, the experimental duration of ouabain infusion was different statistically, but the amount of ouabain infused up to that time was not different. Under the same infusion rate of quabain, the induction of a specific effect of ouabain such as inotropism or arrhythmia depends clearly upon the duration of ouabain infusion. Thus, it would appear that a relatively constant amount of ouabain may be necessary to induce a certain pharmacological effect of ouabain irrespective of the rate of administration of ouabain.

b. Ouabain Accumulation in the Major Subcellular Fractions of Dog Myocardium under the Low and High Doses of Ouabain Infusion.

The accumulation of ouabain in the major subcellular fractions of intact dog myocardial tissue is summarized in table 9. In general, the fractions take up more ouabain when the duration of ouabain infusion is increased at a constant rate of ouabain infusion. That is, the longer the duration of ouabain infusion, the greater amount of ouabain is accumulated in all fractions. Since the total experimental period in the paced heart was relatively short, this time dependent ouabain uptake may be the same phenomenon of dose dependency of ouabain uptake because the longer infusion of ouabain means more ouabain being infused to certain animal with the minimal excretion of ouabain. When the low dose of ouabain is exposed for 15 minutes, the mitochondrial fraction has the least ouabain accumulation, which was about the same as that of the supernate. However, in the rest of experimental period mitochondrial fractions accumulate ouabain next to microsomal and NaK ATPase fractions. In all experiments the highest ouabain accumulation was noted in the NaK ATPase fractions without exceptions, while the microsomal fractions are next highest. The supernates accumulate the least ouabain excluding the 15 minute experiment under the low dose of ouabain infusion. Homogenate, pellet, nuclear fractions accumulate similar quantities of ouabain at any time period.

The data from the time dependent ouabain infusion schedule indicate that ouabain uptake processes in all fractions except the NaK ATPase may reach equilibrium about 40 minutes after the low dose infusion of ouabain. This may be particularly true at least for the accumulation process of pellet, nuclei
Table 9. The accumulation of ouabain in major subcellular fractions under the low and high doses of ouabain infusion

<table>
<thead>
<tr>
<th>Dose schedule</th>
<th>Loading dose</th>
<th>3ug/kg</th>
<th>8ug/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion dose</td>
<td>1ug/kg/min</td>
<td>1ug/kg/minute</td>
<td>2ug/kg/minute</td>
</tr>
<tr>
<td>Infusion time (min)</td>
<td>15</td>
<td>40</td>
<td>Avg 84</td>
</tr>
<tr>
<td>Total dose (ug/kg)</td>
<td>18</td>
<td>43</td>
<td>Avg 87</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1.9±0.3#</th>
<th>3.3±0.3b*</th>
<th>4.6±0.8c</th>
<th>5.2±1.1 n.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.2±0.1</td>
<td>1.5±0.1</td>
<td>2.5±0.4b</td>
<td>2.9±0.6 n.s.</td>
</tr>
<tr>
<td>Supernate</td>
<td>2.0±0.3</td>
<td>4.6±1.2</td>
<td>4.9±0.3</td>
<td>5.9±0.8 n.s.</td>
</tr>
<tr>
<td>Pellet</td>
<td>2.5±0.6</td>
<td>4.4±1.1</td>
<td>4.5±0.4b</td>
<td>6.2±0.9 n.s.</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1.1±0.1</td>
<td>5.0±1.9</td>
<td>6.2±1.2</td>
<td>7.0±0.6 n.s.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>4.2±1.6</td>
<td>12.1±2.2a</td>
<td>13.9±0.8d</td>
<td>12.4±3.5 n.s.</td>
</tr>
<tr>
<td>Microsome</td>
<td>6.0±2.8</td>
<td>18.2±4.4</td>
<td>23.9±3.7c</td>
<td>15.9±2.4 n.s.</td>
</tr>
<tr>
<td>LV</td>
<td>262±52</td>
<td>488±33b</td>
<td>725±41e</td>
<td>685±61 n.s.</td>
</tr>
</tbody>
</table>

@ n indicates number of animal used.

# All values are expressed pm ouabain per mg protein, mean±standard error except left ventricle, which is expressed pm ouabain per gram of heart muscle (wet weight).

* According to unpaired t-test against 15 min ouabain treated group, a, b, c, d, and e indicate that p values are smaller than 0.05, 0.025, 0.01, 0.005 and 0.001 respectively. Last column n.s. (non significant) indicates p > 0.05 based on the unpaired t-test between avg. 84 and 46 minutes ouabain treated group.
and mitochondria because after 40 min additional infusion of the low dose of ouabain hardly increase the ouabain content in these fractions. In the case of NaK ATPase fraction, longer infusion of ouabain increased ouabain binding from 18.2 pm/mg protein in 40 min ouabain treated group to 23.9 pm/mg protein for average 84 min ouabain treated group.

Although ouabain binding to NaK ATPase in vitro is reportedly completed in a matter of minutes, in vivo binding to the partially purified NaK ATPase obtained from dog myocardium apparently is a time dependent and slow process. The high dose of ouabain infusion for relatively short period of time allowed NaK ATPase to bind ouabain only 15.9 ± 2.4 pm/mg protein, which may illustrate this slow in vivo binding process of ouabain to this enzyme.

Table 10 shows the ratio of ouabain accumulated in supernate to pellet. Since the pellet was not obtained by a single centrifugation in this study, ouabain content in the pellet was calculated by substracting the ouabain content in supernate from that in homogenate after correcting the ouabain content in vascular bed as the heart was not washed. The low dose of ouabain infusion either for 40 min or average 84 increased the S/P ratio significantly in comparison to that after 15 min ouabain infusion. The high dose of ouabain infusion raised the S/P ratio, but it was not statistically different from the group treated ouabain for average 84 min with the low dose.

There was similarity in the accumulation of ouabain between the two groups which showed similar pharmacological effect, lethality, by infusing the two different doses of ouabain. According to the result of unpaired Student's t-test, the difference in the accumulation of ouabain between these two groups was not significant in the fractions because p values were always greater than 0.05. In time dependent study there are not many differences between 15 min and 40 ouabain treated groups in the accumulation capability of ouabain, although there are statistically significant differences in homogenate, microsome and left ventricular muscle at the level of 5%. Ouabain accumulation in all fractions of average 84 minutes ouabain infused group was significantly different from that of 15 min ouabain infused group in most of the fractions.

c. Effect of the Low and High Doses of Ouabain on the ATPase Activity.

In 6 paced control dogs the total ATPase activity was 17.1 ± 2.0 um pi/mg protein/hr following the 80 minutes of saline infusion. In these control
Table 10. The supernate to pellet ratio of ouabain after the low and high doses of ouabain infusion

<table>
<thead>
<tr>
<th>Ouabain Loading (ug/kg)</th>
<th>Dose (ug/kg/min)</th>
<th>Duration of ouabain infusion (min)</th>
<th>n</th>
<th>S/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>15</td>
<td>3</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>40</td>
<td>4</td>
<td>0.43±0.03a</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Avg. 84</td>
<td>4</td>
<td>0.45±0.06a</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>Avg. 46</td>
<td>3</td>
<td>0.63±0.16n.s.</td>
</tr>
</tbody>
</table>

@ n indicates number of animals used.

* According to unpaired t-test against 15 min ouabain treated group, a indicates that p value is smaller than 0.05.

# According to unpaired t-test against average 84 min ouabain treated group, n. s. indicate p value is greater than 0.05.
experiments NaK ATPase activity which was obtained by subtracting the Mg-dependent ATPase activity from the total ATPase activity was 14.0 ± 1.8 um pi/mg protein/hr as shown in figure 11 and table 11. In comparison to the control ATPase activity, there was no statistically significant inhibition of either the total or the NaK ATPase activities in 3 dogs which were infused the low dose of ouabain for 15 min when the positive inotropic response to ouabain was significant. As the duration of ouabain infusion or the total amount of ouabain infused is increased, the total and NaK ATPase activities were gradually reduced. The highest inhibition of NaK ATPase activity occurred at the time of lethality (p<0.005). The low dose of ouabain infusion for 40 min induced 55.4 percent inhibition of NaK ATPase (p<0.05).

At the time of lethality induced by two different doses of ouabain, the NaK ATPase activity was inhibited to a similar extent because there is no statistical difference in the NaK ATPase activity between the low and high doses of ouabain infused groups. In order to induce the lethal effect, a relatively constant amount of ouabain may be needed. That is, it appears that ouabain progressively gets bound to the NaK ATPase fraction thereby gradually inhibiting the physiological functional role of this enzyme.

Table 11 also shows the ATPase activities in the microsomal fractions. The total ATPase activity in the microsomal fraction is always greater than that of partially purified NaK ATPase. The Mg-dependent ATPase activity in the microsomal fraction is always considerably greater than that of partially purified NaK ATPase. Consequently, NaK ATPase activity in the microsomal fraction which is obtained from the difference between the total ATPase and the Mg-dependent ATPase activity is consistently lower than that of partially purified NaK ATPase. Therefore, the reduction of Mg-dependent ATPase activity without a considerable loss of the total ATPase activity was achieved by the treatment of 2 M LiBr and a series of purification procedures. In the same table, although the ATPase activity of NaK ATPase prepared from the 40 and average 84 minutes ouabain infused groups are significantly different from the control group, the NaK ATPase activity in the microsomal fraction from the control group was not statistically different from other NaK ATPase activities in the microsomal fraction treated with any doses of ouabain for any time of infusion duration of ouabain.
Figure 11. Effect of the low and high doses of ouabain on the total, Mg-dependent and NaK ATPase activities. Vertical bars indicate standard error and parentheses indicate number of animals used. See table 9 for the meaning of the rest of signs.
Table 11. Effect of the low and high doses of ouabain on the total, Mg-dependent and NaK ATPase activities.

<table>
<thead>
<tr>
<th>Treatment of ouabain</th>
<th>Fraction</th>
<th>n@</th>
<th>Total ATPase activity</th>
<th>Mg(^{++}) dependent NaK ATPase activity</th>
<th>% inhibition of NaK ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NaK ATPase</td>
<td>6</td>
<td>17.1±2.0#</td>
<td>3.3±0.6</td>
<td>14.0±1.8</td>
</tr>
<tr>
<td></td>
<td>Microsome</td>
<td>3</td>
<td>17.5±4.1</td>
<td>10.0±1.6</td>
<td>7.5±2.6</td>
</tr>
<tr>
<td>15 min</td>
<td>NaK ATPase</td>
<td>3</td>
<td>15.5±2.8</td>
<td>5.9±0.5</td>
<td>9.5±2.3^n.s.</td>
</tr>
<tr>
<td></td>
<td>Microsome</td>
<td>3</td>
<td>16.6±2.2</td>
<td>10.7±0.4</td>
<td>5.9±1.9^n.s.</td>
</tr>
<tr>
<td>40 min</td>
<td>NaK ATPase</td>
<td>4</td>
<td>11.4±1.9</td>
<td>5.2±0.5</td>
<td>6.3±2.1^a</td>
</tr>
<tr>
<td></td>
<td>Microsome</td>
<td>4</td>
<td>14.2±3.2</td>
<td>11.0±2.5</td>
<td>3.0±1.1^n.s.</td>
</tr>
<tr>
<td>Avg. 84 min</td>
<td>NaK ATPase</td>
<td>5</td>
<td>8.0±1.6</td>
<td>4.6±0.5</td>
<td>3.4±1.3^d</td>
</tr>
<tr>
<td></td>
<td>Microsome</td>
<td>5</td>
<td>11.8±1.4</td>
<td>9.2±1.7</td>
<td>2.6±0.7^n.s.</td>
</tr>
<tr>
<td>Avg. 46 min</td>
<td>NaK ATPase</td>
<td>4</td>
<td>9.7±0.8</td>
<td>4.6±0.2</td>
<td>4.8±0.8^n.s.*</td>
</tr>
<tr>
<td></td>
<td>Microsome</td>
<td>4</td>
<td>11.6±1.5</td>
<td>8.1±0.6</td>
<td>3.6±0.3^n.s.*</td>
</tr>
</tbody>
</table>

@ indicates number of animal used.

\# Each value is expressed in um pi released per mg protein per hour, mean ± standard error.

* based on unpaired t-test against average 84 min ouabain treated group, and all other symbols or signs were used as indicated in table 9.
d. Effect of the Low and High Doses of Ouabain on the Concentration of Plasma Sodium and Potassium.

Since the primary action of NaK ATPase is the active transport of monovalent ions, chiefly Na and K, it is expected that any significant inhibition of the NaK ATPase activity should be reflected in the plasma Na and K concentrations. With this idea in mind, the effect of the low and high doses of ouabain on the concentration of plasma Na and K was studied. Table 12 and figures 12 and 13 show the results. Plasma Na and K concentrations were 139 ± 0.9 and 3.2 ± 0.1 meq/l respectively at zero time in 15 control animals. There was no significant change in either K or Na concentration in plasma at any time interval throughout the experimental period. Neither the low nor high dose of ouabain infusion affected significantly the plasma Na concentration during the entire experimental period excluding the plasma Na concentration at 40 min after the high dose of ouabain infusion. At that time plasma Na concentration was 121 ± 4.4 meq/l in 3 animals. This one point was statistically significantly different from the control at the level of 5%.

The low dose of ouabain infusion did not increase the plasma concentration of K up to 40 min after ouabain treatment. Following this a continuous infusion of the low dose of ouabain raised the plasma concentration of K from the control 3.2 to 4.0 meq/l at 60 min after the ouabain infusion. This effect was pronounced with the high dose of ouabain infusion schedule, which raised the plasma K concentration up to 5.6 meq/l at 40 min after the high dose of ouabain infusion (p<0.001). Changes in the plasma K and Na concentrations after ouabain infusion may be difficult to interpret in terms of the activity of myocardial NaK ATPase because the plasma K and Na concentrations reflect the overall K and Na metabolism of the whole animal body. However, it is not surprising to see the similar increase of plasma K and decrease of Na at the time of lethality induced by either the low or high dose of ouabain, which caused more than 65% inhibition of NaK ATPase activity.

e. Interrelationship between the Major Myocardial Parameters in Relation to the Development of LV dp/dt Induced by Ouabain in the Paced Heart Preparations.

