CA FLUX IN ISOLATED KITTEN HEARTS DURING THE POSITIVE INOTROPIC EFFECT TO OUABAIN

by

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DEDICATION

This Thesis is Dedicated to the Summer of 1973

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ABSTRACT

This investigation was designed to study the mechanism by which ouabain increases Ca influx into kitten cardiac muscle in the presence of the positive inotropic effect. (PIE). To determine the parameters of the associated Ca kinetics, a group of control hearts were perfused sequentially with Krebs-Henseleit solution (K-H solution) at four different perfusate Ca concentrations: 1.25, 2.5, 5.0 and 10.0 mEq/l. Treated hearts were exposed to the same conditions in the presence of ouabain (5×10^{-8} g/ml). A second group of control and treated hearts followed a similar format to the above with the addition of a Ca-free wash between each Ca uptake. Determination of Ca kinetics based on a two compartment Ca uptake curve included: a) half-time for the Ca uptake to approach a steady state. b) rate of Ca uptake by both Ca compartments. c) Ca content of each compartment. Maximum levels of contractile force and rates of restoration of contractile force were also measured.

Ouabain caused a PIE, although it was not always statistically significant. Rates of restoration of contractile force were increased in all treated preparations, although statistical significance was recorded at only one of the Ca perfusate concentrations.

There were no significant differences in half-time, Ca content, or rate of Ca accumulation into Ca_2 which could be attributed to ouabain, although there were instances in which a consistent trend was evident in the ouabain data. In all groups of hearts, Ca content and rate of Ca accumulation was dependent on perfusate Ca concentration, while half-time values remained reasonably constant.

Of greater interest is the effect of ouabain on the kinetics of Ca_{Tr} (trigger compartment) and the correlation data comparing Ca uptake with contractile force. Although neither the Ca content nor the half-time values of Ca_{Tr} were effected to any extent, the rate of Ca accumulation into Ca_{Tr} was consistently elevated by the ouabain treatment. Analysis of the correlation data suggest that the PIE of ouabain may be caused by an increased exchange of intracellular Ca between Ca_2 and the contractile mechanism (CM).

In light of the compartamental model for excitation-contraction coupling (E-C coupling) proposed by Ong and Bailey (1972), the following E-C coupling pathway is proposed to explain the data of the present study. In the presence of ouabain there is an increased rate of Ca accumulation into Ca_{Tr} accompanied by an increase in the exchange of Ca between Ca_{Tr} and Ca_2. This increased exchange of Ca between Ca_{Tr} and Ca_2 triggers a further elevation in the exchange of intracellular Ca between Ca_2 and the CM. This augmented transfer of Ca between Ca_2 and the CM supports the additional E-C coupling which is necessary to maintain the PIE.

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INTRODUCTION

A. Historical Consideration of Ca in Excitation-Contraction Coupling

The necessity for the presence of Ca ions to maintain contractility in the heart was realized nearly one hundred years ago by Ringer (1882). It was not until 1913 that Mines demonstrated that the contractile activity of the heart was dependent on Ca rather than electrical activity. This finding was based on the observation that a heart bathed in a Ca-free medium would stop beating, but would retain its surface electrical activity. Forty years later, Sandow (1952) introduced the concept of excitation-contraction coupling (E-C coupling) in skeletal muscle with the proposal that the Ca ion somehow completed the circuit between the initial depolarization of the cell membrane and the final mechanical response of the muscle cell. Elaboration of this role of Ca in the E-C coupling process of cardiac tissue comprises a large portion of the present study. However, some consideration of the concept of intracellular Ca compartments and their projected role in the E-C coupling process is necessary.

B. Ca Compartment Models and Their Relation with E-C Coupling

The role of Ca in E-C coupling in cardiac tissue has been studied by numerous investigators (Winegrad,1961; Winegrad and Shanes, 1962; Niedergerke, 1957, 1963 a.b., 1969). However, it was the investigations of Niedergerke (1963), which inspired the concept of an intracellular Ca pool which might be involved with the process of E-C coupling. In 1963 he observed an increased influx of Ca during the depolarization and subsequent contraction of the cardiac muscle cell. Of greater significance was his observation that the influx of Ca continued to increase after the

heart had attained maximal levels of contractility. This observation led Niedergerke to postulate the existence of an intracellular Ca pool somehow involved in the E-C process to account for the continued increase in Ca uptake. Very simply, he proposed that the influx of Ca, after being released from a membrane Ca carrier complex (Ca_R), entered the cell and became the intracellular "Activator" Ca pool, which in turn interacted with the contractile mechanism (CM) to initiate contraction. The suggestion of an intracellular calcium compartment being at least partially responsible for E-C coupling has inspired many investigators to elaborate on such a scheme. Most of these revised models are the result of investigative techniques employing Ca washout and/or Ca uptake studies. Winegrad and Shanes (1962, studying Ca flux in guinea-pig atria have proposed a Ca compartment system comprised of three components:

a) A fast component with a half-time of 4.5 minutes

b) A slower component with a half-time of 86 - 168 minutes

c) An unexchangeable Ca component

An investigation of Ca flux in isolated rabbit atria (Teiger and Farah, 1967), yielded a similar three component model with a slight modification in that the third component was considered to be a slowly exchanging Ca storage compartment.

Further refinement of the Ca compartments of cardiac tissue was proposed by Langer (1964, 1965, 1967), who studied Ca washout curves from arterially-perfused dog papillary muscle. He identified five Ca components, termed Phase 0 to Phase 4 which were described as follows (Langer 1965):

Phase 0 Vascular,

Phase 1 Interstitial,

Phase 2 Sarcotubular system,

Phase 3 Intracellular slowly exchanging and,

Phase 4 Intracellular and/or connective tissue.

More closely related to the current research are the results of Bailey and Dresel (1968), who studied the washout of Ca from gas-perfused Langendorf kitten hearts. They have suggested the following three Ca compartments which are presented schematically in Fig. 1