The main objective of this study was to investigate how the various biochemical parameters such as ouabain binding to the NaK ATPase, its inhibition and the plasma K concentration relate to the development of the left ventricular dp/dt due to ouabain in the paced intact dogs. It is noted that in control animals where there is no ouabain present, the NaK ATPase activity was high
Table 12. Effect of the low and high doses of ouabain on the plasma concentrations of potassium and sodium ions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time in minute after ouabain treatment</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>3.2±0.1</td>
<td>2.9±0.2</td>
<td>2.8±0.3</td>
<td>2.8±0.4</td>
<td>2.9±0.15</td>
<td>2.7±0.3</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Plasma K⁺ (meq/l)</td>
<td>Low dose (3/1 ug)</td>
<td>3.2±0.1</td>
<td>3.4±0.3</td>
<td>3.4±0.2</td>
<td>3.5±0.3</td>
<td>3.5±0.3</td>
<td>4.0±0.3a</td>
<td>4.7±0.3b</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>High dose (8/2 ug)</td>
<td></td>
<td>3.2±0.1</td>
<td>3.4±0.05</td>
<td>3.9±0.1b</td>
<td>4.5±0.3b</td>
<td>5.6±0.2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>139±0.9</td>
<td>140±4.5</td>
<td>139±2.5</td>
<td>139±1.5</td>
<td>140± -</td>
<td>138±6.5</td>
<td>139±6.9</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Plasma Na⁺ (meq/l)</td>
<td>Low dose (3/1 ug)</td>
<td>139±0.9</td>
<td>137±4.5</td>
<td>136±6.3</td>
<td>135±5.8</td>
<td>134±6.6</td>
<td>128±2.8</td>
<td>126±1.8</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>High dose (8/2 ug)</td>
<td></td>
<td>139±0.9</td>
<td>134±3.6</td>
<td>131±3.4</td>
<td>129±4.2</td>
<td>121±4.4a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>15</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

@ Each value represents at least three sample readings, mean ± standard error.

# Number of animal used.

* P < 0.05, based on the t-test computing against the value of 80 minutes ouabain treated group under the low dose of ouabain infusion schedule and all other symbols are used as indicated in table 9.
Figure 12. Effect of the low and high doses of ouabain on the plasma concentration of sodium ion. Vertical bars and parentheses indicate standard error and number of animals used respectively. According to unpaired t-test against the control group, a indicates p is smaller than 0.05.
Figure 13. Effect of the low and high doses of ouabain on the plasma concentration of K⁺. Vertical bars and parentheses indicate standard error and number of animals used respectively. See table 9 for the meaning of the rest of signs.
and the inotropism was reduced over the 80 minute-period of saline infusion with respect to its own control period at equilibrium. As the duration of ouabain infusion is prolonged, that is, an increased amount of ouabain is administered, ouabain binding to the NaK ATPase is gradually enhanced with a progressive decrease of NaK ATPase activity. Along with these changes, an inotropic response to ouabain is increased in a roughly parallel fashion with the increment of ouabain binding to the NaK ATPase. But this was true up to 40 minutes of ouabain infusion because further additional average 44 minutes of ouabain infusion did not increase the positive inotropism, although the inhibition of NaK ATPase and ouabain binding to this enzyme were still increased. It should be born in mind the fact that 40 min and average 84 after the initiation of ouabain infusion were about the time to onset of arrhythmia and lethality, respectively. Up to 40 min after the initiation of ouabain infusion there was no drastic change in plasma K concentration. Following this, plasma K concentration is rapidly increased along with the development of arrhythmia until the lethal point.

Table 13 shows the comparison of the major myocardial parameters obtained from the two groups at the time of lethality induced either by the low or the high dose of ouabain. Effects of the low and high doses of ouabain infusion on the major myocardial changes in the paced heart preparation are summarized in table 14. Figure 14 shows diagrammatically the time dependent ouabain effect.

In the paced hearts, the low and high doses of ouabain induced a considerable changes in many parameters measured in this study. Since the main theme of this study is to test the claimed hypothesis that the inhibition of the NaK ATPase activity induced by ouabain is causally related to the inotropic response to ouabain, correlation studies between these two parameters and/or the analysis of variance for the test of the property of the regression coefficient was found useful to draw a meaningful conclusion. All data obtained from the 22 paced heart-experiments were pooled and, utilizing the biomedical computer program (BMD02D program, UCLA, Calif.), correlation has been made between the 5 variables—the total experimental period, total dose of ouabain, ouabain binding to the NaK ATPase, NaK ATPase activity and the inotropic response to ouabain. Variance-covariance and correlation matrixes
Table 13. Comparison of the major myocardial parameters at the time of lethality induced by the low and high doses of ouabain.

<table>
<thead>
<tr>
<th>Ouabain treatment</th>
<th>@ Total Duration of Ouabain takeup</th>
<th>LV Microsome NaKATPase binding</th>
<th>NaKATPase activity</th>
<th>P,K</th>
<th>P,Na</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ug/kg) (min) (pm/ml) (pm/mg) (pm/mg)</td>
<td>(pm/mg) (pm/mg) (pm/mg)</td>
<td>(pm/mg) (pm/mg) (pm/mg)</td>
<td>(pm/mg) (pm/mg) (pm/mg)</td>
<td>(um pi/mg) (meq/l) (meq/l)</td>
</tr>
<tr>
<td>Control</td>
<td>6 0 80</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>14.0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>±1.2  ±0.1  ±1.7</td>
<td>±3.4  ±0.8  ±3.7</td>
<td>±1.3  ±0.3  ±1.3</td>
<td>±1.3  ±0.3  ±1.3</td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>5 87 84</td>
<td>123 123</td>
<td>725 13.9</td>
<td>23.9</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>±6.7  ±6.8</td>
<td>±12  ±41</td>
<td>±0.8  ±3.7</td>
<td>±1.3  ±0.3  ±1.3</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>4 100,n.s. 46,d</td>
<td>307,d 307,d</td>
<td>685,n.s. 12.4,n.s.</td>
<td>15.9,n.s. 4.8,n.s.</td>
<td>5.6,a 121,a</td>
</tr>
<tr>
<td></td>
<td>±9.0  ±4.5</td>
<td>±35  ±61</td>
<td>±3.5  ±2.4</td>
<td>±0.6  ±0.2  ±4.4</td>
<td></td>
</tr>
</tbody>
</table>

@ n indicates number of animals used.
* All values are expressed in appropriate units, mean ± standard error.
# Unpaired t-test of this group was done against the low dose of ouabain treated group. See table 9 for the implication of other symbols.
Table 14. Effects of the low and high doses of ouabain infusion on the major myocardial parameters.

<table>
<thead>
<tr>
<th>Ouabain treatment</th>
<th>Ouabain LV uptake (pm/ml)</th>
<th>Ouabain uptake (ug/kg heart)</th>
<th>Microsomal NaK ATPase activity (um pi/mg/hr)</th>
<th>NaK ATPase (um pi/mg/hr)</th>
<th>LV dp/dt</th>
<th>RV ct</th>
<th>Plasma K+ (meq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14.0 +2.0 ± 9 +0.9 ± 0.1</td>
</tr>
<tr>
<td>15 min</td>
<td>116 ±9</td>
<td>18 ±52</td>
<td>4.2 ± 1.6</td>
<td>6.0 ± 2.8</td>
<td>9.5 n.s.</td>
<td>116a 108g 3.2 n.s.</td>
<td></td>
</tr>
<tr>
<td>40 min</td>
<td>121 n.s. ±10</td>
<td>43e ±33</td>
<td>12.1a ±2.1</td>
<td>18.2 n.s. 6.3a</td>
<td>128c 125g 3.5 n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. 84 min</td>
<td>123 n.s. ±12</td>
<td>87e ±66</td>
<td>13.9d ±0.3</td>
<td>23.9c ±3.7</td>
<td>3.4d 132c 171g 4.7e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. 46 min</td>
<td>307 n.s. ±35</td>
<td>100 n.s. ±61</td>
<td>12.4 n.s. 15.9 n.s. 4.8 n.s. 158 n.s. 130e 5.6 n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. All values are expressed in appropriate units, mean ± standard error.
2. In case there is no control value, unpaired t-test was made against the value obtained from 15 minutes ouabain treated group.
3. The t-test of this group was made against average 84 minute ouabain treated group.
4. See table 9 for the implication of all other symbols.
Figure 14. The time dependent effect of ouabain infusion on the ouabain binding to the NaK ATPase, its activity, LV dp/dt and plasma K concentration in the paced heart preparations. Vertical bars and parentheses indicate standard error and number of animals used respectively. See table 9 for the meaning of other signs.
obtained from these five variables are shown in Table 15. Since the correlation matrix is calculated mathematically from the variance-covariance matrix by computing the covariances when the variance is normalized to unity, immediate information regarding the relationship between these variables can be obtained directly from the correlation matrix. Column 1, the experimental duration, is not related to any other variables. Total dose of ouabain (column 2) infused up to that time is significantly correlated with ouabain binding to the NaK ATPase, its ATPase activity and the inotropic response to ouabain. Ouabain binding to the NaK ATPase (column 3) is inversely related to NaK ATPase activity and directly correlated to the development of LV dp/dt. A significant correlation was found between the NaK ATPase activity and the inotropic response induced by ouabain in the experiments which were designed to produce various pharmacological effects of ouabain in a short period of time by infusing a high dose of ouabain.

In order to test the hypothesis that the regression coefficient (b, the slope of the regression line) between these variables is zero, a F test has been made by analyzing the variances (used the Omnitab fit command, Iowa State Univ). Since the F value obtained from the relationship between the inotropic response vs. the NaK ATPase activity was 9.89 which is greater than 8.1 (F_{1,20} = 8.096 at 1%), the hypothesis that the regression coefficient between the inotropic response and the NaK ATPase activity, b = 0, is rejected at 1% level. Likewise, in the case of the regression between NaK ATPase activity vs. ouabain binding to the enzyme and the inotropic response vs. ouabain binding to the NaK ATPase, the same hypothesis is rejected (p is less than 0.01).

Figure 15 shows the regression line between NaK ATPase activity and ouabain binding to the NaK ATPase obtained by the least squares method. The 22 points are plotted as scatter diagram from the 22 paced heart experiments under the low and high doses of ouabain infusion along with the regression point. In the same figure, the regression line between NaK ATPase activity in the microsomal fraction and ouabain binding to the fraction is also plotted along with 17 individual observations as scatter diagram. The two regression lines are paralleled and the regression coefficient, b, is similar, although the intercepts are different. The regression line between the inotropic response to ouabain and the NaK ATPase activity is plotted on figure 16.
Table 15. Correlations between the major myocardial parameters and the analysis of variance in the paced heart preparations.

**VARIANCE-COVARIANCE MATRIX**

<table>
<thead>
<tr>
<th>Experimental duration (min)</th>
<th>Total dose of ouabain (ug/kg)</th>
<th>Ouabain binding to NaK ATPase activity (pm/mg protein)</th>
<th>NaK ATPase activity (um pi/mg/hr)</th>
<th>Inotropic response (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>707.2031</td>
<td>88.7165</td>
<td>0.0595</td>
<td>12.1105</td>
</tr>
<tr>
<td>2</td>
<td>88.7165</td>
<td>1758.3730</td>
<td>333.5488</td>
<td>-162.9410</td>
</tr>
<tr>
<td>3</td>
<td>0.0595</td>
<td>333.5488</td>
<td>119.1396</td>
<td>-31.0963</td>
</tr>
<tr>
<td>4</td>
<td>12.1105</td>
<td>-162.9410</td>
<td>-31.0963</td>
<td>30.1135</td>
</tr>
<tr>
<td>5</td>
<td>-230.5383</td>
<td>986.8059</td>
<td>196.8212</td>
<td>-103.8961</td>
</tr>
</tbody>
</table>

**CORRELATION MATRIX**

<table>
<thead>
<tr>
<th>COL.</th>
<th>COL.</th>
<th>COL.</th>
<th>COL.</th>
<th>COL.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0000</td>
<td>0.0796</td>
<td>0.0002</td>
<td>0.0830</td>
</tr>
<tr>
<td>2</td>
<td>0.0796</td>
<td>1.0000</td>
<td>0.7287*</td>
<td>-0.7077</td>
</tr>
<tr>
<td>3</td>
<td>0.0002</td>
<td>0.7287*</td>
<td>1.0000</td>
<td>-0.5192</td>
</tr>
<tr>
<td>4</td>
<td>0.0830</td>
<td>-0.7077</td>
<td>-0.5192*</td>
<td>1.0000</td>
</tr>
<tr>
<td>5</td>
<td>-0.2634</td>
<td>0.7151*</td>
<td>0.5479*</td>
<td>-0.5753*</td>
</tr>
</tbody>
</table>

**ANALYSIS OF VARIANCE**

**REGRESSION COEFFICIENT**

<table>
<thead>
<tr>
<th>NaK ATPase activity vs. ouabain binding to NaK ATPase</th>
<th>a</th>
<th>b</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.16</td>
<td>-0.26</td>
<td>7.38</td>
<td>&lt;0.025</td>
<td></td>
</tr>
<tr>
<td>1.58</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inotropic response vs. NaK ATPase activity</th>
<th>a</th>
<th>b</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>145.71</td>
<td>-3.45</td>
<td>9.89</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>10.48</td>
<td>1.10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inotropic response vs. ouabain binding to NaK ATPase</th>
<th>a</th>
<th>b</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>97.84</td>
<td>1.65</td>
<td>8.58</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>9.24</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n indicates number of animals used.
* indicates p is smaller than 0.05.
Experiments with the paced hearts

- NaK ATPase activity (um pi/mg protein/hr)
- Microsome

Experiments with the paced hearts

- NaK ATPase
  \[ y = 11.16 - 0.26x \]
  \[ r = -0.5192 \]
  \[ n = 22 \]
- Microsome
  \[ y = 6.61 - 0.24x \]
  \[ r = -0.5045 \]
  \[ n = 17 \]

Figure 15. Correlations of the cardiac NaK ATPase activity in the NaK ATPase and the microsomal fractions with their binding to ouabain. The orthogonal regression lines were calculated by least squares method. See the text for the explanations.
Figure 16. Correlation between the inotropic response to ouabain and the NaK ATPase activity. The orthogonal regression line was calculated by least squares method from 22 paced dog experiments. The big dot indicates the regression point.
2. EXPERIMENTS WITH THE SPONTANEOUSLY BEATING HEART PREPARATION.

In previous experiment with the paced heart preparation, the greatest inhibition of the NaK ATPase activity was observed at the time of lethality induced by either the low or high doses of ouabain infusion and the least effect on the activity of NaK ATPase was noted on administration of the therapeutic dose of ouabain. Since the inotropic effect with these dose-schedules was only transitory and was converted with further administration of ouabain into the toxic effect, it was necessary to determine the relationship between the inotropic effect and the NaK ATPase activity using a dose regimen that maintains an inotropic response to ouabain without leading to any toxic symptoms for a sufficient experimental period. As shown in table 3, the inotropic dose of ouabain (loading dose, 20ug/kg; infusion dose, 0.036 ug/kg/min) produced a significant positive inotropy within 10 minutes and maintained it fairly well up to 300 minute experimental duration without any occurrence of arrhythmia in 5 animals. Doubling the loading and infusion doses—designated as the arrhythmic dose—induced persistent arrhythmias within 60 min and the arrhythmia continued to the termination of experimental period (300 min) without leading to lethality in 5 dogs.

a. Effect of the Inotropic and Arrhythmic Doses of Ouabain Alone or with either KCl or DPH on the Heart Rate.

Since relatively high loading doses of ouabain were used in this group of experiments, it was of great concern whether the treatment of ouabain alone or in combination with either DPH or KCl would affect on heart rate to such an extent that it might be difficult to evaluate an inotropic response to ouabain. The effect of the inotropic and arrhythmic doses of ouabain on the change of heart rate is summarized in table 16 and figure 17. The heart rate was determined by measuring the RR interval when it was appropriate. After the development of arrhythmia, the heart rate was determined by counting the total number of beats for at least 6 seconds and expressed as per cent change of the control.