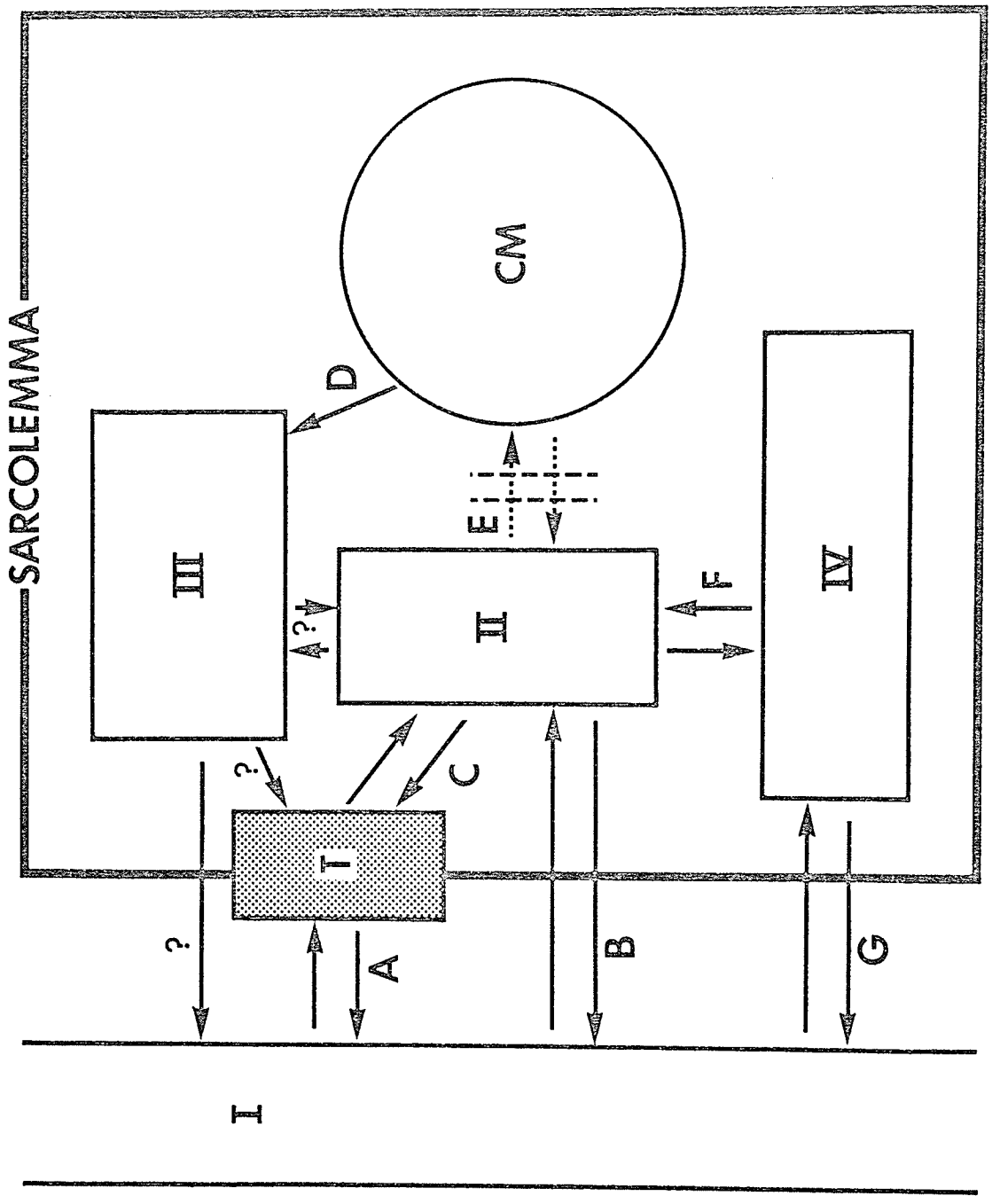
CA_I Vascular Ca

CA_{II} Intracellular Ca pool responsible for the maintenance of contractile force.

CA_{III} Slowly exchanging or non-exchangeable tissue bound Ca.

This model has since been modified by Ong and Bailey (1972), to include an additional "trigger-pool" (depicted in Fig. 1 as T), which is believed to initiate the release of Ca from Ca_2, which then interacts with the CM to initiate the contractile response. Further evidence from Ong and Bailey (1972), using Ca uptake curves as opposed to Ca washouts, suggests the presence of intracellular Ca compartments labelled Ca_{Tr} and Ca_2 which may or may not be equvalent to Ca_I and Ca_{II} of Bailey and Dresel (1968). On the basis of the data from Ong and Bailey (1972), Ca_{Tr} is probably the "trigger-pool" and will be referred to as Ca_{Tr}, while Ca_I of Bailey and Dresel represents vascular Ca. On the other hand, because of the correlation between Ca uptake into Ca_2 and the restoration of contractile force, Ca_2 probably represents the same maintenance pool as does Ca_{II}

Figure 1: A schematic diagram of the hypothetical model for Ca exchange processes occuring during contraction in liquid and gas perfused hearts. See text for details. I, II and III represent the Ca compartments depicted in the Ca-free washout analysis.
I - vascular Ca
II - 'activator Ca'
III - storage pool
IV - residual tissue Ca
T - 'trigger Ca'
CM - contractile elements
The arrows indicate the probable pathway of Ca movement.


SARCOLEMMA
CM
D
E
III
II
IV
F
?
C
I
?
A
B
G
I

although we do not have conclusive proof for this. Further modification of the Ca compartment (Ong, Ph.D. Dissertation) included the proposed Ca_{III} pool as a storage pool rather than non-exchangeable Ca. The investigations of Bailey and co-workers (1972) with gas-perfused kitten hearts, suggests that there is no direct exchange of Ca between Ca_{II} and Ca_{III} during the period of gas perfusion. To complete this description of the Ca compartment model, Bailey and Ong (1973), have proposed a fourth Ca compartment, which is believed to be the non-exchangeable portion of the tissue Ca. Because of the close relationship of Ca to the E-C coupling process, it is probable that Ca plays a role in the PIE to ouabain.

C. The Positive Inotropic Response to Ouabain and its Effect on Ca Kinetics

An excellent review of the inotropic response initiated by ouabain and its relationship with the subcellular components of the myocardium is presented by Lee and Klaus (1971). In addition, a full consideration of the proposed ionic interactions associated with ouabain is found in a review by Glynn (1964).

The PIE to ouabain has long been associated with a requirement for Ca. Loewi (1918) reported that ouabain could not elicit a PIE in the absence of Ca in the bathing medium. However, the sequence of events involving ouabain and Ca to produce the PIE is still a matter of conjecture.

1. Sequence of events involving ouabain, Ca flux and the PIE.

There are at least two possibilities: a) Does ouabain stimulate Ca movement into the cell which in turn increases the force of contracture?

or, b) Is the augmented Ca influx secondary to the increased contractile force which was a direct consequence of ouabain treatment?

Investigations by Holland and Sekul (1959), on the effect of ouabain on ^{45}Ca influx in rabbit atria showed that toxic doses of ouabain increased the exchange of ^{45}Ca. They suggested that at therapeutic levels, ouabain should increase the turnover rate of Ca without disturbing the levels of tissue Ca. Further clarification of this issue was offered by Govier and Holland (1965), who examined the influence of ouabain on contractile force and Ca exchange in rabbit atria. They concluded that ouabain was responsible for the influx of Ca which in turn yielded a positive inotropic effect. This interpretation is logical when one considers experiments in which the external Ca concentration had been increased and a concomitant increase in contractile force was recorded in the absence of ouabain (e.g., Niedergerke, 1963 a). Having agreed upon the general premise that Ca was responsible or at least implicated in the PIE, investigators began to consider more of the specific details such as the source of this Ca involved in the PIE.

2. Source of Ca involved in the PIE.

Three theories evolved regarding the source of Ca necessary for the PIE: a) The PIE was dependent on intracellular stores of Ca which were stimulated by ouabain. Govier and Holland (1965); Klaus (1963); Bailey and Krip (1972), b) The PIE was initiated directly by the influx of extracellular Ca across the cell membrane which was enhanced in the presence of ouabain. Holland and Sekul (1959); Sabatini-Smith and Holland (1967); Bailey and Harvey (1969), or c) or a combination of the two.

As a generalization it appears that ouabain achieves the PIE through an increased influx of external Ca, plus an influence on the intracellular Ca stores, although most compartamental models favour the influx of external Ca, (Langer, 1968).

3. Mechanism by which ouabain influences the transport of extracellular Ca into the cell.

Once again, there are two alternatives to be considered: a) Ouabain increases Ca influx by increasing the permeability of the membrane. b) Ouabain increases Ca influx by increasing the number of Ca carriers in the membrane.

A number of investigators, Holland and Sekul (1959); Sabatini-Smith and Holland (1967); and Bailey and Harvey (1969), on the one hand have observed an increase in the exchange of Ca in the presence of ouabain and have attributed this finding to an increase in Ca permeability of the sarcolemma. On the other hand, there are also some convincing reports in favour of some form of carrier-mediated Ca transport. Reuter and Seitz (1968), demonstrated that the efflux of Ca from cardiac tissue was proportional to the ratio of $\frac{[Ca]_o}{[Na]_o^2}$ which implies some form of competition between the Ca and Na ion for carrier-mediated Ca transport. Additional evidence for such a Ca transport system was presented by Glitsch et al. (1969), who employed double reciprocal plots to study the effect of internal Na concentration on Ca efflux and influx in isolated guinea-pig atria. They found that a high [Na]i or a low [Na]o yielded an increase in Ca accumulation in the atria, which suggests competition between Ca and Na for a carrier complex.

On the basis of these findings it is not unreasonable to speculate that ouabain increases the Ca influx at least partially, by some action on the Ca carrier system. To determine whether ouabain influenced the permeability of the membrane, or a Ca carrier system, or both, Bailey and Sures (1971), did a series of Ca uptake and washout experiments. Their findings showed that ouabain did not alter the half-times to achieve a steady state of Ca uptake while it did increase the amount and the rate of Ca accumulation by the heart. On the basis of this observation, they proposed that ouabain increased Ca exchange through the activation of additional Ca carriers in the membrane, which in the absence of ouabain would be inactive. Although this proposal formed the main substance of their argument, they were unable to rule out completely the possibility that ouabain may also have altered Ca permeability of the cardiac tissue. Thus there remains some degree of uncertainty associated with the possible activation of additional Ca carriers as opposed to an increase in Ca carrier mobility or a change in membrane permeability to explain the mechanism of the action of ouabain on Ca exchange. It is this element of uncertainty regarding carrier activation vs increased permeability or carrier mobility which provides the basis for the present study.

D Statement of the Problem

As a sequel to the study by Bailey and Sures (1971), it was hoped that current investigations might offer further elucidation of the mechanism by which ouabain enhances Ca exchange, either specifically through an increase in the mobility of the available Ca carriers, or through the activation of previously inactive Ca carriers.