In 5 control dogs the heart rate did not change statistically significantly over the 300 minute experimental period. The inotropic dose of ouabain also did not affect heart rate significantly. Practically there was no great fluctuation in heart rate from the control period to 300 min experimental period. However, the arrhythmic dose of ouabain induced a slight positive
Table 16. Effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the heart rate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time in minute after ouabain treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Inotropic dose</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>Arrhythmic dose</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>Arrhythmic dose + DPH</td>
<td></td>
</tr>
<tr>
<td>Arrhythmic dose + KCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

@ all values are expressed in per cent change of heart rate to that of control period, mean ± standard error.

# a indicates that P is lesser than 0.05 based on unpaired t-test between the control and arrhythmic doses of ouabain treated groups.

* see the descriptions under Materials and Method for the doses of ouabain, DPH and KCl.
Figure 17. Effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the heart rate. Vertical bars indicate standard error and parentheses indicate number of animals used. See table 9 for the meaning of other signs.
chronotropic effect within 30 minutes after the ouabain administration. This chronotropic effect of ouabain appeared not statistically significant except about 180 and 300 min after the injection of the arrhythmic dose, when the p was barely less than 0.05.

As will be presented later, with the arrhythmic dose of ouabain various types of arrhythmia were induced. However, the arrhythmia was usually mild so that quantitative evaluation of the inotropic response to ouabain was possible. At 180 min after the start of the arrhythmic dose of ouabain infusion, either KCl or DPH was used to counteract the arrhythmia induced by ouabain. Neither continuous infusion of KCl(1.2 meq/kg/hr) nor DPH(see the description under "Materials and Method" for the DPH dosage) significantly affected the heart rate.

b. The Plasma Concentration of Ouabain After the Inotropic and Arrhythmic Doses of Ouabain Infusion Alone or with either KCl or DPH.

The plasma ouabain concentration was rapidly increased by the loading inotropic and arrhythmic doses of ouabain administration in a few minutes. Following this period it declined gradually until a relatively constant plasma concentration of ouabain was obtained within 40 min to 50 after the start of ouabain maintenance infusion. The steady-state plasma concentrations of ouabain were approximately $10^{-8}$ M with the inotropic dose and $3\times10^{-8}$ M with the arrhythmic dose of ouabain. Both of these plasma concentrations were well maintained throughout the 300 min experimental period with very little fluctuation. Table 17 and figure 18 show the pattern of plasma concentration of ouabain after the inotropic or arrhythmic dose of ouabain treatment. With the arrhythmic infusion dose of ouabain, the plasma concentration of ouabain after KCl or DPH treatment did not change.

c. Effect of the Inotropic and Arrhythmic Doses of Ouabain Alone or with either KCl or DPH on the development of LV dp/dt and RVct.

Effect of the inotropic and arrhythmic doses of ouabain on the LV dp/dt and RVct is summarized in figure 19 and table 18. In 15 control dogs both dp/dt of left ventricular pressure and RV contractile tension did not fall significantly until 60 min after the onset of infusion of saline. Following this period, these parameters gradually fell so that at the time of termination of the experiment(300 min) the LV dp/dt and RVct were about 79% and 76% of their respective control values at $t_0$. In general, it appeared that the rate
Table 17. The plasma concentration of ouabain after the inotropic or arrhythmic dose of ouabain alone or either KCl or DPH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>40</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>210</th>
<th>240</th>
<th>270</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inotropic dose</td>
<td>0</td>
<td>33*</td>
<td>±3.3</td>
<td>±0.9</td>
<td>±1.2</td>
<td>±1.0</td>
<td>±1.0</td>
<td>±1.1</td>
<td>±0.7</td>
<td>±0.8</td>
<td>±0.9</td>
</tr>
<tr>
<td>Arrhythmic dose</td>
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<td>±5.6</td>
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<td>±2.1</td>
<td>±1.5</td>
<td>±2.4</td>
<td>±2.8</td>
<td>±3.0</td>
<td>±4.9</td>
<td>±5.9</td>
</tr>
<tr>
<td>Arrhythmic dose</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(DPH)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All values are expressed in picomoles of ouabain per ml of plasma, mean ± standard error.
Figure 18. The time course changes in the plasma ouabain concentration after the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH. Vertical bars and parentheses indicate standard error and number of animals used respectively. See table 9 for the meaning of other signs.
Figure 19. Effect of the inotropic and arrhythmia doses of ouabain alone or with either KCl or DPH on the development of LV dp/dt and RVct. Vertical bars and parentheses indicate standard errors and numbers of animals used respectively. See Table 9 for the meaning of other signs.
Table 18. Effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the development of LV dp/dt and RVct.

Time in minute after treatment of drugs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>210</th>
<th>240</th>
<th>270</th>
<th>300</th>
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</thead>
<tbody>
<tr>
<td>Control dp/dt</td>
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<td>100</td>
<td>96</td>
<td>99</td>
<td>93</td>
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<td>95</td>
<td>85</td>
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<td>84</td>
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<td></td>
<td></td>
<td>±2.0</td>
<td>±2.6</td>
<td>±3.5</td>
<td>±1.5</td>
<td>±0.6</td>
<td>±9.6</td>
<td>±7.6</td>
<td>±10</td>
<td>±6.6</td>
</tr>
<tr>
<td>RVct</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>97</td>
<td>99</td>
<td>97</td>
<td>93</td>
<td>88</td>
<td>84</td>
<td>80</td>
</tr>
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<td>±1.8</td>
<td>±1.6</td>
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<td>±10</td>
<td>±8.6</td>
<td>±9.0</td>
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<tr>
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<td>122</td>
<td>118</td>
<td>117</td>
<td>122</td>
<td>115</td>
</tr>
<tr>
<td>dp/dt</td>
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<td>121</td>
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<td>±5.5</td>
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<td>±4.8</td>
<td>±6.2</td>
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<td>±16</td>
<td>±18</td>
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<td>±3.8</td>
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<td></td>
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<td>100</td>
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</tr>
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<td>±5.0</td>
<td>±15</td>
<td>±21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are expressed in % change in LV dp/dt or RVct with respect to their control values at t₀.
of fall of RVct was a little faster than that of LV dp/dt with respect to
time. Also it can be further noted that the spontaneously beating heart
preparations used in this study deteriorated more slowly than the paced
pearts used in the previous experiments (compare table 18 with 7). The
upper two panels of figure 20 show an example of the changes in LV dp/dt
and LWP of intrinsically beating control dog.

Eight dogs were used for the study of the inotropic dose of ouabain,
but three of them were sacrificed at 120 min instead of 300 min to
determine the effect of infusion time on the accumulation of ouabain under
the steady-state condition of inotropy. In the remaining 5 dogs in which
the experiment continued to 300 min, the inotropic response to ouabain,
evaluated by both LV dp/dt and RVct, was significantly greater at all
time intervals comparing to the control preparations (see the lower 4
panels of figure 20). In these 5 dogs the inotropic response of ouabain
was well maintained throughout the 300 min experimental duration without
leading to arrhythmia. It was of particular interest that the inotropic
response measured by RVct was fluctuated more than that assessed by the
change in LV dp/dt in the same dog.

With the arrhythmic dose of ouabain both dp/dt and RVct increased
rapidly. In 13 dogs various types of arrhythmia were induced within 60
min following the start of ouabain infusion. The most prevalent type of
arrhythmia was ventricular premature beats. A prolongation of PR
interval (longer than 200 msec) was produced in some dogs and second or
third degree atrioventricular block was not unusual. A burst of
unidirectional ventricular extrasystolic activity in many cases was
followed by multifocal ventricular premature beats as shown in figure 22.
In most cases the arrhythmia induced by ouabain was not severe enough to
interfere with the measurement of the inotropic response of ouabain.
Initially, with the inotropic dose of ouabain both LV dp/dt and RVct
developed rapidly in approximately parallel fashion. However, after the
induction of arrhythmia RVct continued to increase while LV dp/dt gradually
fell as seen in table 18 and figure 19.

A typical response to the arrhythmic dose of ouabain is shown in figure
21. In this specific experiment a significant positive inotropic response
was observed within 8 minutes after ouabain treatment, resulting a slight
Figure 20. Effect of the control saline infusion and the inotropic dose of ouabain infusion on the change or development of LV dp/dt or RVct. Numbers at bottom indicate time in minute after equilibrium period.
Figure 21. A sample of the effect of the arrhythmic dose of ouabain on the myocardial hemodynamic parameters. Numbers at bottom indicate time in minute after ouabain treatment.
increase in the femoral arterial blood pressure. Onset of arrhythmia was induced in 57 min after the ouabain administration. There was no significant changes in the end diastolic pressure (EDP) throughout the 300 min experimental period as shown in the bottom panel of figure 21, which observation was also similar to the control and the inotropic dose of ouabain treated groups.

In order to determine the effect of KCl or DPH on the ouabain-induced arrhythmia, in 8 dogs either KCl or DPH was administered along with the arrhythmic dose of ouabain for the rest of experimental period(120 min) after the confirmation of the persistency of the arrhythmia up to 180 min. Both KCl and DPH were very effective in counteracting the arrhythmia induced by ouabain. A typical example of the antiarrhythmic effect of KCl is shown in figure 22. In this specific dog the normal sinus rhythm was back at 212 min after the beginning of experiment, which took 32 minutes after KCl infusion and thereafter remained free from the arrhythmia by a continuous infusion of KCl up to the termination of experiment. It was of interest to observe the process of electrocardiographic pattern changes back toward normal sinus rhythm after a slow KCl infusion because the process looks like the reversion of the induction of arrhythmia by ouabain in many cases. That is, for a while after KCl infusion began, there were no notable changes in EKG. As more KCl was infused, the burst of ventricular extrasystolic activity reduced along with a decrease of dropped beats and gradually the normal sinus rhythm was re-established.

In this study continuous infusion of KCl with the maintenance dose of ouabain initially increased significantly the RVct after reversion of the persistent arrhythmia induced by ouabain as shown in figure 19. However, near the termination of experiment the RVct fell gradually as the increase of plasma K concentration as indicated in figure 25. DPH did not increase either the LV dp/dt and RVct in 4 animals. The antiarrhythmic effect of DPH was more rapid as shown in figure 23. After 8 mg per kg of DPH as a loading dose, the antiarrhythmic effect of DPH usually could be induced in 4 to 5 minutes. The effect of DPH was so sudden that sometimes it was difficult to make an EKG recording on expanded scale. Extrasystolic activity was reduced along with the decrease of EKG amplitude. Ventricular premature beats might reappear occasionally for a while. In
Figure 22. A sample of the effect of the arrhythmic dose of ouabain and the antiarrhythmic effect of KCl on the myocardial hemodynamic parameters. Numbers at bottom indicate time in minute after drug treatment.
Figure 23: A sample of the effect of the arrhythmic dose of ouabain and the antiarrhythmic effect of DPH on the myocardial hemodynamic parameters. Numbers at bottom indicate time in minute after drug treatment.
this dog the ventricular tachycardia was induced after 37 minutes after ouabain treatment, which basically continued up to 180 min when DPH reversed the arrhythmia back to normal sinus rhythm in 6 min after DPH administration. Even after the reversion of arrhythmia back to normal sinus rhythm, in many cases the electrocardiographic pattern was not alway exactly the same as control one. It was also necessary to continuously administer either KCl or DPH because in pilot experiment arrhythmias reappeared when antiarrhythmic drug administration was stopped.

Table 19 summarizes the time of ouabain infusion required to produce a specific pharmacological effect of ouabain using the inotropic and arrhythmic doses of ouabain. This table also shows the total amount of ouabain infused up to that time. The time required to induce a significant (p<0.05) inotropic response of ouabain was 9.4 ± 0.6 minutes and the total dose of ouabain infused up to that time was 20.3 ± 0.1 ug/kg using the inotropic dose schedule. With the arrhythmic dose of ouabain both dp/dt and RVct were increased significantly within 6.3 ± 0.7 minutes in 13 animals and 30.2 ± 4.2 minutes were required to induce the arrhythmia. The time required to reverse arrhythmia back to normal sinus rhythm after DPH treatment was 4.7 ± 0.4 minutes in 4 dogs. However, since a continuous KCl infusion was made without a loading dose of KCl, 64.5 ± 8.8 minutes of KCl infusion were required to reverse back to normal sinus rhythm in 4 animals.

d. Effect of the Inotropic and Arrhythmic Doses of Ouabain Alone or either with KCl or DPH on the Plasma Concentration of K and Na.

The effect of the inotropic and arrhythmic doses of ouabain on the plasma Na and K concentrations is summarized in figures 24 and 25. In 25 control animals the plasma concentration of Na and K were 141 ± 0.6 and 3.5 ± 0.1 meq/l, respectively. Throughout the 300 minute experimental period there was practically no change in either plasma K or Na concentration in control dogs. Neither the inotropic nor arrhythmic dose of ouabain significantly affected the plasma K or Na concentration with respect to increase in the duration of ouabain infusion. However, an exception was the plasma concentration of K in the arrhythmic group at the time near the termination of the experiment, which appeared statistically significant compared to control group at a comparable time.
Table 19. Time to a significant positive inotropism, onset of arrhythmia and the reversion of arrhythmia back to regular rhythm after either KCl or DPH treatment.

<table>
<thead>
<tr>
<th>Treatment of ouabain, DPH or KCl</th>
<th>Positive inotropy</th>
<th>Onset of arrhythmia</th>
<th>Antiarrhythmic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Dose (ug/kg)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>Inotropic dose ±0.6 ±0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n3</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Arrhythmic dose ±0.7 ±0.1</td>
<td>6.3 ±0.7</td>
<td>40.4 ±0.1</td>
<td>30.2 ±4.2</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

Arrhythmic dose + DPH or KCl

| n | 4  | 4 |

@ indicates number of animals used.

# All values are expressed in indicated units, mean ± standard error.

* see the descriptions under "Materials and Method" for the doses of ouabain, DPH and KCl.
Figure 24. Effect of the inotropic and arrhythmic doses of ouabain alone or with either DPH or KCl on the plasma concentration of Na⁺. Vertical bars and parentheses indicate standard error and number of animals used respectively.
Figure 25. Effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the plasma concentration of K⁺. Vertical bars and parentheses indicate standard error and number of animals used, respectively. See table 9 for the meaning of other symbols.
The arrhythmic dose of ouabain infusion in combination with either KCl or DPH also did not affect on the plasma concentration of Na at any time period. As expected from the results of KCl infusion at the rate of 1.2 meq/kg/hr, the plasma K concentration rose rapidly. Within 30 minutes after KCl infusion the plasma K concentration was significantly elevated to a value of 5 meq/l. It was not until the plasma concentration of K reached approximately 6.5 meq/l that the antiarrhythmic effect was evident. Near 300 minutes (120 min after KCl infusion) after the arrhythmic dose of ouabain infusion along with the KCl, the plasma concentration of K was approximately 8 meq/l and at that time myocardial contractility in general was somewhat depressed.

e. Effect of the Inotropic and Arrhythmic Doses of Ouabain Alone or with either KCl or DPH on the Accumulation of Ouabain.

The accumulation of ouabain using the inotropic and arrhythmic doses of ouabain and the effect of KCl and DPH on the accumulation process are summarized in table 20 and figure 26. As in the case of the paced heart-experiments, there was a general trend that the longer the duration of ouabain infusion, the greater accumulation of ouabain in most of the fractions of the heart. The highest ouabain accumulation was noted in the NaK ATPase fraction and in most cases the supernates accumulated the least quantity of ouabain. With the inotropic dose of ouabain, the accumulation of ouabain in the fractions after 300 min of ouabain infusion was not significantly different from that of 120 min ouabain treated group, though the mean values were greater for LV and for microsomal fraction. This may suggest that equilibrium may have virtually been completed at 120 minutes using the inotropic dose.

With the arrhythmic dose of ouabain infusion, most of the fractions took up more ouabain, so that the ouabain accumulation was significantly different from that of the inotropic dose of ouabain. Particularly, the NaK ATPase and the microsomal fraction accumulated about 3 fold more than with the inotropic dose. As shown in table 17, with the arrhythmic dose of ouabain the plasma ouabain level(28pm/ml) was about three times that of the inotropic dose of ouabain(9pm/ml). This may indicate that the uptake of ouabain by the heart and its subcellular fractions have been affected by the three fold higher plasma concentration.

Ouabain accumulation in most subcellular fractions in the presence of DPH used in this study appears to be not significantly different from
Table 20. The accumulation of ouabain in the major myocardial subcellular fractions under the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH.

<table>
<thead>
<tr>
<th>Treatment of ouabain</th>
<th>LV^2</th>
<th>Homogenate</th>
<th>Supernate</th>
<th>Pellet</th>
<th>Nuclei</th>
<th>Mitochondria</th>
<th>Microsome</th>
<th>NaK ATPase</th>
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</thead>
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<tr>
<td>Inotropic dose, 120min</td>
<td>176</td>
<td>1.5</td>
<td>0.5</td>
<td>1.3</td>
<td>1.4</td>
<td>0.9</td>
<td>1.7</td>
<td>3.6</td>
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<tr>
<td></td>
<td>96</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
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<td>0.4</td>
</tr>
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<td>Inotropic dose, 300min</td>
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<td>0.7</td>
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<td>1.9</td>
<td>1.5</td>
<td>2.9</td>
<td>4.7</td>
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<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Arrhythmic dose, 300min</td>
<td>669^*</td>
<td>3.6^a</td>
<td>1.8^a</td>
<td>3.4</td>
<td>3.4^b</td>
<td>2.9^a</td>
<td>9.2^b</td>
<td>13.6^e</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Arrhythmic dose, DPH</td>
<td>587</td>
<td>3.6</td>
<td>3.0^b</td>
<td>3.8</td>
<td>3.8</td>
<td>2.2</td>
<td>7.7</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Arrhythmic dose, KCl</td>
<td>577</td>
<td>2.9</td>
<td>2.3</td>
<td>2.9</td>
<td>2.5</td>
<td>2.2</td>
<td>6.6</td>
<td>9.3^a*</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>1.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

@ expressed pm ouabain per g of heart (wet weight), and all other values are expressed as pm ouabain per mg protein, mean ± standard error.

# Unpaired t-test was made against the ouabain treated group with an inotropic dose.

* Unpaired t-test was made against the ouabain treated group with an arrhythmic dose.

+ see table 9 for the meaning of all other symbols and figure 26 for the replication of experiment.
Figure 26. The accumulation of ouabain in the major myocardial subcellular fractions under the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH. Vertical bars and parentheses indicate standard error and number of animals used respectively.
that of the arrhythmic dose of ouabain alone according to the statistical analysis except one fraction—the supernate. In the case of the supernate (3.0 ± 0.3 pm of ouabain per mg of protein), it appears that DPH treatment raised significantly the concentration of ouabain in this fraction. However, the S/P ratio calculated on the muscle weight basis is not statistically different from that of the group used the arrhythmic dose alone as shown in table 21. Overall slight decrease of ouabain accumulation in most of the fractions may contribute to the increase of ouabain concentration in the supernate. But the exact implication of this result is not clear at this time.

It appears that KCl infusion along with the arrhythmic dose of ouabain reduced the ouabain accumulation in most of the fractions, except for the supernate. The most prominent effect of KCl infusion is the reduction of the binding of ouabain to the NaK ATPase from the control 13.6 ± 1.0 to 9.2 ± 1.9 pm/mg protein in 4 dogs. This reduction due to KCl is statistically significant. As in the case of DPH treatment, it was noted that KCl infusion also raised the ouabain content in the supernate from 1.8 to 2.3 pm/mg protein. However, the S/P ratio did not increase significantly compared to the control group treated with the arrhythmic dose of ouabain alone.

f. Effect of the Inotropic and Arrhythmic Doses of Ouabain Alone or with either KCl or DPH on the ATPase Activity.

In 5 control dogs after 300 minutes of saline infusion, the total ATPase and NaK ATPase activities were 14.9 and 9.6 um Pi/mg protein/hr, respectively. As presented earlier, in 80 minutes control experiment with 6 paced dog hearts the NaK ATPase activity was 14.0 ± 2.0 um Pi/mg protein/hr (see table 22). Although the total ATPase activity was not different in these two preparations, the decrease of the NaK ATPase activity was significant. However, the total as well as the NaK ATPase activities in the microsomal fraction obtained from the paced hearts (80 min) were not significantly different from those of intrinsically beating preparation for 300 minutes. Under anesthesia, to keep the animal for such long time (300 min) in open-chest situation might affect the enzyme activity by changing factors involved in the regulations of the NaK ATPase activity. But it may not be fair to compare these two groups because one
Table 21. The supernate to pellet ratio of ouabain after the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH.

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Ouabain dose</th>
<th>n</th>
<th>S/P ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loading (ug/kg)</td>
<td>Infusion (ug/kg/min)</td>
<td></td>
</tr>
<tr>
<td>Inotropic dose 120 min</td>
<td>20</td>
<td>0.036</td>
<td>3</td>
</tr>
<tr>
<td>Inotropic dose 300 min</td>
<td>20</td>
<td>0.036</td>
<td>5</td>
</tr>
<tr>
<td>Arrhythmic dose 300 min</td>
<td>40</td>
<td>0.072</td>
<td>5</td>
</tr>
<tr>
<td>Arrhythmic dose 300 min + DPH</td>
<td>40</td>
<td>0.072</td>
<td>4</td>
</tr>
<tr>
<td>Arrhythmic dose 300 min + KCl</td>
<td>40</td>
<td>0.072</td>
<td>4</td>
</tr>
</tbody>
</table>

@ n indicates number of animals used.
* expressed as mean ± standard error.
Table 22. Comparison of the total and NaK ATPase activities between paced and spontaneously beating heart preparations after saline infusion in control dogs.

<table>
<thead>
<tr>
<th>Animal condition</th>
<th>Experimental duration (min)</th>
<th>Myocardial fractions</th>
<th>n</th>
<th>Total ATPase activity (um pi/mg protein/hr)</th>
<th>NaK ATPase activity (um pi/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paced heart</td>
<td>80</td>
<td>NaK ATPase Microsome</td>
<td>6</td>
<td>17.1 ± 2.0</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td>Intrinsic heart</td>
<td>300</td>
<td>NaK ATPase Microsome</td>
<td>5</td>
<td>14.9 ± 0.6</td>
<td>9.6 ± 0.3a</td>
</tr>
</tbody>
</table>

@ indicates number of animal used.

# Each value represents at least two determinations, mean ± standard error.

* a indicates that P is lesser than 0.05, based on unpaired t-test between paced and spontaneously beating groups.
is paced and another is spontaneously beating.

Effect of the inotropic and arrhythmic doses of ouabain on the ATPase activity of partially purified NaK ATPase and microsomal fraction is illustrated in table 23 and figure 27. In general, the total ATPase activity in microsomes is always greater than that of partially purified NaK ATPase. Mg-dependent ATPase activity did not change drastically with respect to ouabain dosage. It was also true that Mg-dependent ATPase activity in the microsomal fractions are always at least 2 to 3 times greater than that of the NaK ATPase prepared from the same animals, which all suggest the greater impurity of the microsomal fraction. In other words, in the microsomal fractions there may be greater portion of Mg-dependent ATPase exists compared to the NaK ATPase preparations which were partially purified by the treatment with 2.0 M LiBr and a series of centrifugation procedure.

Neither 120 min nor 300 min infusion of ouabain with the inotropic dose changed the 3 ATPase activities significantly from the control hearts. However, 300 min infusion of the arrhythmic dose of ouabain significantly reduced the total ATPase activity from $14.9 \pm 0.6$ to $10.7 \pm 0.5 \text{ um Pi/mg protein/hr}$ and the NaK ATPase activity from $9.6 \pm 0.3$ to $6.0 \pm 0.6 \text{ um Pi/mg protein/hr}$. The NaK ATPase and total ATPase activities in microsomes were not different statistically from the control, however.

Table 23 and figure 27 also show the effect of DPH and KCl along with the arrhythmic dose of ouabain on the three ATPase activities. According to statistical analysis, KCl infusion together the arrhythmic dose of ouabain did not affect any of these three ATPase activities. However, in 4 dogs DPH raised the Mg-dependent ATPase and the total ATPase activities. Thus, the NaK ATPase activity which was obtained by subtracting Mg-dependent ATPase activity from the total ATPase activity remained unchanged from the arrhythmic dose of ouabain treated group. Although the NaK ATPase and Mg-dependent ATPase are different enzymes, it is interesting to note the stimulating effect of DPH on the Mg-dependent ATPase activity. However, DPH did not increase significantly the Mg-dependent or the total ATPase activity in the microsomal fractions.

g. Interrelationship between Ouabain Binding and the Change of the NaK ATPase in Relation to Pharmacological Effect of Ouabain in the Spontaneously Beating Heart.

The change in the major myocardial cellular events after the administra-
Table 23. Effect of the inotropic and arrhythmic doses of ouabain alone or either with KCl or DPH on the activity of the NaK ATPase in microsomal and partially purified NaK ATPase fractions.

<table>
<thead>
<tr>
<th>Treatment of ouabain</th>
<th>Myocardial fractions</th>
<th>Mg\textsuperscript{++} dependent NaK ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NaK ATPase 5</td>
<td>14.9±0.6 # 5.3±0.4</td>
</tr>
<tr>
<td>300 min</td>
<td>Microsome 5</td>
<td>17.6±0.6 10.8±1.1</td>
</tr>
<tr>
<td>Inotropic dose, 120 min</td>
<td>NaK ATPase 3</td>
<td>15.0±0.3 4.5±1.3</td>
</tr>
<tr>
<td></td>
<td>Microsome 3</td>
<td>16.5±0.3 8.7±3.0</td>
</tr>
<tr>
<td>Inotropic dose, 300 min</td>
<td>NaK ATPase 5</td>
<td>12.8±1.3 4.3±0.3</td>
</tr>
<tr>
<td></td>
<td>Microsome 5</td>
<td>13.8±1.9 9.5±1.6</td>
</tr>
<tr>
<td>Arrhythmic dose, 300 min</td>
<td>NaK ATPase 5</td>
<td>10.7±0.5 * 4.7±0.3 * 6.0±0.6 *</td>
</tr>
<tr>
<td></td>
<td>Microsome 5</td>
<td>16.5±1.1 11.8±2.0 *</td>
</tr>
<tr>
<td>Arrhythmic dose, DPH</td>
<td>NaK ATPase 4</td>
<td>14.4±1.0 * 7.1±0.6 *</td>
</tr>
<tr>
<td></td>
<td>Microsome 4</td>
<td>19.0±1.8 14.2±1.4 *</td>
</tr>
<tr>
<td>Arrhythmic dose, KCl</td>
<td>NaK ATPase 4</td>
<td>11.9±1.7 4.6±0.4</td>
</tr>
<tr>
<td></td>
<td>Microsome 4</td>
<td>18.5±1.5 11.9±1.5</td>
</tr>
</tbody>
</table>

\* indicates number of animal used.
\# All values are expressed in umol released per mg protein per hour, mean ± standard error, and each value represents at least two measurements.
* based on unpaired t-test between inotropic and arrhythmic doses of ouabain treated groups.
+ based on unpaired t-test between arrhythmic dose of ouabain and DPH treated groups.
\* see the table 9 for the meaning of other signs.
Figure 27. Effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the total, Mg-dependent and NaK ATPase activities in the spontaneously beating hearts. Vertical bars and parentheses indicate standard error and number of animals used respectively. See table 9 for the meaning of other signs used.
tion of the inotropic and arrhythmic doses of ouabain alone or with either DPH or KCl is summarized in table 24. All values represent the observations made at the end of experimental period prior to the sacrifice of dogs. As presented under an appropriate heading, the rate of ouabain infusion after a loading dose of ouabain determined the plasma concentration of ouabain, which increased both LV dp/dt and RVct. The most distinct effect of ouabain treatment was reflected in the ouabain accumulation in LV, the microsomal and the NaK ATPase fractions along with the changes in the plasma concentration of K and the NaK ATPase activity. Although both KCl and DPH treatments removed the arrhythmia induced by ouabain, the effect of DPH or KCl on these parameters was not statistically significant with some reservations as pointed out earlier.

Table 24 also shows the effect of the inotropic and arrhythmic doses of ouabain by itself or with either KCl or DPH on the binding of ouabain to the NaK ATPase, its activity and the changes in the plasma K concentration together with the development of LV dp/dt and RVct. The administration of the inotropic dose of ouabain either for 120 or 300 min produced a significant increase in LV dp/dt without a significant change in the NaK ATPase activity and the plasma K concentration. Under this inotropic dose schedule, there was no arrhythmia and the inotropic response to ouabain continued up to 300 minute experimental period. The administration of either KCl or DPH was effective in reversing the arrhythmia induced by the arrhythmic dose of ouabain, but they failed to reverse the inhibition of the NaK ATPase activity induced by ouabain, though KCl reduced the binding of ouabain in the NaK ATPase when the plasma K concentration was about 7.5 meq/l.

h. Comparison of the Major Myocardial Parameters at the Time of Significant Positive Inotropism and Arrhythmia Induced by Ouabain with Respect of the Dose Regimen or Animal Preparation.

Experiments with the paced and the intrinsically beating hearts were designed by entirely different experimental approaches. The basic differences were the animal preparation (paced vs. intrinsically beating), ouabain dose regimen (a high infusion rate after a little loading dose vs. a slow infusion rate after a high loading dose) and the total experimental duration (under 100 min vs. 300 min). However, these two experimental approaches induced the same pharmacological actions of ouabain—inotropism and arrhythmia. Therefore, it may be meaningful to correlate the similarity and dissimilarity, if any, in the accumulation of ouabain and the change in the NaK ATPase
Table 24. The effect of the inotropic and arrhythmic doses of ouabain alone or with either KCl or DPH on the major myocardial parameters in the spontaneously beating heart.