To evaluate the effect of ouabain on the uptake of Ca into the two Ca pools involved in E-C coupling, i.e. Ca_{Tr} and Ca_2, contractile force will be restored in Ca-depleted hearts by reperfusion with K-H solution at 1.25, 2.5, 5.0 and 10.0 mEq Ca/l. The rate of Ca accumulation by both pools, and the rate of restoration of contractile force will be determined at each concentration. Data obtained will be analyzed by the Scatchard Format (Koshland, 1970), to determine if ouabain produced an increase in the number of binding sites in either of the Ca pools. The velocity of Ca uptake at each perfusate Ca concentration will be plotted as a function of the perfusate Ca concentration. Since Ca uptake by both Ca pools is saturable, the intercept of the line with the abscissa yields an index of the binding capacity of each pool. A shift to the right of the intercept after ouabain treatment suggests that ouabain increased the number of binding sites available in one or both of the pools.

Because of some shortcomings in the computer analysis of our data, much of the data may not satisfy the requirements of the Scatchard Format analysis. With this qualification in mind, an alternative approach should offer some insight into the mechanism of the influence of ouabain on Ca uptake into the Ca compartments described above.

This alternative consists of an analysis of the kinetic parameters governing the Ca flux of Ca_{Tr} and Ca_2, such as:

a) rate of Ca uptake into the Ca compartment

b) half-time for Ca uptake to approach steady state

c) Ca content of each Ca compartment

In addition, correlation of the rate of Ca uptake in these Ca compartments with the rate of restoration of contractile force in the Ca-depleted hearts will lend additional support to the evidence from the Ca kinetics analysis.

SECTION II

METHODS

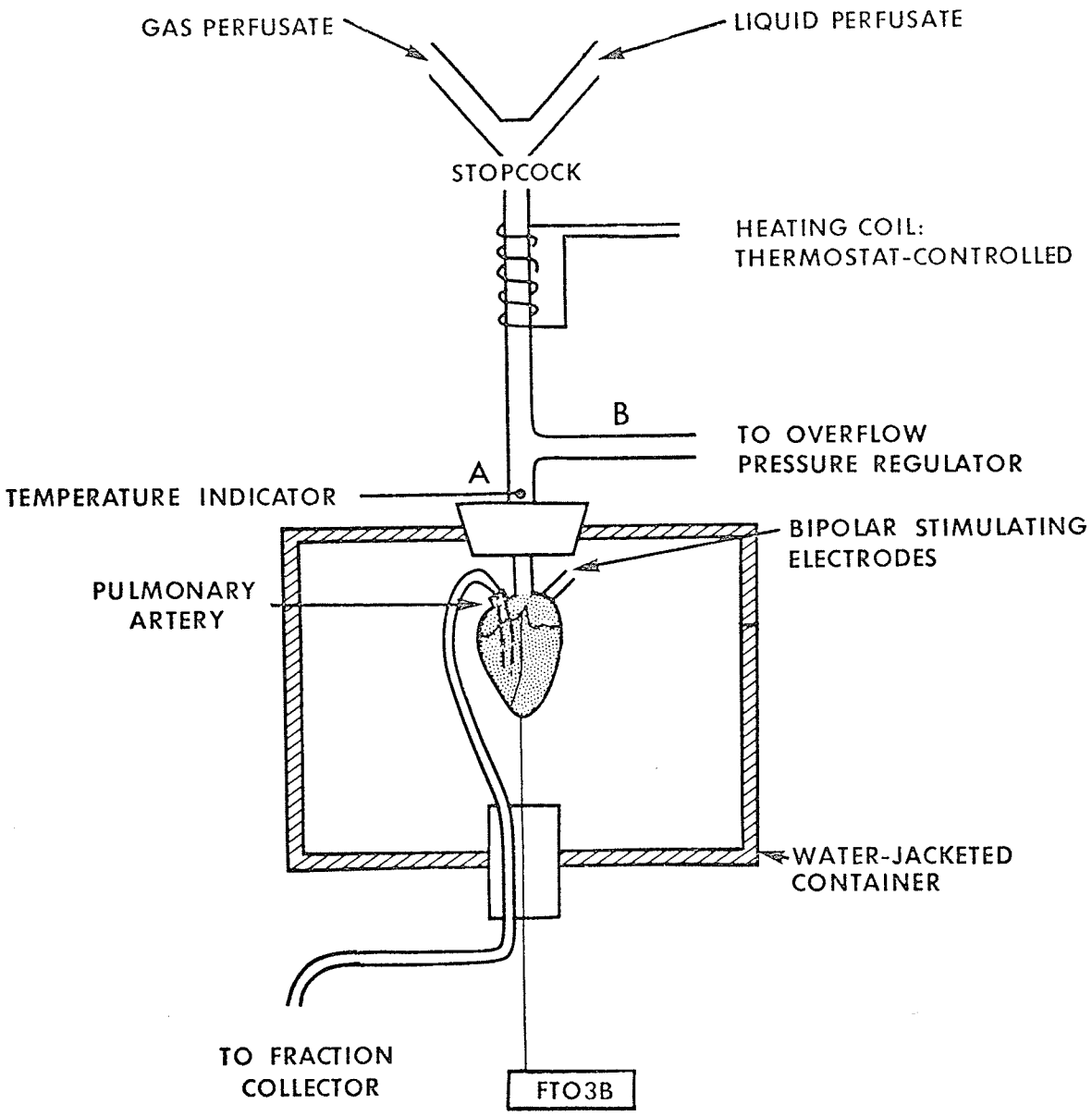
A. Experimental Preparation

1. Perfusion apparatus

A complete description of the gas perfusion apparatus has been published by Gabel and colleagues (1966), and by Bailey and Dresel (1968). The preparation is similar to that shown schematically in Fig. 2. The different perfusates entered the heart through a Y-shaped cannula (one side for gas - one side for liquid) with a thermistor probe (YSI Model 403) in place near the neck of the Y. The flow of current in the heating coil around the glass tubing leading to the cannula was controlled by a thermoregulator (YSI Model 73). The heating coil was employed for the final temperature adjustment of the gas perfusate which was maintained at 37.0°C $\pm$ 0.5°C. Regulation of the perfusion pressure was achieved by immersing a glass tube in a column of water which corresponded to a pressure of 60 mm Hg. Whenever a change from liquid to gas perfusion occurred, the excess liquid perfusate in the cannula overflowed into a side-arm flask.

Liquid perfusates of various compositions were equilibrated with 95% O_2 - 5% CO_2 in glass reservoirs. Perfusion pressure was maintained by exhausting the overflow gases into a column of water at a depth corresponding to 60 mm Hg (Gabel et al, 1966). Heating of the liquid perfusate to 37.0° $\pm$ 0.5°C was achieved by spiral condensers located just prior to the cannula. The gas mixture was heated in a similar fashion, and then humidified in a water-jacketed scrubbing bottle filled with saline.

Figure 2: Schematic drawing of the perfustion apparatus.
See text for details.


GAS PERFUSATE
LIQUID PERFUSATE
STOPCOCK
HEATING COIL:
THERMOSTAT-CONTROLLED
B
TO OVERFLOW
PRESSURE REGULATOR
A
TEMPERATURE INDICATOR
BIPOLAR STIMULATING
ELECTRODES
PULMONARY
ARTERY
WATER-JACKETED
CONTAINER
TO FRACTION
COLLECTOR
FTO3B

The perfused heart was maintained at a temperature of 37.0° ± 0.5°C and high humidity in a water-jacketed plexiglass box. Both the cannula in the pulmonary artery and the monofilament attached to the apex of the heart were passed through a hole in the bottom of the box. To prevent dripping of perfusate liquid onto the Grass FT03B force displacement transducer, the hole was sealed with Plastibase^{R} (Squibb, New York), which was held in place by attachment of a finger from a rubber glove. Friction of the Plastibase against the monofilament damped the contractile force recordings to a small extent.

2. Preparation of the heart

Kittens of either sex weighing 0.7 - 1.2 kg were killed by a blow to the head. To prevent formation of thrombi in the coronary arteries after the animal was killed, 1000u/kg Heparin (Connaught Labs, Toronto, Canada) was injected one hour prior to sacrifice. After removal of the heart, it was placed immediately in cold (4°C) Krebs-Henseleit solution (Krebs and Henseleit, 1932), and all extraneous tissue was removed. The heart was then attached by the aorta to the perfusion cannula. An incision was made into the pulmonary artery, and a cannula for effluent collection was inserted through this opening into the right ventricle. To prevent loss of perfusate, both the venae cavae and the pulmonary vein were ligated. At the apex of the left ventricle, a small incision was made to allow excess gas and liquid to escape, and also to serve as a point of attachment for the stainless steel clip. A monofilament line attached to this clip was affixed to a Grass FT03B force displacement

transducer. Changes in isometric tension were recorded on a Brush Mark 220 polygraph. Resting tension was adjusted to 10 g which produced a developed force of contraction which was approximately 50% of the maximum on the basis of the length-tension relationship in a majority of hearts. Resting tension was maintained during all experiments. Electrical stimulation at twice threshold voltage and at a frequency of 180 beats/min. was supplied by a Grass S6 stimulator by one electrode attached to the right atrium and the clip in the apex which served as the indifferent electrode.