<table>
<thead>
<tr>
<th>Treatment of Ouabain</th>
<th>P_ouabain (pm/ml)</th>
<th>Total dose ouabain (ug/kg)</th>
<th>LV uptake (pm/g heart)</th>
<th>Supernate uptake (pm/mg protein)</th>
<th>Microsomal uptake (pm/mg protein)</th>
<th>NaK ATPase binding (pm/mg protein)</th>
<th>NaK ATPase activity (um Pi/mg protein/hr)</th>
<th>LV dp/dt</th>
<th>RV_{ct}</th>
<th>Plasma K^+ (meq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.6</td>
<td>79</td>
<td>76</td>
<td>76</td>
<td>3.4</td>
</tr>
<tr>
<td>Inotropic dose, 120 min</td>
<td>9.1</td>
<td>24.32</td>
<td>176</td>
<td>0.5</td>
<td>1.7</td>
<td>3.6</td>
<td>10.5</td>
<td>122</td>
<td>122</td>
<td>3.6</td>
</tr>
<tr>
<td>dose, 300 min</td>
<td>9.1</td>
<td>30.80</td>
<td>288</td>
<td>0.7</td>
<td>2.8</td>
<td>4.7</td>
<td>8.7</td>
<td>124</td>
<td>134</td>
<td>3.9</td>
</tr>
<tr>
<td>Arrhythmic dose, 300 min</td>
<td>28.0</td>
<td>61.60</td>
<td>669.e</td>
<td>1.8</td>
<td>9.2.b</td>
<td>13.6.e</td>
<td>6.0.d</td>
<td>105.a</td>
<td>141.b</td>
<td>4.2.a</td>
</tr>
<tr>
<td>DPH</td>
<td>1.7</td>
<td>61.60</td>
<td>684</td>
<td>3.0.a</td>
<td>7.7</td>
<td>12.4</td>
<td>7.2</td>
<td>103</td>
<td>136</td>
<td>4.4</td>
</tr>
<tr>
<td>Arrhythmic dose, KCl</td>
<td>24.0</td>
<td>61.60</td>
<td>577</td>
<td>2.3</td>
<td>6.6</td>
<td>9.3.a</td>
<td>7.3</td>
<td>96</td>
<td>137</td>
<td>7.9.e</td>
</tr>
</tbody>
</table>

@ All values are expressed in appropriate units, mean ± standard error.
# Unpaired t-test was made against the inotropic dose of ouabain treated group.
* Unpaired t-test was made against the arrhythmic dose of ouabain treated group.
+ see table 9 for the meaning of all other signs.
activity, etc at the time of the two distinct pharmacological end points. Table 25 shows the overall picture of the ouabain accumulation, the NaK ATPase activity and other important parameters obtained at those end points with the two animal preparations. At the time of a significant response induced by either the low dose or the inotropic dose of ouabain with the paced or intrinsically beating hearts, the ouabain binding to the NaK ATPase, NaK ATPase activity and the inotropic response to ouabain were strikingly comparable, though the plasma concentration of ouabain and the total experimental duration were very different.

This sort of comparison is also very well fit for the parameters obtained at the time of manifestation of arrhythmia. Both ouabain binding to the NaK ATPase and the NaK ATPase activity of the enzyme prepared from either the paced or intrinsically beating hearts were not different from each other when comparing hearts at the arrhythmic stage. If we consider the ouabain concentration in the extracellular space (since the heart was not washed out), the difference in the ouabain content in LV at the time of induction of the arrhythmia is significant at 5% level instead of 0.1% as indicated in table 25. After the induction of arrhythmia, there is a considerable difference in the magnitude of LV dp/dt and RVot. Since either the inotropism or arrhythmia can be induced in the paced or spontaneously beating animals by the manipulation of ouabain dose regimen and the duration of ouabain infusion, it may be understandable that a certain extent of ouabain binding to cellular structures and a certain fraction of the inhibition of the NaK ATPase activity may characterize the inotropism or arrhythmia induced by ouabain in intact dogs without respect to the experimental procedures.

1. Correlation of NaK ATPase Activity with the Inotropic Response to Ouabain in the Spontaneously Beating Heart Preparations.

The ultimate objective of this study with the intrinsically beating heart is to correlate the change of the NaK ATPase activity and the inotropic action of ouabain under a steady-state positive inotropic response to ouabain without its adverse effects for sufficient time interval. Since the steady-state positive inotropic response to ouabain was achieved throughout the 300 minute experimental period without leading to arrhythmia in the intrinsically beating hearts, it may be justified to attempt to correlate the NaK ATPase activity and the inotropism induced by ouabain, to determine
Table 25. Comparison of the major myocardial parameters at the time of inotropism and arrhythmia irrespective of their methods of induction.

<table>
<thead>
<tr>
<th>Effect of ouabain</th>
<th>Animal condition</th>
<th>Experiment duration (min)</th>
<th>Ouabain (pm/ml)</th>
<th>Total dose of ouabain (ug/kg)</th>
<th>LV uptake (pm/g heart)</th>
<th>NaKATPase binding activity (pm/mg protein)</th>
<th>dp/dt (um Pi/mg protein/hr)</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inotropism</td>
<td>Intrinsic heart</td>
<td>300</td>
<td>9.1</td>
<td>30.8</td>
<td>176</td>
<td>4.7</td>
<td>8.1</td>
<td>124</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Paced heart</td>
<td>15</td>
<td>116 n.s.</td>
<td>18.0</td>
<td>262</td>
<td>6.0 n.s.</td>
<td>9.5 n.s.</td>
<td>116 n.s.</td>
<td>108 n.s.</td>
</tr>
<tr>
<td></td>
<td>Paced heart</td>
<td>40</td>
<td>121 n.s.</td>
<td>43.0</td>
<td>488 d</td>
<td>18.2 n.s.</td>
<td>6.5 n.s.</td>
<td>128 e</td>
<td>125 n.s.</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>Intrinsic heart</td>
<td>300</td>
<td>28.0</td>
<td>61.6</td>
<td>669</td>
<td>13.6</td>
<td>6.0</td>
<td>105</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Paced heart</td>
<td>15</td>
<td>121 n.s.</td>
<td>43.0</td>
<td>488 d</td>
<td>18.2 n.s.</td>
<td>6.5 n.s.</td>
<td>128 e</td>
<td>125 n.s.</td>
</tr>
<tr>
<td></td>
<td>Paced heart</td>
<td>40</td>
<td>10.0</td>
<td>33</td>
<td>4.4</td>
<td>2.1</td>
<td>8.9</td>
<td>6.8</td>
<td></td>
</tr>
</tbody>
</table>

*All values are expressed in appropriate units, mean ± standard error.

* see the description under Materials and Method for the ouabain dose and treatment.

# see table 9 for the meaning of other signs used.
if they are causally related.

Excluding the data from the 8 dogs used for the study of the antiarrhythmic action of KCl or DPH, all relevant data obtained from the remaining 18 dogs were utilized for the statistical analysis under the same computer programs used in the paced group experiments (BMD02D for correlation problem and Omnitab fit command for the analysis of variance). For the purpose of comparison with the paced group experiments, the results of statistical analysis are arranged and presented in a similar manner in table 26 and figure 28.

As in the case of the paced group experiments, the total experimental duration (column 1) is not correlated with any other variables. The total dose of ouabain (column 2) infused up to that time is significantly correlated with ouabain binding to the NaK ATPase as well as with the NaK ATPase activity, but not with the inotropic response to ouabain measured by LV dp/dt (p > 0.05). Ouabain binding to the NaK ATPase activity (column 3) is well correlated with the NaK ATPase activity (r = -0.69). However, it is not correlated with the inotropic response to ouabain because the correlation coefficient between these two parameters is 0.137, which is not significant at all. The correlation coefficient obtained from the relationship between the NaK ATPase activity (column 4) and the inotropic response (column 5) is only 0.045. Therefore, the NaK ATPase activity is not correlated with the inotropic response to ouabain under the steady-state inotropic condition and during the persistent arrhythmia in the spontaneously beating hearts.

A similar conclusion was drawn from the analysis of variance by testing the regression coefficient against the null hypothesis that the regression coefficient, b = 0. At 0.5% level the null hypothesis was rejected for the b obtained from the relationship between the NaK ATPase activity and ouabain binding to the NaK ATPase because F value is 14.91 which is greater than 10.58 (F_{1, 16} = 10.575 at 0.5%). The same hypothesis against the regression coefficient derived from the relationship between the inotropic response and the NaK ATPase or ouabain binding to the NaK ATPase is accepted since F values are not significant (p > 0.05).

Figure 28 shows the regression line between the NaK ATPase activity and ouabain binding to the NaK ATPase obtained by the least squares method in 18 spontaneously beating hearts. The 18 points obtained from the 18
Table 26. Correlations and the analysis of variance of the major myocardial parameters obtained in the spontaneously beating hearts.

### VARIANCE-COVARIANCE MATRIX

<table>
<thead>
<tr>
<th>Experimental duration (min)</th>
<th>Total dose of ouabain (ug/kg)</th>
<th>Ouabain binding to NaK ATPase (pm/nm protein)</th>
<th>NaK ATPase activity (pm pi/mg/hr)</th>
<th>Inotropic response (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL. 1</td>
<td>4764.6875</td>
<td>171.5312</td>
<td>57.1767</td>
<td>-61.9411</td>
</tr>
<tr>
<td>COL. 2</td>
<td>171.5312</td>
<td>564.1900</td>
<td>115.8372</td>
<td>-34.6603</td>
</tr>
<tr>
<td>COL. 3</td>
<td>57.1767</td>
<td>115.8372</td>
<td>25.3153</td>
<td>7.8047</td>
</tr>
<tr>
<td>COL. 4</td>
<td>-61.9411</td>
<td>-34.6603</td>
<td>-7.8047</td>
<td>4.9885</td>
</tr>
<tr>
<td>COL. 5</td>
<td>-624.7039</td>
<td>180.4274</td>
<td>19.8529</td>
<td>2.9001</td>
</tr>
</tbody>
</table>

### CORRELATION MATRIX

<table>
<thead>
<tr>
<th>COL. 1</th>
<th>COL. 2</th>
<th>COL. 3</th>
<th>COL. 4</th>
<th>COL. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0000</td>
<td>0.1046</td>
<td>-0.4018</td>
<td>-0.3148</td>
</tr>
<tr>
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<td>1.0000</td>
<td>0.9693</td>
<td>0.2642</td>
</tr>
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<td>-0.6945*</td>
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<td>0.2642</td>
<td>0.1373</td>
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</tbody>
</table>

### ANALYSIS OF VARIANCE

<table>
<thead>
<tr>
<th></th>
<th>REGRESSION COEFFICIENT F  p</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaK ATPase activity vs. ouabain binding to NaK ATPase</td>
<td>10.18 -0.31 14.91 &lt;0.005</td>
</tr>
<tr>
<td>Inotropic response vs. NaK ATPase activity</td>
<td>106.05 -0.58 0.03 &gt;0.05</td>
</tr>
<tr>
<td>Inotropic response vs. ouabain binding to NaK ATPase</td>
<td>106.75 -0.78 0.31 &gt;0.05</td>
</tr>
</tbody>
</table>

n indicates number of animals used.
* indicates p is smaller than 0.05.
Figure 28. Correlation between NaK ATPase activity and ouabain binding to the NaK ATPase. The orthogonal regression line was calculated by least squares method from 18 intrinsically beating hearts. The big dot indicates the regression point.
individual experiments using the inotropic and arrhythmic doses of ouabain are plotted as a scatter diagram along with the regression point and the regression equation.
VI. DISCUSSION

1. ADEQUACY OF THE EXPERIMENTAL APPROACHES AND METHODS USED.

Animal preparations including open-chest surgery, pacing the heart, insertion of catheter into the left ventricular lumen and suturing a strain gauge on the right ventricle was usually completed within 30 minutes without causing any appreciable loss of blood. Moderate doses of ouabain used in this study did not affect significantly the end diastolic pressure (EDP) so that the per cent change of LV dp/dt instead of \( \frac{dp}{dt} \) was justified to represent the true increase or decrease of inotropic response to ouabain. The heart rate and the temperature also did not change significantly in both experiments with the paced and spontaneously beating hearts, so that the changes of dp/dt are probably to be valid responses to ouabain rather than produced by cardiac hemodynamic changes. Similar observations have been reported by Alberti et al(1971) and Williams et al(1966) in dogs with comparable doses of ouabain.

The primary objective of the experiments with the paced hearts was to induce one of three distinct pharmacological end points of ouabain action— inotropism, onset of arrhythmia and lethality by adjusting the duration of ouabain infusion. A continuous infusion of ouabain(1ug/kg/min) after a 3ug per kg as a loading dose induced these three distinct pharmacological effects of ouabain depending upon the duration of ouabain infusion. With the continuous infusion rate, the time required to induce inotropism, arrhythmia and lethality were distinctly different so that correlation between these pharmacological effects of ouabain with the NaK ATPase activity or ouabain accumulation was theoretically possible. A similar experimental design can be found in the experiments of Akera et al(1970) and Besch et al(1970). However, this experimental design failed to provide the basis for the clear-cut comparison between individual pharmacological effect of ouabain and the biochemical parameters studied. The main reason is that, under such experimental conditions, the inotropic or arrhythmic response to ouabain is progressively converted to other stages of ouabain action and thereby the
change in the NaK ATPase activity could not be correlated with the transient pharmacological effects of ouabain. Therefore, it was necessary to formulate another experimental approach which was not subject to this disadvantage.

With the intrinsically beating hearts, the inotropic dose calculated on the basis of ouabain clearance and plasma half life did induce a significant positive inotropic response to ouabain and maintain this steady-state of inotropism for 300 minutes of experimental period without leading to ouabain-induced adverse effects. Thus, it is justified to refer to this as the inotropic response to ouabain and to correlate it with biochemical parameters such as the NaK ATPase activity or the accumulation of ouabain, which is the integral part of this study. Likewise, the arrhythmic dose of ouabain induced arrhythmia within a predictable time period which was persistent without leading to lethality for a number of hours. A similar dose of ouabain required to induce arrhythmia in dogs was noted by other investigators (Alberti et al., 1971; Williams et al., 1966). This provided the experimental basis for the comparison of ouabain-induced arrhythmia with the inhibition of the NaK ATPase. The persistency of arrhythmia was also served for the study of the antiarrhythmic action of KCl or DPH.

As far as the measurement of ouabain content in the myocardial subcellular fractions is concerned, most of the fractions were diluted enough to count with relatively little quenching effect (usually counting efficiency was greater than 17%) and sufficient net counts were usually obtained. It was relatively easy to measure the radioactivity bound to the partially purified NaK ATPase because the binding affinity is very high. As will be discussed later, since there is no quick way to wash out the extracellular space in these in situ hearts, the final concentration of plasma ouabain may contribute to the ouabain content of the heart.

For the preparation of the NaK ATPase, which is basically followed the method of Akera et al. (1969) with some modifications, it was noted that either Virtis 45 or Polytron homogenizer did not significantly affect the enzyme yield or activity in control experiments because in several early control experiments NaK ATPase was prepared by Virtis 45, of which results are not different from the rest of control experiments. Especially Polytron Pt-20 was impressive for the speed of homogenization of the tough myocardial tissue of the dog. Since the standard procedure established in this labora-
tory was used for the preparation of nuclear, mitochondrial and microsomal fractions (see figure 4), the microscopic verification of the purity was not carried out.