B. Composition of Perfusion Solutions

1. Modified Krebs-Henseleit Solution

The liquid perfusate used to equilibrate the hearts prior to the experiment, was modified Krebs-Henseleit solution (K-H solution). The composition of the mofified K-H solution is shown in Table 1. After equilibration with 95% O_2 - 5% CO_2 at 37.0°C the pH of the solution was 7.4.

2. Modification of control perfusate

a. Ca-free perfusate: Composition was changed by omission of the $CaCl_2 \cdot 6H_2O$.

b. Different Ca concentrations: Appropriate amounts of the stock solution $CaCl_2 \cdot 6H_2O$ were added to make four different Ca concentration perfusates: 1.25 mEq/l, 2.5 mEq/l, 5.0 mEq/l, and 10.0 mEq/l.

c. Addition of ouabain: In the treated hearts a solution of aqueous ouabain (Fluka, Switzerland) was added to all perfusates in the amount 50 ul/l, yielding a final concentration of 5×10^{-8} g/ml (8.5×10^{-8} M)

Figure 3: Perfusion protocol to determine the effect of different perfusate Ca concentrations on Ca uptake and restoration of contractile force, without calcium-free wash (CNW and DNW). In the DNW hearts, ouabain (5×10^{-8} g/ml) was added to all perfusates.

KREBS-HENSELEIT SOLUTION	Equilibration	(30 min.)
↓		
GAS PERFUSION	Equilibration	(30 min.)
↓		
Ca-free wash		(1-2 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
FIRST CALCIUM UPTAKE	1.25 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
SECOND CALCIUM UPTAKE	2.5 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
THIRD CALCIUM UPTAKE	5.0 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
FOURTH CALCIUM UPTAKE	10.0 mEq/l.	(2-3 min.)

TABLE I

COMPOSITION OF MODIFIED KREBS-HENSELEIT SOLUTION

Component	Concentration	
	mM	g/l
NaCl	112.5	6.56
KCl	4.5	0.33
$NaH_2PO_4 \cdot 2H_2O$	1.2	0.18
$MgSO_4 \cdot 7H_2O$	1.2	0.29
$NaHCO_3$	26.2	2.20
$CaCl_2 \cdot 6H_2O$	2.5	0.55
Glucose	11.2	2.00

in the perfusate solution. Calculated osmolarity of the above modified solutions ranged from between 300 and 320 mOsm which did not differ significantly from the physiological osmolarity of 290-320 mOsm (Ruch and Patton, 1965). Perfusate solutions were prepared on the day of the experiment from four stock solutions as follows:

A. NaCl plus KCl

B. $NaH_2PO_4 \cdot 2H_2O$ plus $NaHCO_3$

C. $MgSO_4 \cdot 7H_2O$

D. $CaCl_2 \cdot 6H_2O$

which were stored at 4°C.

Solution B was bubbled with 100% CO_2 for an hour prior to the experiment as stipulated by Krebs and Henseleit (1932). The four solutions were stored separately to prevent formation of microcrystals of insoluble Ca carbonate and Ca phosphate which have been shown to obstruct coronary vessels (Young, 1968).

C. Experimental Protocol

1. Perfusion scheme

Two experimental designs were employed in this study, one in which Ca-free washes were interpolated between each Ca uptake (CW), and one group in which no Ca-free wash was used (CNW). In the CNW hearts, the experimental design was further divided into untreated and ouabain treated hearts (DNW). A flow chart for these two groups of hearts is shown in Fig. 3. A contractile force tracing corresponding to this experimental protocol is shown in Fig. 6a and 6b. The second group of hearts was also divided into two sets: controls with a Ca-free wash (CW), and ouabain

treated test hearts, (DW), also exposed to the Ca-free wash. The experimental protocol is shown in Fig. 4, while typical contractile force tracings are presented in Fig. 7a and 7b.

a. A Ca uptake without Ca-free wash (CNW and DNW hearts).

Initially, the hearts were equilibrated for 30 min. with 5.0 mEq Ca/l. K-H solution, followed by 30 min. of perfusion with 95% O_2 -5% CO_2 gas mixture. Next, the heart was washed out with a Ca-free perfusate until contractility was reduced to less than 1 gram. At this level of contractility, it was considered that there was little endogenous Ca available for contraction, and that minimal damage was done to the functional integrity of the cardiac muscle cell. Ca-free washout was followed by a brief 20 sec period of gas perfusion to remove any fluid remaining in the coronary vasculature after washout which might interfere with the Ca-uptake perfusion.

After gas perfusion, the first of the sequence of Ca uptake perfusions was initiated with the 1.25 mEq Ca/l. perfusate. This was followed by a second brief period of gas perfusion to clear the liquid perfusate out of the coronaries. The remaining Ca perfusates (2.5, 5.0, and 10.0 mEq Ca/l) were introduced into the heart in order of increasing concentration. A brief gas perfusion was always interpolated between each of these Ca uptake perfusates. During each Ca perfusion, samples of the effluent were collected at 6 sec intervals from the cannula in the pulmonary artery. A record of contractile force was maintained throughout the experiment. The treated hearts (DNW) were exposed to a concentration of 5×10^{-8} g/ml ouabain in all perfusates.

Figure 4: Perfusion protocol for the second group of hearts comprised with Ca-free wash between each perfusate (CW) and ouabain-treated hearts with a Ca-free wash (DW). In addition to the Ca-free wash, the perfusate sequence was also altered.

KREBS-HENSELEIT SOLUTION	Equilibration	(30 min.)
↓		
GAS PERFUSION	Equilibration	(30 min.)
↓		
Ca-free wash		(1-2 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
FIRST CALCIUM UPTAKE	5.0 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
Ca-free wash		(1-2 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
SECOND CALCIUM UPTAKE	2.5 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
Ca-free wash		(1-2 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
THIRD CALCIUM UPTAKE	10.0 mEq/l.	(2-3 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
Ca-free wash		(1-2 min.)
↓		
Brief gas perfusion		(20 sec.)
↓		
FOURTH CALCIUM UPTAKE	1.25 mEq/l.	(2-3 min.)

b. Ca uptake with a series of Ca-free washes (CW, DW)

The second group of hearts received a similar series of Ca perfusates to the above, with the addition of a Ca-free wash between each perfusate. The initial equilibration on K-H solution, gas perfusion and Ca-free washout was unchanged. However, the sequence of the perfusates was changed to: 5.0 - 2.5 - 10.0 - 1.25 mEq Ca/l. (Fig. 4). The interjection of a Ca-free wash between each Ca uptake was done to eliminate the possibility of partial filling of Ca compartments under the initial protocol (CNW, DNW) between each Ca uptake. It was felt that partial filling of the Ca compartments could produce Ca efflux during uptake and distort the values of the kinetic parameters governing Ca movement, e.g., the half-time to approach a steady state of Ca influx or the rate of filling the Ca compartments. The DW hearts were exposed to ouabain (5×10^{-8} g/ml).

2. Analytical procedures

a. Determination of ion concentrations

Collection of the sample effluent through the cannulated right pulmonary artery was timed at 6 sec intervals, using a Model 272 (ISCO) Fraction collector (ISCO, Lincoln, Nebraska). The average flow rate for the perfusate was 4.5 $\pm$ 0.2 ml/min/g wet weight of heart tissue, and was reasonably constant in both groups of hearts throughout all phases of an experiment. The strength of contractile force was measured from the tracings at 6 sec intervals to coincide with the collection time of the effluent samples.