2. CORRELATION OF THE MAGNITUDE OF THE OUABAIN CONTENT IN THE MYOCARDIAL TISSUE WITH THE PHARMACOLOGICAL EFFECTS OF OUABAIN.

Considerable effort has been directed to correlate the plasma concentration of digitalis or its content in the myocardial tissue with its pharmacological effects. It would be ideal to maintain a certain constant plasma digitalis concentration which would allow the induction of a specific digitalis effect such as positive inotropism or different degrees of digitalis toxicity. In this study with the paced heart, the infusion of the low dose of ouabain induced various effects of ouabain as a function of the duration of ouabain infusion without much fluctuation in the plasma ouabain concentration. Under this experimental condition, the ouabain concentration in the tissue and plasma may not be in equilibrium so that it may be difficult to correlate these with other parameters such as ouabain effects. In other words, since the plasma concentration of ouabain was 120 pm/ml at this dose, about 250 pm of ouabain was cleared for each minute per kg while the infusion rate was 1710 pm/min per kg body weight. The experiments with the high dose of ouabain infusion explain the situation, in which the ouabain infusion rate is so great that it far exceeded the clearance of ouabain including tissue ouabain binding and other distribution processes.

However, the experiments with the intrinsically beating hearts have provided a justification for comparison of the ouabain effect and its distribution and its effect on the NaK ATPase activity. The dominant plasma half life of ouabain representing the excretion of ouabain was 260 minutes and tissue binding was 16 minutes. Under similar experimental conditions, Doherty and Perkins (1966) reported 23 hours and 30 minutes respectively as the slow and fast half lives of digoxin in dogs. The inotropic dose calculated from the half life and ouabain clearance data was expected to produce about 30 pm/ml, which is about three time higher than the actual value (9.1 pm/ml). However, considering the variations in the plasma concentration of ouabain in individual experiment value, this deviation may be insignificant. The infusion of ouabain was also started at 10 minutes after the loading dose of ouabain injection instead at to. Although little is known
about the nature of ouabain accumulated in different cellular compartments or subcellular fractions, ouabain in the plasma and probably in the extracellular space is directly related to the renal excretion. The approximated value, 40 pm/g of tissue estimated on the basis of the study on the digoxin distribution in 15 organs of the dogs might be underestimated. However, the amount of ouabain from this estimation contributed less than 24% of the total dose calculated from the ouabain distribution volume.

In this study, at the time of induction of a significant positive inotropic response to ouabain, the left ventricle accumulated 262 pm/g wet weight in the paced heart and 288 in the intrinsically beating hearts (see tables 9, 20 and 25). Similarly, between these paced and intrinsically beating groups there was a similar extent of ouabain accumulation to other myocardial subcellular fraction including the microsome and mitochondria, which may characterize the inotropic effect of ouabain in terms of the ouabain content to the various fractions.

In the cardiopulmonary bypass preparation of dog heart, Luchi et al. (1971) observed an inotropic response to ouabain when the mean myocardial content of ouabain was 115 pm/g wet weight and 280 pm/g was noted at the time of ouabain toxicity (ventricular tachycardia). These values seem lower compared to the observation made in the current study. As pointed out elsewhere (Dutta et al., 1972), the difference in the ouabain dose regimen (single injection vs. continuous infusion) may be one of the major reasons for this discrepancy. In their study an inotropic effect was defined as a 10% or greater increase in strain gauge amplitude which may be still under subinotropic effect according to the definition of inotropic effect of ouabain used in this study. In the present study inotropic effect is referred to a statistically significant (p<0.05) increase in LV dp/dt.

Another important difference is the fact that they assessed the myocardial ouabain content from the right ventricular biopsy samples, which are known to accumulate significantly (p<0.001) less digitalis than the LV as demonstrated by Deutscher et al. (1971). They showed that, 240 minutes after a single injection of digoxin (80ug/kg) in dog, the right ventricle accumulated only 130 pm/g wet weight while the left ventricle accumulated 250, which means at that time the right ventricle accumulated about 50% of that of LV.
In their study with digoxin they also showed that the peak inotropic effect of digoxin was induced in 60 minutes after a single injection of digoxin. At that time the LV and the septal tissue accumulated about the same amount of digoxin (250 pm/g tissue) and the plasma digoxin concentration was 11 pm/ml. The digoxin plasma concentration and LV digoxin content are very much comparable to the ouabain accumulation observed in the current study. However, the plasma concentration of digoxin is still at least 30 to 3 times higher than that under the therapeutic condition in human subjects reported by Smith and Haber (1971).

In this study the amount of ouabain required to produce the onset of arrhythmia was relatively constant either for the paced preparation (58 ug/kg) or intrinsically beating hearts (42 ug/kg), which is in agreement with Alberti et al (1971) who reported 53 ug/kg ouabain was required to induce various types of arrhythmia in dogs. In the study of Williams et al (1966) 56 ug/kg of ouabain was needed to produce a ventricular tachycardia. Since the accumulation of ouabain in the myocardial fractions including the NaK ATPase at the time of onset of arrhythmia was comparable between the paced and intrinsically beating hearts, the ouabain-induced arrhythmic effect may be characterized in terms of fixation of ouabain molecules to various subcellular fractions.

The infusion time required to induce death was 84 min with the low dose infusion and 46 with the high dose infusion schedule. At those times with the low and high doses of ouabain infusion the plasma concentration of ouabain was 123 and 307 pm/ml respectively. As expected, the total dose of ouabain infused up to the lethal point either by the low or high dose infusion was the same and this lethal dose is in good agreement with Zelis et al (1970) who showed that at the infusion rate of 1 ug/kg/min without a loading dose lethality was induced with 94.6 ug/kg of ouabain. In the dog heart-lung preparation Farah and Maresh (1948) noted that 100 ug/kg was required to induce a lethal effect. One of the most interesting characterization of lethality induced by ouabain in intact dog may be the degree of the NaK ATPase inhibition and the extent of ouabain accumulation to various subcellular fractions. At the time of lethality induced by either the low or high dose of ouabain, there is no difference in the accumulation of ouabain in the various fractions. However, at that time the plasma concentration of Na and K between these two
groups was significantly different, which means the changes of plasma Na and K induced by ouabain may not be the direct cause of death.

3. THE BINDING OF OUABAIN TO THE PARTIALLY PURIFIED NaK ATPase AND ITS IMPLICATION.

The NaK ATPase has attracted a considerable attention not only because of its unique physiological functional role involved in the transport of monovalent cations, but also because it serves as an excellent molecular model system for the study of drug and receptor interaction. One of the most important characteristics of the NaK ATPase is its ability to specifically bind ouabain or other active cardiac glycosides. Ouabain and the NaK ATPase complex resulting from an in vitro interaction between the drug and the enzyme prepared from ouabain sensitive species is stable and under physiological condition is practically irreversible (Allen and Schwartz, 1969; Albers et al., 1968).

Generally it requires extensive isolation and purification procedures to obtain partially purified NaK ATPase from the intact organ. Therefore, it has been wondered whether the ouabain binding to the NaK ATPase and its inhibition took place during the artificial manipulation for the preparation of this enzyme. Several comments may be pertinent in this regards. The high content of K in cell homogenate caused by disruption of the cell may not be a favorable condition for the interaction between ouabain and the NaK ATPase, especially at low temperature near 0°C. as shown by Albers et al (1968). Allen et al (1970a) showed that the NaK ATPase treated with ouabain once binds comparably less amount of this drug on subsequent in vitro exposure of ouabain, which suggests that once bound ouabain is stable so that it is not dislodged by the isolation or purification procedures. In the current studies with the paced dog heart, about the 40 minute infusion of either the low or high dose of ouabain resulted in the accumulation of a similar amount of ouabain in the microsome and the NaK ATPase (see table 9). But with the high dose of ouabain more accumulation of this drug was observed in the rest of myocardial fraction including LV, homogenate and supernate. This probably suggests that, when ouabain is presented to the biophase, it is taken up by the plasma and sarcoplasmic membrane structures of the cardiac cells preferentially and retained it by these sites with high affinity, so that ouabain cannot be taken up by other nonspecific sites until the specific
sites are saturated. It may also point to the fact that at the time of homogenization ouabain probably is not transferred from one myocardial fraction to another. This fact is in agreement with the known specific requirements for the interaction between ouabain and the NaK ATPase in vitro system such as optimal temperature, the concentrations of ATP, Na and K (Albers et al., 1968; Schwartz et al., 1969). Further, it is understandable that the high concentration of ouabain in the extracellular space at the time of cell fractionation may increase ouabain content in the homogenate or cardiac muscle in general.

In this study 20% myocardial homogenate of the left ventricle (30g) produced usually 10 to 15 mg of partially purified NaK ATPase at the last stage of purification procedures. The yield of enzyme is 0.3 to 0.5 mg enzyme as protein per g of heart (wet weight), which was a little higher yield than that recently reported by Spain and Chidsey (1972) from the same species. However, the total ATPase activity of these two preparations was about the same (17 um Pi/mg protein/hr). Under similar experimental conditions Akera et al (1970) reported 23.8 um Pi/mg protein/hr and Besch et al (1970) found 16.2 as the NaK ATPase activity. In comparison to cardiac tissue, different tissues from different species have a great variation in the magnitude of ouabain binding and the ATP hydrolyzing activity. Rivas et al (1972) reported NaK ATPase from electroplax membrane bound ouabain 4310 pm/mg protein at the ouabain concentration of 8.3 x 10^-7 M. The enzyme yield was 5.7 mg for each g of electric organ and molecular weight was estimated under 40,000. However, with the same organ under similar experimental conditions Albers et al (1968) reported 655 pm of ouabain bound per each mg protein.

Regard the purity of the NaK ATPase used in this study, the NaK ATPase activity was greater than 64% of the total ATPase activity because in control experiment the total ATPase activity was 14.9 um Pi/mg protein/hr and the specific activity of the NaK ATPase was 9.6. This is about two fold increase of the NaK ATPase activity in comparison to that in the microsome. Post et al (1965) obtained 85 to 90% purified NaK ATPase from the guinea pig kidney. More than 99 per cent pure NaK ATPase from bovine brain of which specific activity was 400 to 750 um Pi/mg protein/hr (Uesugi et al., 1971) and near homogenous NaK ATPase from the rectal gland of the dog fish (Hokin et al., 1973).
have been reported prepared. In the present study, at the time of lethality ouabain binding to the NaK ATPase was about 30 pm/mg protein, which is about the same as the in vitro ouabain binding to the NaK ATPase from dog (Allen et al., 1970a). If one molecule of ouabain or P^32 is assumed to bind to a molecule of ATPase (Post et al., 1965; Matsui and Schwartz, 1968; Wilson et al., 1970; Rivas et al., 1972), since the molecular weight of near homogenous NaK ATPase has been estimated as 304,000 (Hokin et al., 1972), the enzyme used in this study is only 0.9% pure. Since the maximum binding of ouabain to this enzyme is about 3300 pm/mg protein based on the estimated molecular weight, recent value of 4310 pm of ouabain bound per mg NaK ATPase from electroplax organ (Rivas et al., 1972) is in quite good agreement with the calculation.

The purity of the NaK ATPase may be expressed as a turnover number, which is defined as the ratio of unit of the enzyme activity to the number of P^32 incorporated in the enzyme. Since the control NaK ATPase preparation used in this study had about 0.23 unit as activity and the maximum ouabain binding was about 30 pm/mg protein based on in vivo as well as in vitro binding study, the turnover number is 7667 per minute. On the basis of similar calculation Post et al. (1965) reported the turnover number of the NaK ATPase from the guinea pig kidney as 20,000 per minute.

In this study, in general, at the time of ouabain-induced inotropism there was a similar degree of ouabain binding in the NaK ATPase irrespective to the ouabain dose regimen or animal preparation. In experiments with either the paced or the spontaneously beating hearts, there was a good parallelism between ouabain binding to the NaK ATPase and its activity (see tables 15 and 26), which confirms the previous observation (Albers et al., 1968; Matsui and Schwartz, 1968). Although a number of recent studies indicate the binding of ouabain by the partially purified NaK ATPase in vitro or by the cultured cell (Okarma et al., 1972), it is not clear from the present study the exact cytological site of ouabain binding during the arrhythmic or inotropic action of ouabain. It should be emphasized that the NaK ATPase used in the present study is a heterogenous light particulate fraction treated with DOC and LiBr. In addition to the fragmented plasma membrane, the enzyme in all probability contains in considerable amount of other light fragments which have a similar density such as mitochondrial and sarcoplasmic reticular membranes including cardiac lysosomes.
Dutta et al (1968b) considered the digitalis receptor may reside in the light particulate fraction, which is known to be a rich source of the NaK ATPase. Thus, it may be of interest to look into the possibility that what extent bound ouabain to the NaK ATPase is derived from the microsome. In this study about 500 to 700 pm of ouabain were recovered from the total 200 to 400 mg microsome depending upon the dose of ouabain employed. This microsome was further subjected to purification by the treatment of LiBr and a series of washing procedures. At the final stage of purification about 10 to 15 mg NaK ATPase were obtained with the total ouabain content of 300 to 400 pm. Therefore, about the half of ouabain content in the microsome was lost by this purification procedure and more than 20 times of protein was washed out, which suggests practically most of ouabain-enzyme complex in the NaK ATPase is derived from the microsome. This can be visualized in different way. As shown in figure 15, the regression lines obtained from the relationship between the ATPase activities in the microsome or the NaK ATPase and their binding of ouabain are in parallel because the slopes of the regression lines are similar.

4. CORRELATION OF THE ACTIVITY OF NaK ATPase WITH THE INOTROPIC OR ARRHYTHMIC EFFECT OF OUABAIN IN DOGS.

In the present study, an attempt has been made to answer the questions as to 1) the magnitude of inhibition of the cardiac NaK ATPase in vivo upon administration of the positive inotropic, arrhythmic and lethal doses of ouabain and 2) the effect of ouabain on the enzyme in vivo under a dose regimen that allows only a positive inotropy and maintains it for a long period of time without leading to arrhythmia or death. The first question might be answered satisfactory from the experiments where the heart was subjected to pacing. That is, the low dose of ouabain infusion for 15 minutes produced a significant increased LV dp/dt(p<0.025) and RVct(p<0.01) as shown in figures 9 and 10 without a significant(p>0.05) inhibition of the NaK ATPase activity. Further infusion of the same dose for 40 minutes increased both LV dp/dt and RVct and also induced various types of arrhythmia and lethality. At the time of manifestation of the arrhythmia or death there was a significant(p<0.005) inhibition of the enzyme.
In the present study, in general, as the duration of ouabain infusion or the total amount of ouabain infused is increased, the total and NaK ATPase activities were gradually reduced in most experiments. Because of this time and dose dependent inhibitory effect of ouabain on the NaK ATPase activity, Dutta et al (1972) suggested that the mathematical products of the plasma ouabain concentration and the duration of ouabain infusion determined the inhibition of the NaK ATPase activity by ouabain in the intact dog.