At the completion of the perfusion sequence, the heart was removed from the apparatus and a sample of approximately one g was cut from the

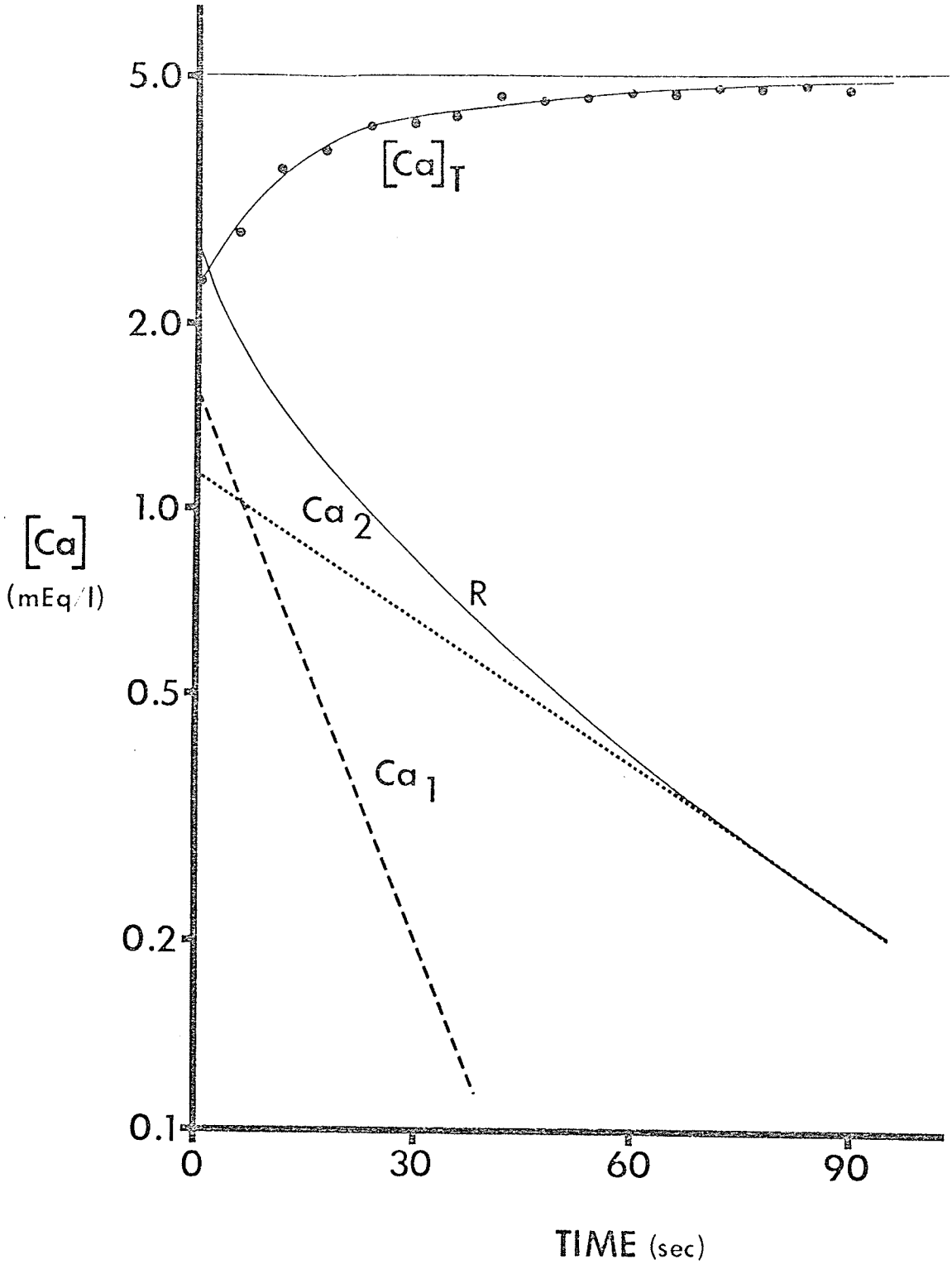
ventricle, which was then partially dried by blotting, and weighed. Remaining moisture was removed from the tissue sample by drying for 24 hours at 80°C in vacuo and the dry weight was noted. Ca ion concentrations for the effluent and tissue samples were determined using a Perkin-Elmer 303 Atomic Absorption Spectrophotometer (Norwalk, Connecticut). The ashed samples of heart tissue and aliquots of effluent were prepared for Ca determination by dilution with 1% lanthanum in 5% (v/v) HCl.

3. Graphical analysis of Ca uptake data and contractile force

a. Ca uptake data

The Ca concentration measured in the effluent during perfusion with 1.25, 2.5, 5.0, and 10.0 mEq Ca/l was plotted as a logarithmic function of time. Best fit Ca uptake curves were drawn with a french curve through the data points. The asymptote which this curve approached was the Ca concentration measured in the perfusate. This curve smoothing method was employed because the different data points when plotted on the expanded scale of semi-logarithmic paper were generated disproportionately large error values. Further justification of this procedure is given in the Discussion. Each value of Ca concentration was subtracted from the asymptote value and the resultant points were then plotted against time on semi-logarithmic paper (See Fig. 5). In essence, this procedure converts an uptake curve into a corresponding washout curve which was then analyzed by computer. The computer analysis for these washout curves was based on the graphical analysis as described by Riggs (1963).

Figure 5: The uptake of Ca in a typical experiment during reperfusion with 5.0 mEq Ca/l. The heart was first depleted of Ca by Wash I. The logarithm of Ca concentration in the effluent is plotted as a function of time. The curve, $[Ca]_T$, is the least squares best fit line for the data points (⊙). The horizontal line indicates the concentration of Ca in the perfusion medium, 5.0 mEq Ca/l. The curve, R, is the least best fit line for the difference between the Ca concentration measured in the effluent and the reperfusion Ca concentration. The broken lines are the two compartments resolved by graphical analysis.


5.0
2.0
1.0
0.5
0.2
0.1
[Ca]
(mEq/l)
[Ca]T
Ca 2
R
Ca 1
0
30
60
90
TIME (sec)

In its simplest form, this computerized compartamental analysis operated as follows: The least squares best-fit line was calculated for the last five data points, after which each subsequent data point was tested. If four subsequent data points were above the best-fit line, and if both the third and fourth points were more than one standard deviation away from the line, another best-fit line was drawn through the four data points and the slopes of the two lines compared. This procedure yielded two alternatives: if there was no significant difference ($P > 0.05$) between the two slopes, then a new best-fit line through all the data points (9) examined became a tentative compartment while subsequent data points were analyzed the same way. On the other hand, if there was a significant difference between slopes ($P < 0.05$) then the second best-fitting line became another tentative compartment and subsequent data points were tested as above until all data points in the uptake were analyzed.

The uptake of Ca by Ca-depleted hearts was described by the following general equation:

$$[Ca]_T = 5.0 - [Ca]_{1t=0}\exp(-k_1 t) - [Ca]_{2t=0}\exp(-k_2 t) \qquad (1)$$

(The symbols used are defined in Table 2)

Note: Substitute 1.25, 2.5, 10.0 in place of 5.0 for the appropriate perfusate Ca concentration.

The unknowns in equation 1, $[Ca]_{1t=0}$ $[Ca]_{2t=0}$ k_1 and k_2 were determined by the computer program for the analysis of Ca washout curves, after the Ca uptakes had been subtracted from the asymptote and plotted as Ca washout curves. When the influx of Ca into the heart was equal to

the Ca efflux from the heart, the Ca concentration in the perfusate was equal to the Ca concentration of the effluent. On this basis, the slopes (k_1 and k_2 equation 1) are indicative of the rate of approach to a steady state of Ca influx into Ca_{Tr} and Ca_2, respectively (Fig. 5). In other words, these slopes are considered to be rate constants for the approach to steady state of Ca uptake into the appropriate compartment Ca_{Tr} and Ca_2). Thus, the slopes, k_1 and k_2 can be interpreted as rates of filling of these Ca pools in the heart.

i. Half-time

The mathematical relationship between the half-time for Ca uptake ($T_{1/2}$) and the rate constants is determined by equation 2.

$$T_{1/2} = 0.693.\ k_i^{-1} \qquad (2)$$

(See Table 2 for definition of symbols)

ii. Ca content

The quantity of Ca accumulated by each compartment during the first 6 seconds of reperfusion was calculated by multiplying $[Ca]_{NT=0}$ by the volume of the effluent sample and dividing by the weight of the heart. The total quantity of Ca taken up by either compartment was the product of the effluent volumes and $[Ca]_N$ summed over N samples and divided by heart weight. It was expressed in terms of mEq/kg Tissue Wet Weight by the following equation:

$$Ca_n \text{ content } = \frac{\sum_{i=1}^{N} [Ca]_{ni}.Vi}{W} \qquad (3)$$

(See Table 2 for definition of symbols)

TABLE II

DEFINITION OF SYMBOLS

Symbols	Definition
Ca_n	The n^{th} compartment where n = 1 or 2 for uptake data.
$[Ca]_T$	Total Ca concentration in effluent (mEq/l).
$[Ca]_{ni}$	Ca concentration in the i^{th} sample of effluent from the n^{th} compartment (mEq/l).
$[Ca]_{nt=O}$	Ca concentration for the n^{th} compartment in the first sample (mEq/l).
k_n	Rate constant or slope for the n^{th} compartment (sec^{-1}).
V_i	Volume of i^{th} sample of effluent (ml).
W	Wet weight of ventricles (g).
t	Time of collection of i^{th} sample (seconds).

b. Ca content based on a one compartment analysis.

To compare our Ca content data with those of Bailey and Sures, (1971), the Ca content data from the DW and CW groups at 5.0 mEq Ca/l were re-analyzed on the computer using a one-compartment analysis. This was done (using the same program as previously described) by fixing the boundaries of a single compartment; (rather than allowing the computer to determine the boundaries of a two compartment system) and changing them when necessary until a single compartment analysis with the lowest standard deviation had been analyzed. The Ca contents were calculated as described by equation 3 and compared with the Ca contents of the same experiment which had been analyzed as a two component system. This one component Ca content analysis was also compared with the Ca content data of Bailey and Sures (1971). Further explanation of this comparison is presented in the Discussion.

c. Contracile force.

i. Maximum contractile force

Levels of contractile force were measured directly from the contractile force tracings. In instances where recording sensitivity had been altered, the appropriate conversion factors were employed.

ii. Rate of restoration of contractile force

Rate of restoration of contractile force was also measured directly from the contractile force tracings. A line was drawn by eye through the steepest portion of the tracing (usually near the beginning), and the slope calculated from it was considered to be the rate of restoration of contractile force. Conversion factors were employed where the

sensitivity of the tracings had been changed.

D. Statistical Analysis

Correlation analysis (Steel and Torrie, 1960) of the rate of Ca uptake with the rate of restoration of contractile force in Ca-depleted hearts was done by the same method as employed by Bailey and Dresel (1968) for Ca washout studies.

Tests of significance between means of various parameters of ion kinetics were analyzed by Student's unpaired t-Test (Dixon and Massey, 1957). For all analyses the criterion for statistical significance was pre-selected at a probability of 0.05.

SECTION III

RESULTS

A. Effect of Perfusate Ca Concentration on Restoration of Contractile Force and Ca Kinetics.

1. Restoration of contractile force.

a. Maximum contractile force

Reperfusion of hearts previously depleted of Ca by a Ca-free wash restored contractile force to a new steady level in both CW and CNW hearts. This is illustrated in Fig. 6 a and b and Fig. 7 a and b showing typical contractile force tracing of a CW and CNW treatment. These tracings correspond with the protocol outlined in Fig. 3 and 4, respectively. Table 3 shows that the maximum force of contraction developed by each group of hearts was related to the different perfusate Ca concentrations. Furthermore a comparison of the CW and CNW hearts indicated no significant difference in maximal contractile force achieved at the four perfusate Ca concentrations.

b. Rate of restoration of contractile force

The rate of restoration of contractile force in CW and CNW hearts previously depleted of Ca varied directly with the perfusate Ca concentration as shown in Table 4. It should be noted that in both groups of hearts, (CNW and CW), the rate of restoration of contractile force did not increase further at the perfusate Ca concentration above 5.0 mEq/l. In comparing the rates of restoration of contractile force between CW and CNW hearts, no significant differences were observed at any perfusate Ca concentration.

2. Kinetics of Ca uptake into compartment two (Ca_2)

Three parameters were used to describe the kinetics of Ca uptake

TABLE III

The Effect of Different Perfusate Ca Concentrations (1.25, 2.5, 5.0 and 10.0 mEq/l) on Maximum Contractile Force in CW and CNW Hearts.

CONTRACTILE FORCE

(g)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	1.1 ± 0.2 * (3)	4.1 ± 0.8 (4)	8.2 ± 2.0 (6)	13.6 ± 6.4 (3)
CW	-	4.8 ± 1.1 (4)	11.0 ± 1.9 (4)	12.4 ± 2.2 (3)

Number in parentheses indicates number of hearts

* Mean ± S.E.

TABLE IV

The Effect of Different Perfusate Ca Concentrations on the Rate of Restoration of Contractile Force in CW and CNW Hearts.

RATE OF RESTORATION OF CONTRACTILE FORCE

(g/sec)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.10 ± 0.02 * (2)	0.20 ± 0.06 (4)	0.23 ± 0.07 (6)	0.15 ± 0.06 (3)
CW	-	0.23 ± 0.1 (3)	0.67 ± 0.30 (4)	0.66 ± 0.34 (4)

Number in parentheses indicates number of hearts

* Mean ± S.E.

Figure 6a: The effect of increment in perfusate Ca concentrations (1.25, 2.5, 5.0, 10.0 mEq/l) on the restoration of contractile force in Ca-depleted hearts (Group CNW).

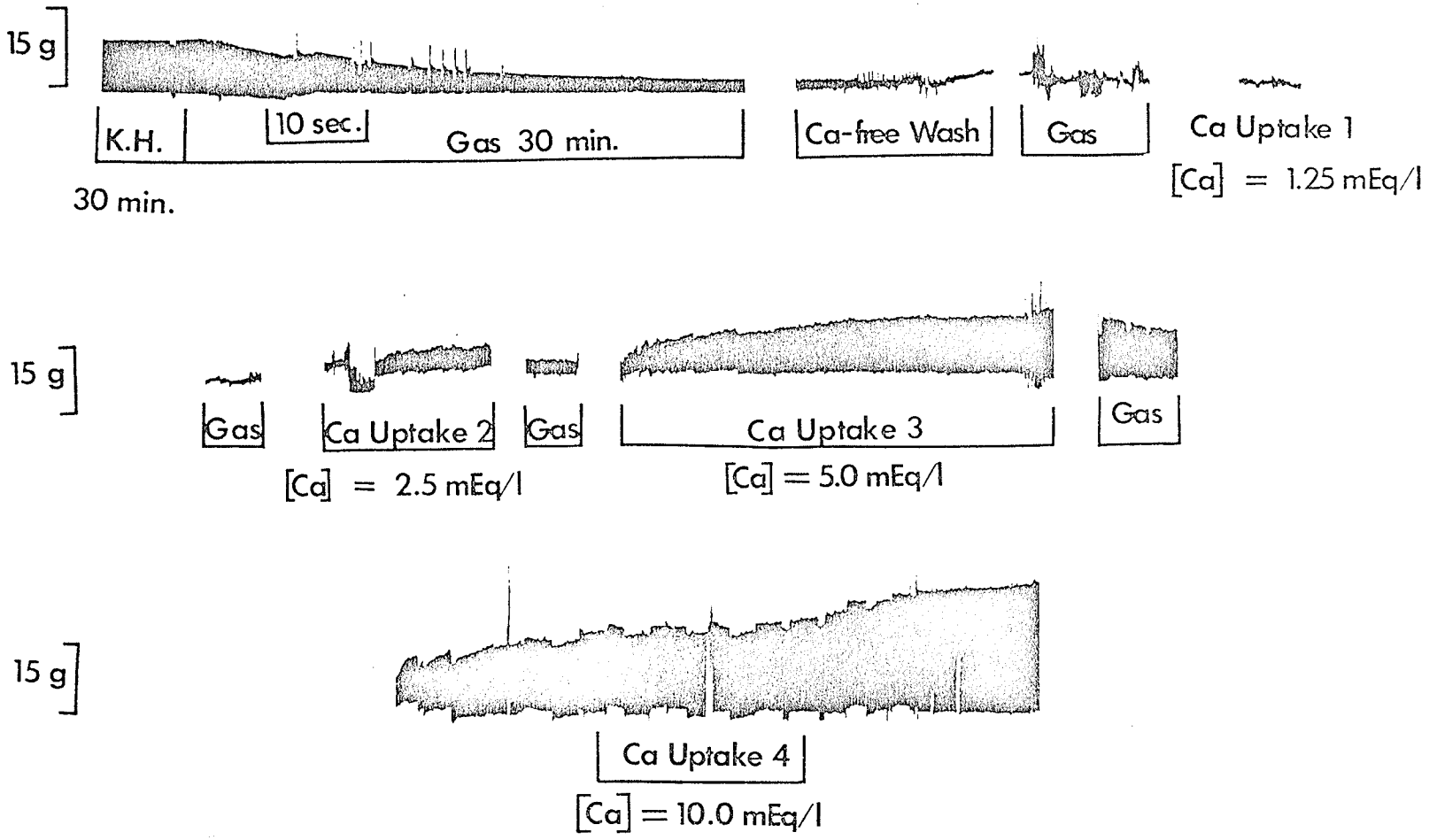

15 g
K.H.
10 sec.
Gas 30 min.
Ca-free Wash
Gas
Ca Uptake 1
[Ca] = 1.25 mEq/l
30 min.
15 g
Gas
Ca Uptake 2
Gas
Ca Uptake 3
Gas
[Ca] = 2.5 mEq/l
[Ca] = 5.0 mEq/l
15 g
Ca Uptake 4
[Ca] = 10.0 mEq/l

Figure 6b: The effect of ouabain and increments in perfusate Ca concentrations (1.25, 2.5, 5.0, 10.0 mEq/l) on the restoration of contractile force in Ca-depleted hearts (Group DNW).