In the dog cardiac bypass preparation Besch et al (1970) reported that both the "partial and full" doses of ouabain increased significantly both LV dp/dt and RVct with a concomitant and statistically significant (p<0.02) inhibition of the NaK ATPase. However, the plasma concentration of ouabain was 1000 pm/ml in their study (see table 27 for comparison) and it should be pointed out that their "full" ouabain effect implied not only the maximum positive inotropic effect but also a few premature ventricular contraction. Therefore, the present study only clarifies the point that was not made in the study of Besch et al (1970) who failed to mention a significant inhibition of the NaK ATPase activity took place at the appearance of ouabain-induced toxicity.

In the spontaneously beating dog hearts Akera et al (1970) also made a similar observation. However, it is not clear from their study whether their "low dose" of ouabain which increased 26.1 per cent of LV contractile force caused a statistically significant inhibition of the NaK ATPase, although they clearly indicated that their "high dose" reduced it significantly (p<0.05) with cardiac irregularities marked by extrasystoles or a lengthening of the P-Q interval progressing to atrioventricular block, which observations are in agreement with the present study. In the isolated rabbit heart Okita (1972) failed to demonstrate a significant difference in ATPase activities between ATPase isolated before and after washout of positive inotropic effect of strophanthidol-3-bromoacetate, which further supports the fact that inotropic response to digitalis can be shown without a significant inhibition of the NaK ATPase activity.

However, experiments with the paced hearts failed to provide the basis for justifiable comparison between ouabain-induced inotropy and the inhibition of the NaK ATPase because of the transitory response to the high dose of
Table 27. Comparison of various studies on the relationship between ouabain-induced inhibition of NaK ATPase activity and the positive inotropic effect of ouabain in the dog.

<table>
<thead>
<tr>
<th>Author and classification of experiments</th>
<th>Plasma Conc. of ouabain (ps/ml)</th>
<th>Total Dose of ouabain (ug/kg)</th>
<th>Expt Duration (min)</th>
<th>Positive Inotropic Response</th>
<th>NaK ATPase Activity (um F1/ug/hr)</th>
</tr>
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<tbody>
<tr>
<td>Altera et al (1970)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>up to 200</td>
<td>--</td>
<td>23.8±3.1</td>
</tr>
<tr>
<td>Low dose</td>
<td>--</td>
<td>20 to 100</td>
<td>--</td>
<td>26.1±1.8</td>
<td>17.2±3.6</td>
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<tr>
<td>High dose</td>
<td>--</td>
<td>20 to 100</td>
<td>--</td>
<td>54.0±11.0</td>
<td>12.6±1.7</td>
</tr>
<tr>
<td>Besch et al (1970)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1000 (10^-6 M)</td>
<td>--</td>
<td>20 to 25</td>
<td>23.1±3.2</td>
<td>16.2±1.2</td>
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<tr>
<td>Partial</td>
<td>1000 (10^-6 M)</td>
<td>20 to 25</td>
<td>--</td>
<td>8.9±3.2</td>
<td>11.1±1.0</td>
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<tr>
<td>Full</td>
<td>1000 (10^-6 M)</td>
<td>20 to 25</td>
<td>--</td>
<td>49.3±17.6</td>
<td>6.6±1.4</td>
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<tr>
<td>Present study</td>
<td></td>
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</tr>
<tr>
<td>1) Paced Control heart</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>The low dose</td>
<td>121(1.1x10^-7 M)</td>
<td>18</td>
<td>15</td>
<td>116±9.0</td>
<td>9.5±2.3ns</td>
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<tr>
<td>The low dose</td>
<td>121(1.2x10^-7 M)</td>
<td>43</td>
<td>40</td>
<td>128±8.7</td>
<td>6.3±1.4</td>
</tr>
<tr>
<td>2) Spontaneously beating heart</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Inotropic dose</td>
<td>9.1(9.1x10^-9 M)</td>
<td>30.8</td>
<td>300</td>
<td>124±5.5</td>
<td>8.7±1.1ns</td>
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<td>Arrhythmic dose</td>
<td>28.0(2.8x10^-8 M)</td>
<td>61.6</td>
<td>300</td>
<td>105±5.5</td>
<td>6.0±0.6d</td>
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<tr>
<td>Deutscher et al (1971) used digoxin instead of ouabain.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>240</td>
<td>Approx. 86</td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>Approx. 4</td>
<td>80</td>
<td>240</td>
<td>&quot; 115</td>
<td>&quot; 136</td>
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</tbody>
</table>

@ % change from control, mean ± s.e.; # % change from the perfusion period of approx. 10 min. * % change from the control value(100%). According to statistical analysis, a indicates p is less than 0.05 and b, c, d, and e indicate p is less than 0.02, 0.01, 0.005 and 0.001 respectively. N. s. indicates p is greater than 0.05 and ? indicates that the p is not available.
ouabain employed for a short period of time. As Glynn (1964) also pointed out once, not many studies on the NaK ATPase activity or the inhibition of the active translocation of the cations have been designed properly to evaluate the slowly developing effect of digitalis with the therapeutic dose. In order to answer the second question, a different experimental approach was used as presented under the description of "Materials and Method" with the spontaneously beating hearts. It may share a common problem with Glynn's proposition in the sense that this study employed the inotropic dose of ouabain to induce a therapeutic effect of ouabain free from the undesirable toxic symptoms.

In the study with the spontaneously beating hearts, it was noted that the time course of the development of LV dp/dt maximum was well parallel to that assessed by RVct with the inotropic dose of ouabain throughout the 300 min experimental period. Both LV dp/dt and RVct were always significantly greater than the control at any time intervals for 300 min, of which situation was ideal for the comparison of it with other biochemical parameter. Under this steady-state inotropic response to ouabain with the inotropic dose there was no statistically significant inhibition of NaK ATPase activity. This result seems to be surprising. But, since the total dose of ouabain infused for 300 minute experimental period using the inotropic dose of ouabain was only 30.8 ug/kg, it further supports ouabain-dose dependent NaK ATPase inhibition observed in the experiment with the paced hearts. Likewise, the observations reported by Akera et al (1970) and Besch et al (1970) could be the same ouabain-dose related NaK ATPase inhibition rather than the claimed causal relationship between the inhibition of NaK ATPase activity and the inotropic response induced by ouabain.

However, with the arrhythmic dose of ouabain LV dp/dt maximum was not parallel at all time interval with RVct, especially after onset of arrhythmia as shown in table 18 and figure 19. That is, the arrhythmic dose of ouabain infusion increased both dp/dt and RVct in parallel fashion up to 60 min. After that (about the time of induction of a persistent arrhythmia) the LV dp/dt fell gradually despite of the continuous increase of RVct due to continuous infusion of ouabain. A similar result has been reported in the study of Deutscher et al (1971) with digoxin in dogs (see table 27). In their study the RV contractile force measured by strain gauge was increased with respect to time up to 240 min experimental period along with the increase of
RV content of digoxin. However, the parallelism was true with the LV dp/dt only for a short experimental period (60 min in their experiment). At 120 min after digoxin injection, the LV dp/dt was considerably decreased even though the LV content of digoxin remained practically unchanged up to 240 minutes irrespective of decrease of plasma digoxin concentration. Since the parameter, dp/dt is more sensitive to the hemodynamic changes than the contractile tension measured by strain gauge, long term experiment with open-chest dog which probably affected any of hemodynamic factors such as vascular resistance or venous return may result in this difference after onset of arrhythmia.

In the spontaneously beating hearts the arrhythmic dose of ouabain induced various types of arrhythmia within 60 min after the start of ouabain infusion and the arrhythmia was persisted the rest of experimental period (at least 240 min) without leading to fatality. Under such condition the inhibition of the NaK ATPase activity was highly significant in comparison to the control. Thus, whenever the toxic symptoms induced by ouabain are evident, the observation made in this study that there is a significant inhibition of the NaK ATPase activity is in good agreement with Goldstein et al (1971) who observed a significant (p < 0.001) inhibition of the NaK ATPase at onset of arrhythmia induced by digoxin (10^{-6}M) in dogs.

The results of the "full ouabain" experiment of Besch et al (1970) and the "high dose" experiment of Akera et al (1970) are not against this hypothesis because they had also observed a significant inhibition of the NaK ATPase at the initial manifestation of ouabain-induced toxicity. In the intact dog Spain and Chidsey (1971) also reported that the NaK ATPase activity was inhibited significantly (p < 0.01) by the in vivo administration of toxic doses of ouabain to a value of less than 70% of the control activity. From the present study, together with other growing evidence (Mason et al., 1971; Okita, 1972) the causal relationship between the NaK ATPase activity and the arrhythmia induced by ouabain more likely exists rather than the claimed causal relationship between ouabain-induced positive inotropism and the inhibition of NaK ATPase.

5. THE ANTIARRHYTHMIC ACTION OF KCl IN CONNECTION WITH THE ACCUMULATION OF OUABAIN AND THE INHIBITION OF THE NaK ATPase ACTIVITY.

For the management of digitalis toxicity many chemical agents have been tested with varying degree of success. In cardiovascular clinic patients
usually have multiple therapy including diuretics which likely enhance the
digitalis toxicity in those patients due to K depletion. It is generally
agreed that toxic dose of digitalis decrease the myocardial content of K.
Thus, the simple explanation of the antiarrhythmic effect of K may be in
the replenishment of K lost from myocardium induced by the arrhythmic doses
of digitalis.

A slow infusion of K(1.2 meq/kg/hr) without a loading dose of K along
with ouabain had no effect initially on EKG, arterial blood pressure and LV
dp/dt. It took about an hour to reverse the arrhythmia to normal sinus
rhythm. At that time the LV dp/dt was significantly enhanced over that with
ouabain by itself and RVct was increased more than 40 per cent over its
comparable control value. Similar observations have been made by Williams
et al(1966) who reported that KCl allowed further increase of inotropic
also observed a similar effect of KCl in the intact dog, but in their exper­
iment, the protocol was such that more ouabain was infused along with KCl
for a longer period than the comparable control experiment.

In the present study further infusion of KCl at the same rate for a total
of two hours reduced the LV dp/dt 20 to 30 min before the termination of the
experiment. At that time the plasma K concentration was 7.9 meq/l and thus,
the high plasma concentration of K may explain the reduction of LV dp/dt.
At the time of onset of antiarrhythmic effect, the plasma K concentration
had increased to 6.5 meq/l from the control 3.2 in 22 control animals. The
result is in good agreement with Williams et al(1966) who reported that at
least 5.6 meq/l of K was needed to reverse the ouabain-induced arrhythmia in
dogs. Zelis et al(1970) also indicated the requirement of high plasma conc­
centration of K for the reversal of ouabain-induced arrhythmia. However, they
also noted that KCl infusion or pacing the heart failed to raise the dose of
ouabain to induce lethality although they counteracted successfully the arrhy­
thmia induced by ouabain.

Little is known about the mechanism of the antiarrhythmic effect of KCl.
Dutta and Marks(1969) showed an inverse relationship existed between the
accumulation of ouabain and the concentration of K in perfusion medium in the
isolated guinea pig heart. From this observation, Baskin(1971) explored the
effect of KCl on the accumulation of ouabain in relation to its antiarrhyth­
mic effect in the isolated guinea pig heart preparation. Perfusion of $10^{-6}$ M ouabain in Krebs-Henseleit solution containing high K (11.6 mM) reduced significantly the accumulation of ouabain with the manifestation of antiarrhythmic action. In the same experiment he could not see the effect of 8.7 mM KCl on the accumulation of ouabain in most fractions. In the present study with the intact dog KCl infusion at the rate of 1.2 meq/kg/hr did not affect the accumulation of ouabain in most fractions except the partially purified NaK ATPase. However, the ouabain content in the supernate was raised so that the S/P ratio was enhanced from the control 0.47 to 0.72, as was also observed in the case of DPH treatment to be discussed later.

A reduced ouabain binding to the NaK ATPase due to the antiarrhythmic dose of KCl may have a specific implication because of the known affinity of the enzyme for digitalis. The antagonizing effect of KCl on the in vitro inhibition of NaK ATPase by digitaloids has been claimed by various workers with different degree of purity of enzymes and sources (Post et al., 1965; Hoffman, 1969; Dunham and Glynn, 1961; Judah and Ahmed, 1964). This antagonizing effect of KCl may be achieved by interfering with the interaction between digitalis and the NaK ATPase, which may be the cause of the well known antiarrhythmic effect of KCl. The effect of KCl on the binding of digoxin to myocardial NaK ATPase has been studied in vitro (Matsui and Schwartz, 1967). In their study K behaved as an allosteric activator of the NaK ATPase so that KCl reduced the digoxin binding to the enzyme; the current data are in basic agreement with this. Similar observations have been made in an in vitro binding study of ouabain to dog myocardial NaK ATPase with 1 mM KCl (Akera and Brody, 1971).

Although ouabain binding to the partially purified NaK ATPase fraction was reduced with KCl infusion, the infusion failed to relieve the NaK ATPase inhibition by ouabain. This may suggest that ouabain was dislodged by the KCl treatment from the part of the ATPase fraction which may not be related to the ATP hydrolizing activity of the enzyme. That is, the considerable impurity which existed in this partially purified NaK ATPase may have contributed to the nonspecific binding to ouabain which is dissociated by KCl. The failure of KCl to antagonize the ouabain-induced inhibitory effect on NaK ATPase in vivo may be explained by the fact that KCl stabilizes the ouabain and enzyme complex as demonstrated in vitro experiment (Akera and
Brody, 1971) with KCl concentration up to 80 mM. After KCl infusion along with ouabain Akera et al.(1970) noted a further inhibition of the NaK ATPase instead of an increase of the activity. In their case they infused more ouabain for longer period along with KCl until the reversion of arrhythmia to normal sinus rhythm. Thus, the more inhibition of the NaK ATPase is very likely as pointed out by Dutta et al.(1972).

In considering the hypothesis of the possible dissociation between digitalis induced inotropic and arrhythmic actions as claimed by various workers(Scherlag et al., 1968; Helfant et al., 1968; Williams et al., 1966; Roth-Schechter et al., 1970), one may assume that the NaK ATPase has at least two different ouabain binding sites -- a binding site related to the catalytic activity of NaK ATPase and another binding site related to the arrhythmia or toxicity of digitalis. In this study, since relatively a constant amount of ouabain binding to NaK ATPase in connection with the comparable degree of NaK ATPase inhibition characterized the arrhythmia induced by ouabain, it may be reasonable to assume further that a certain extent saturation of these two sites with ouabain may be responsible for the induction of arrhythmia. The present data are befitting if KCl supposes to dislodge specifically ouabain bound to the site related to digitalis-induced arrhythmia without affecting ouabain molecules attached to the site related to catalytic activity. This specific displacement of ouabain by KCl from the site which is responsible for the induction of arrhythmia could be achieved by an allosteric or conformational transition of this enzyme induced by this physiological ligand as reported by Nagai et al. (1970) Lindenmeyer and Schwartz (1970) and Yoda and Hokin (1970b) so that the dissociation energy of enzyme-ouabain complex of one site is far less than that of another site.

Recent study(Hokin et al., 1973) may substantiate this possibility because of the successful demonstration of two subunits of the enzyme. Basically they found that an almost homogenous NaK ATPase from the rectal gland of dogfish consists of a dimer, of which a monomer has a definite catalytic subunit identified by labeling it with p-32 on incubation with ATP-γ-32P, Mg and Na and a carbohydrate containing subunit of which molecular weight of 55,000. However, since the NaK ATPase fraction used in this study was not homogenous by all means, it can be further speculated
that the antiarrhythmic effect of KCl might be achieved by displacing ouabain attached on the same identities existed as impurity in the NaK ATPase fraction. Also it should not be ruled out the possibility that the antiarrhythmic action of KCl is accomplished by the restoration of ionic imbalance which might be created by the infusion of ouabain.

6. ANTIARRHYTHMIC ACTION OF DPH IN CONNECTION WITH ITS EFFECT OF OUABAIN ACCUMULATION AND THE INHIBITION OF THE NaK ATPase ACTIVITY.

The effectiveness of DPH on arrhythmia, especially induced by digitalis has been well established (Helfant et al., 1967a, 1968; Bigger et al., 1970; Katzung et al., 1970). Helfant et al (1968) showed that DPH reversed the negative difference between the a-v K concentration induced by high dose of acetyl strophanthidin in dog. In their study the inotropic effect of ouabain was further developed by counteracting the arrhythmia induced by acetyl strophanthidin with the DPH treatment. From this observation they suggested the dissociation of digitalis effect on K efflux from its inotropic response. Baskin (1971) also reported a similar observation in the isolated guinea pig heart, in which DPH allowed further the inotropic response to ouabain without leading to arrhythmia.

In the present study DPH was very rapidly effective as reported (Bigger et al., 1970) in eliminating the arrhythmia induced by ouabain and increased the LV dp/dt by 5 to 10 per cent above its control value. This increase was statistically not significant. This difference may be attributed to the fact that in this present study DPH was administered after three hours of ouabain treatment, which had effected maximum positive inotropy, leaving no room for further improvement of inotropy once the arrhythmia was removed by DPH. If DPH had been administered earlier with high ouabain infusion rate, the augmented inotropic effect of ouabain after elimination of arrhythmia by DPH might have been seen in this study.

In this study DPH injection with diluent over 1 minute period did not affect femoral arterial blood pressure, LV pressure and its dp/dt. In contrast to this observation Mixter et al (1966) reported myocardial depression after DPH treatment and its effect on the dilatation of peripheral vessels. But it seems purely matter of injection techniques because the fast injection of high dose of DPH is known to depress the heart. In this study it was noted that an accidental fast injection of DPH did depress the heart in a
dog, but the LV pressure and its dp/dt returned to their control value in 5 to 10 min in this dog.

An important objective to this investigation was to study the effect of DPH on the accumulation of ouabain in major myocardial subcellular fractions in intact dog. It may be reasonable to test the hypothesis that the antiarrhythmic effect of DPH may be achieved by reducing the accumulation of ouabain from a specific myocardial fraction. Little is known about the nature of the molecular interaction between DPH and the NaK ATPase in connection with the binding of cardiac glycoside. In view of the possible three-point interaction between cardiac glycosides and the NaK ATPase postulated by Wilson et al(1970), DPH may have a property of a high affinity to the enzyme because the imidazole nucleus of DPH might have an equal or even greater affinity to the component "C" of the NaK ATPase than cardenolide or butyro-lactone as predicted by Wilson et al(1970). The similarity of the ring size and shape between imidazole and crotonolactone including their similar planarity suggests the possibility of structural competition between DPH and active cardiac glycosides.

In the isolated guinea pig heart Baskin(1971) showed that DPH reduced the ouabain accumulation in all myocardial fractions with similar extent. But the supernate accumulated more ouabain so that the supernate to pellet ratio was increased. From this observation he concluded that the antiarrhythmic effect of DPH is due to the displacement of ouabain from the particulate to the supernate. However, the effect of DPH was dose dependent and at least 10^{-4} M of DPH was required to reduce ouabain accumulation. In the present study with intact dog DPH did not significantly decrease ouabain accumulation in any fractions including the partially purified NaK ATPase. However, a significant increase of ouabain content in the supernate was observed in this study which may represent the overall slight decrease of ouabain content in most fractions. Since in this study the plasma concentration of ouabain was 3 \times 10^{-8} M rather than 10^{-6} M used by Baskin(1971) in isolated guinea pig, less dose of DPH may be required to reverse the arrhythmia induced by ouabain, which explains the dose dependent DPH effect. If the density of entire body mass is assumed to be about the same as water, the DPH dose used in this study(8 mg/kg) is equivalent to 3 \times 10^{-5} M.
The antiarrhythmic dose of DPH used in this study failed to increase the activity of partially purified NaK ATPase under the experimental conditions described under "Materials and Method". Similar observation has been reported in dog myocardial NaK ATPase (Spain and Chidsey, 1971; Goldstein et al., 1971) and in human myocardial microsome with DPH concentration ranging from $10^{-2}$ M to $10^{-5}$ M (Gibson and Harris, 1969). In the present study a significant increase of the Mg-dependent ATPase activity with DPH is unexpected because in most experiments of this study the Mg-dependent ATPase activity remained practically unchanged for the wide range of ouabain dose regardless the different experimental procedures. To the best of our knowledge, no systemic in vivo or in vitro investigation has been made on the effect of DPH on the Mg-dependent ATPase activity. Although this enzyme is different from the NaK ATPase, little is known about its characteristics and function at the present time and the implication of the stimulatory effect of DPH on this enzyme observed in this study remains to be answered.

Attempt has been made to explain the action of DPH in terms of stimulation of the NaK ATPase in various tissues. In rat brain tissue Woodbury (1955) showed that DPH treatment reduced the intraneuronal Na content, which was widely cited to justify the concept that DPH stimulates the transport enzyme. DPH induced the increase of K influx (Helfant et al., 1968) may be in agreement with Woodbury's hypothesis. The stimulatory effect of DPH was demonstrated in rat brain cortex synaptosomal preparation (Festoff and Appel, 1968). However, it was true only in medium containing a high Na and low K (50 : 1). In the low ratio (10 : 1) medium DPH had no effect and even further depressed at lesser than 5 : 1. Similar observation has been made by Spain and Chidsey (1971) in dog myocardial NaK ATPase. Other investigators have also suggested the possible stimulatory effect of DPH on the NaK ATPase with various techniques in different tissues such as dog kidney (Kock et al., 1962) and frog skin (Watson and Woodbury, 1972). But, from the observation on the redistribution of cation such as decrease of intracellular Na after DPH treatment, it is difficult to associate it entirely with the stimulatory effect of DPH on the transport ATPase without a direct demonstration of the stimulatory effect of DPH because of the errors involved in the direct measurement of intracellular Na concentration.

The significance of the stimulatory effect of DPH only under the high Na
to K ratio is questionable. However, this does not rule out the possibility that an abnormally high Na-K ratio does not exist in certain specific tissues. Indeed, Danielson (1964) showed that the distribution of water and ions between conductile and contractile tissues of various mammalian species was not the same. Davis et al. (1952) reported a high concentration of Na in the conduction system of bovine cardiac muscle, a low value in the atria and the least in the ventricle; while the distribution of K was the reverse of that of sodium. A similar observation has been made by Mazel and Holland (1958) in the frog and turtle hearts. In cultured HeLa cell Vaughan and Cook (1972) noted that the 86Rb influx rate was raised by 40% in the cells cultured in low K medium (0.7 mM) or in a medium containing ouabain. Their interpretation of the increase of 86Rb influx due to ouabain is that the low K or ouabain containing medium induced the synthesis of new binding sites for ouabain or transport enzyme. This may suggest that such unique ionic concentration (high Na-K ratio, this case at least 35:1) may be created by high concentration of ouabain (10^{-2} M) so that an increase of ion movement could be accomplished by an increase of the synthesis of transport enzyme rather than the direct stimulation of the enzyme.

However, in considering the known fact that most arrhythmia is due to a-v or intraventricular impulse conduction abnormality rather than an impairment of contractile machinery, effect of DPH on ionic fluxes in connection with the NaK ATPase activity in conductile tissues will be one of fruitful areas to be explored. The in vitro interaction between digitalis and the NaK ATPase is known to be slow and parallel to its binding to this enzyme (Albers et al., 1968). To the best of our knowledge, no study has been made on the effect of DPH on the rate of the dissociation of this enzyme-ouabain complex. In order for DPH to reactivate the enzyme inhibited by ouabain, the effect of ouabain upon the enzyme has to be eliminated. This could be achieved by displacing ouabain from the active site or modification of the affinity of K for the enzyme. However, the digitalis-NaK ATPase complex is known to be very stable (Albers et al., 1968; Matsui and Schwartz, 1968; Post et al., 1965) so that the rate of its dissociation does not compare with the fast antiarrhythmic effect of DPH observed in this study in intact dog. Therefore, the fast antiarrhythmic action is likely better correlated with
other cellular events which may exist in the process of impulse formation
or its transmission. In this sense, Brian Hoffman's model (1970) may be
appealing. He assumes the arrhythmia is due to injured zone in cardiac
muscle, which permits reentry of impulses that reexcite the ventricle
culminating in arrhythmia. His explanation of the antiarrhythmic effect of
DPH is associated with the termination of the reentry of irregular rhythm
by increasing the responsiveness of the injured zone based on his observation
that DPH enhanced the $V_{\text{max}}$ of action potential amplitude.
VII. SUMMARY AND CONCLUSIONS

The accumulation of ouabain in major subcellular fractions of intact dog myocardium and the effect of ouabain on the NaK ATPase activity in relation to the various pharmacological effects of ouabain have been investigated in 22 paced and 18 intrinsically beating anesthetized open-chest dogs. By varying the duration of ouabain infusion or its concentration, various pharmacological effects of ouabain were induced in order to correlate them with various biochemical parameters. The effect of ouabain was manifested within predictable infusion periods, so that inotropy, arrhythmia and lethality were produced as a function of ouabain infusion time. Higher doses of ouabain shortened the infusion time for the induction of these effects. However, a relatively constant amount of ouabain was required to produce onset of arrhythmia or death induced by ouabain.

The least ouabain was accumulated in the dog hearts at the time of inotropic response to ouabain and the highest ouabain accumulation was noted at the time of lethality. Comparison of ouabain accumulation among different myocardial fractions revealed that the highest ouabain binding was noted in the NaK ATPase in all experiments. The next highest accumulation of ouabain was recorded in the microsomal fraction. The other fractions, such as nuclear or mitochondrial, showed relatively much less ouabain accumulation compared to that of the microsomal or the NaK ATPase fraction. At the time of onset of arrhythmia or death induced by ouabain, irrespective to the duration of ouabain infusion or its concentration, similar accumulation of ouabain was noted in myocardial subcellular fractions along with comparable inhibition of the NaK ATPase activity.

The specific activity of the NaK ATPase used in this study was about 14 um Pi/mg protein/hr. The Mg-dependent ATPase activity was not changed significantly in most of preparations with various doses of ouabain. The NaK ATPase activity was decreased as the ouabain dose or duration of ouabain infusion increased. At the same time, the ouabain binding to this enzyme progressively increased. The greatest inhibition of the NaK ATPase
activity was noted in the enzyme prepared at the lethal time. A significant inhibition of the NaK ATPase was also observed for the enzyme prepared at the time of onset of arrhythmia. However, a slight but statistically non significant inhibition of the NaK ATPase was observed when a positive inotropic response to ouabain was noted. Overall correlation study based on 22 individual observations and F test indicate that the NaK ATPase activity, ouabain binding to the NaK ATPase and the inotropic effects were well correlated one to another under the experimental design used for the paced heart preparation.

Since the inotropic effect of ouabain observed was not in a steady-state, it may be irrational to correlate inotropic response to ouabain with the NaK ATPase activity under these experimental conditions which induced various effects in a short period of time due to high infusion rate of ouabain. In spontaneously beating heart, two doses (inotropic and arrhythmic) of ouabain induced a steady-state positive inotropic response and a persistent arrhythmia respectively for a number of hour. Under this experimental protocol the ouabain distribution pattern was basically similar to that of the study with the paced hearts except that relatively much less ouabain was accumulated by the various fractions. The arrhythmic dose of ouabain increased the accumulation of ouabain in most fractions compared to the inotropic dose. The NaK-ATPase bound the largest amount of ouabain while the supernate accumulated the least amount of ouabain. The most significant observation is the fact that, under the steady-state positive inotropic response to ouabain induced by the low therapeutic loading and infusion doses without any complications of arrhythmia, there was no significant inhibition of the NaK ATPase activity. On the other hand, with the arrhythmic loading and infusion doses of ouabain which affected a sustained arrhythmia did cause a significant inhibition of the NaK ATPase. Therefore, it is concluded that a steady-state positive inotropic action of ouabain can be demonstrated without a significant inhibition of the NaK ATPase, but whenever ouabain-induced toxicity is evident, a significant inhibition of the NaK ATPase was observed.

In an attempt to understand the problem of digitalis toxicity and the rationale for the use of KCl or DPH against arrhythmia induced by digitalis, effect of KCl or DPH on the accumulation of ouabain and on the inhibition of
the cardiac NaK ATPase was investigated. Both KCl and DPH were very effective in reversing the arrhythmia induced by the arrhythmic dose of ouabain. Initially KCl or DPH increased the LV dp/dt to a varying degree. However, the KCl infusion for a longer period depressed the dp/dt at the terminal stage of experiment because of the high plasma K level.

The dose of KCl used in this study did not reduce the ouabain accumulation by any myocardial fraction except the NaK ATPase. However, KCl raised the ouabain content in the supernate. KCl infusion failed to alter ouabain-induced in vivo inhibition of the NaK ATPase activity. Therefore, it is concluded that the antiarrhythmic effect of KCl is probably not due to the direct reactivation of the inhibited enzyme by digitalis. The possibility that the antiarrhythmic effect of KCl may be achieved by the displacement of ouabain attached to hypothetical arrhythmia-related sites has been discussed.

DPH at the dose tested did not affect ouabain accumulation by the myocardial subcellular fractions, but it raised the ouabain content in the supernate as did KCl infusion. DPH had no effect on the activity of the NaK ATPase inhibited by ouabain, but unlike KCl it raised the Mg-dependent ATPase activity of the enzyme treated with ouabain and partially purified. This study does not support the hypothesis that the antiarrhythmic action of DPH is mediated by stimulation of the NaK ATPase. However, this does not deny the possible stimulatory effect of DPH in certain specialized cellular environments. The action of DPH in counteracting the arrhythmia induced by ouabain is so rapid that it is unlikely that during the short time interval DPH would be able to dissociate the stable ouabain-enzyme complex. Therefore, it tentatively suggests the possibility that the antiarrhythmic site of DPH may reside somewhere in the series of events involved with the generation of the impulse and its transmission for the rhythmic excitation, contraction and relaxation of the heart.
VIII. BIBLIOGRAPHY


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