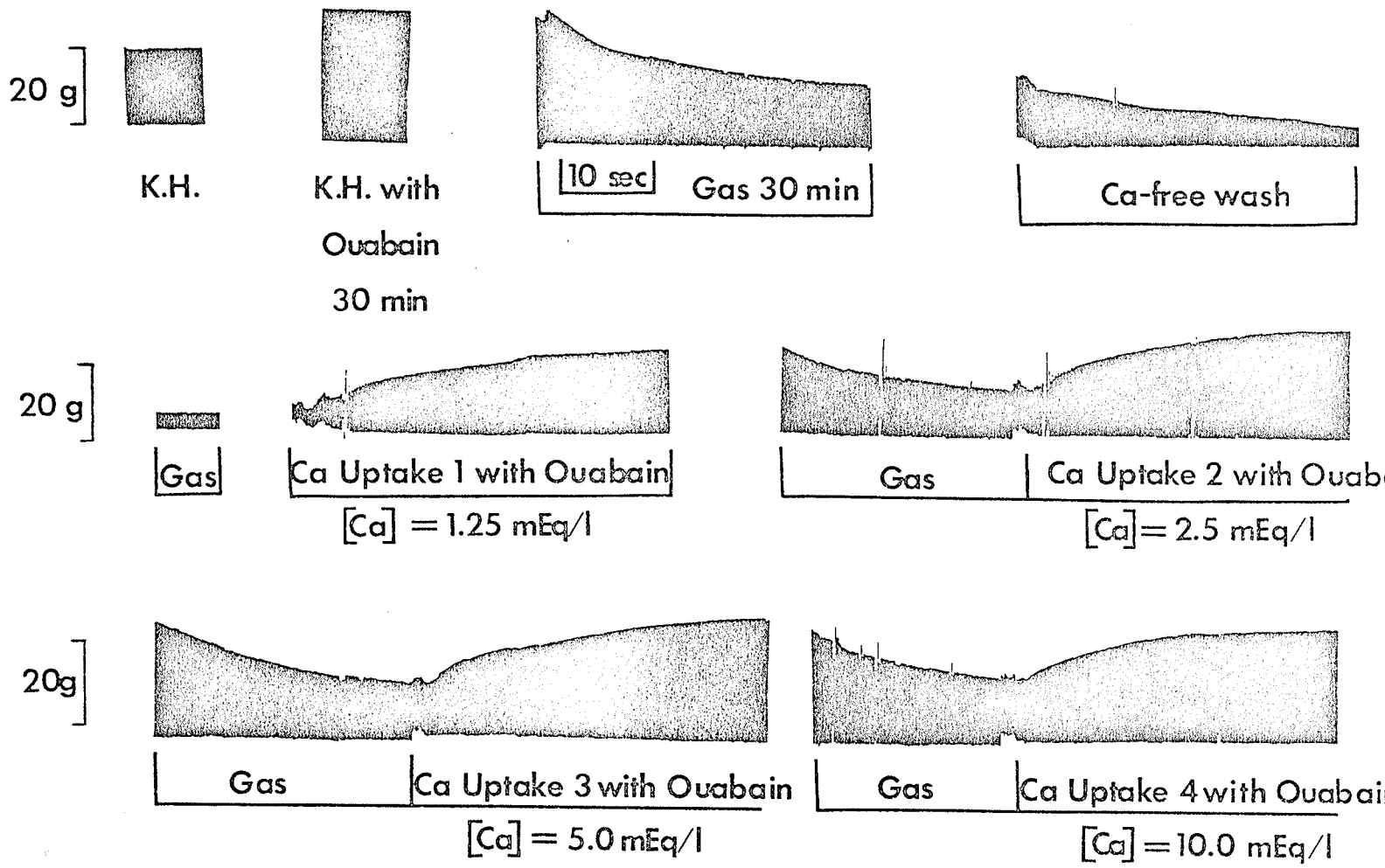

20 g
K.H.
K.H. with
Ouabain
30 min
10 sec
Gas 30 min
Ca-free wash
20 g
Gas
Ca Uptake 1 with Ouabain
[Ca] = 1.25 mEq/l
Gas
Ca Uptake 2 with Ouabain
[Ca] = 2.5 mEq/l
20g
Gas
Ca Uptake 3 with Ouabain
[Ca] = 5.0 mEq/l
Gas
Ca Uptake 4 with Ouabain
[Ca] = 10.0 mEq/l

Figure 7a: The effect of different perfusate Ca concentrations on the restoration of contractile force in Ca-depleted hearts (Group CW) after a Ca-free wash between each Ca uptake.

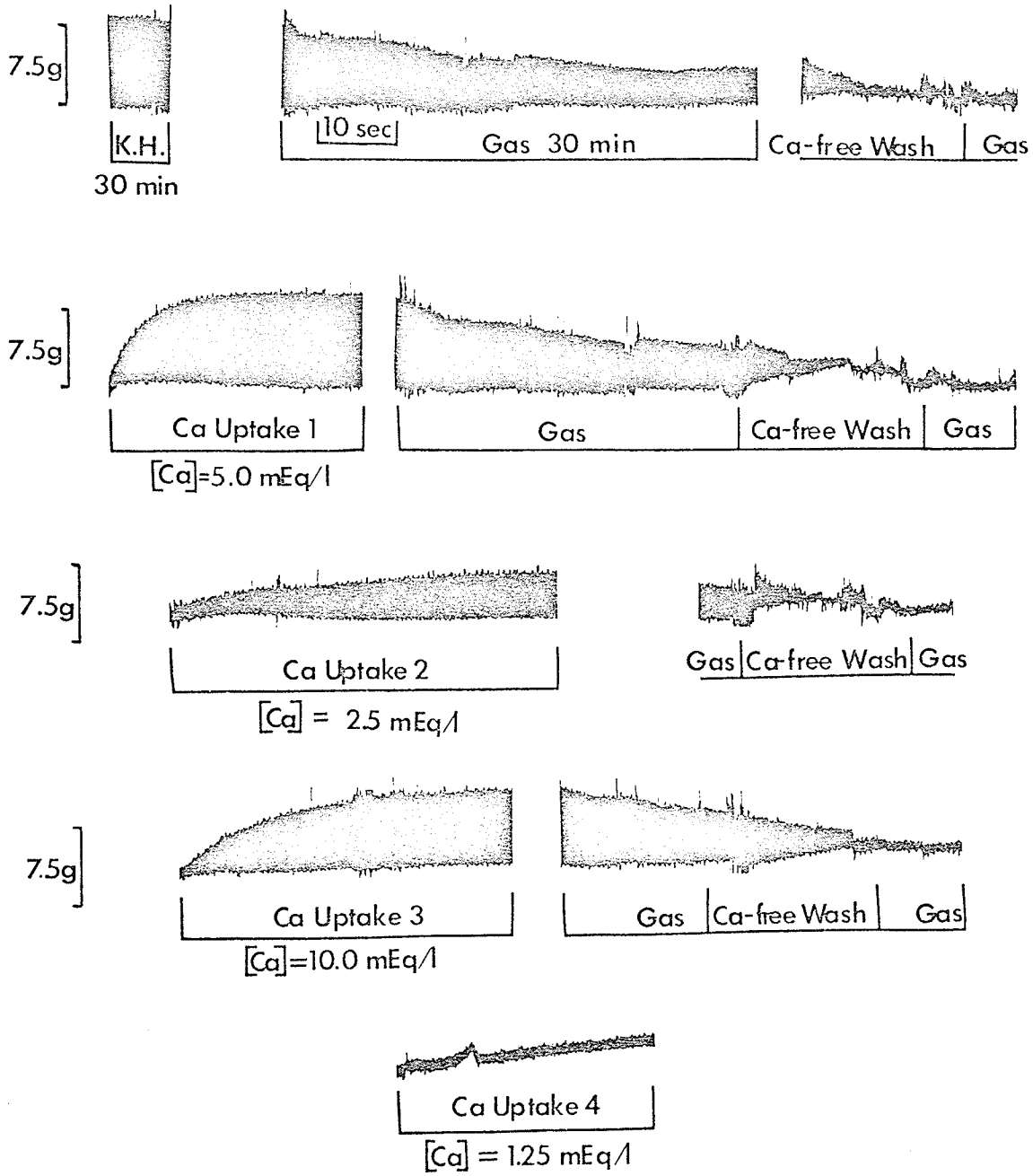
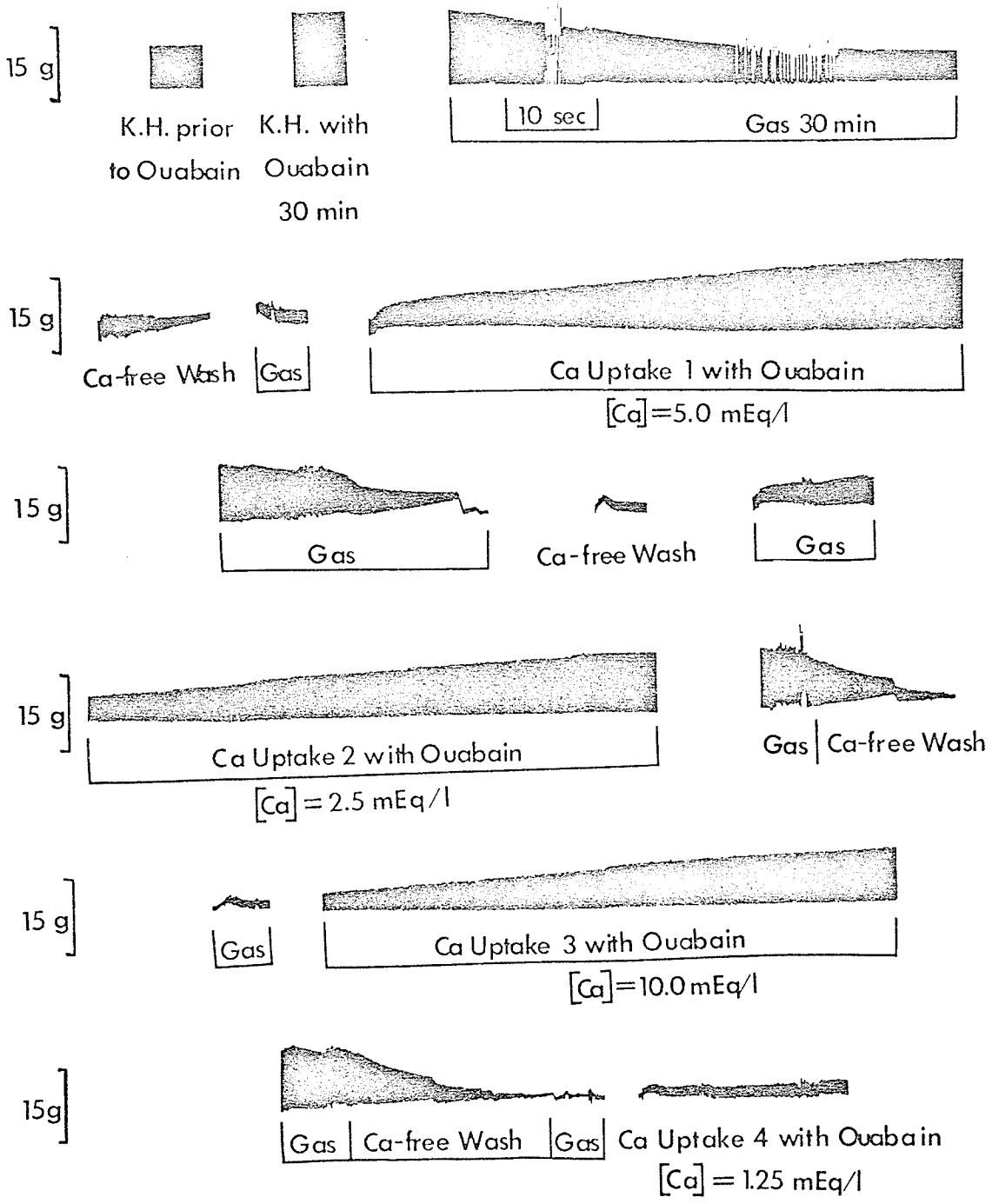

7.5g
K.H.
30 min
10 sec
Gas 30 min
Ca-free Wash
Gas
7.5g
Ca Uptake 1
[Ca]=5.0 mEq/l
Gas
Ca-free Wash
Gas
7.5g
Ca Uptake 2
[Ca] = 2.5 mEq/l
Gas
Ca-free Wash
Gas
7.5g
Ca Uptake 3
[Ca]=10.0 mEq/l
Gas
Ca-free Wash
Gas
Ca Uptake 4
[Ca] = 1.25 mEq/l

Figure 7b: The effects of different perfusate Ca concentrations (ouabain-treated) with a Ca-free wash between each Ca uptake, on the restoration of contractile force in Ca-depleted hearts (Group DW).


15 g
K.H. prior to Ouabain
K.H. with Ouabain 30 min
10 sec
Gas 30 min
15 g
Ca-free Wash
Gas
Ca Uptake 1 with Ouabain
[Ca]=5.0 mEq/l
15 g
Gas
Ca-free Wash
Gas
15 g
Ca Uptake 2 with Ouabain
[Ca] = 2.5 mEq/l
Gas
Ca-free Wash
15 g
Gas
Ca Uptake 3 with Ouabain
[Ca]=10.0 mEq/l
15g
Gas
Ca-free Wash
Gas
Ca Uptake 4 with Ouabain
[Ca] = 1.25 mEq/l

into Ca_2. These parameters have been derived in the methods section, and are as follows: a. Half-time for the approach to steady state by the Ca uptake, b. Rate of accumulation of Ca by Ca_2, and c. Total Ca content of Ca_2.

a. Half-time

A comparison of the half-times for the uptake of Ca into Ca_2 to approach a steady state in CW and CNW hearts is shown in Table 5. A significant difference between the CW and CNW hearts was recorded only at the 5.0 mEq Ca/l. Further comparison on a group basis suggests a trend towards an extended half-time in the CW group which may implicate the Ca-free wash.

b. Rate of Ca accumulation by Ca_2

The rate at which Ca was accumulated by Ca_2 at the four perfusate Ca concentrations is presented in Table 6. Both the CNW and CW hearts exhibited rates of Ca uptake which increased progressively with the perfusate Ca concentration. The sharp increase in rate of Ca uptake at the 10.0 mEq/l in both groups of hearts is difficult to reconcile with the contractile force data which are relatively level at perfusate Ca concentrations above 5.0 mEq/l. Although there were no significant differences between the CW and CNW hearts at any perfusate Ca concentration, the rate of Ca accumulation in the CNW hearts was greater than that of the CW group at the first three perfusate Ca concentrations.

c. Ca content of Ca_2

Table 7 compares the Ca content of Ca_2 measured after perfusion

TABLE V

The Effect of Different Perfusate Ca Concentrations on the Half-time of Ca Uptake into Ca_2 to Approach Steady State in CW and CNW Hearts.

HALF-TIME (sec)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	3 .9 ± 6.3* (7)	24.8 ± 2.6 (10)	23.5 ± 2.4 (10)	19.6 ± 2.0 (6)
CW	–	27.4 ± 5.7 (6)	32.8 ± 2.6† (6)	25.6 ± 2.4 (7)

Number in parentheses indicates number of hearts

* Mean ± S.E.

† Significant difference $P < 0.05$

TABLE VI

The Effect of Different Perfusate Ca Concentrations on the Rate of Ca Accumulation into Ca_2 in CW and CNW Hearts.

RATE OF Ca ACCUMULATION (mEq/Kg/sec)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.015 ± 0.003 (5)	0.047 ± 0.020 (7)	0.068 ± 0.010 (7)	0.147 ± 0.030 (6)
CW	0.004 ± 0.002 (2)	0.026 ± 0.004 (5)	0.043 ± 0.010 (5)	0.249 ± 0.100 (5)

Number in parentheses indicates number of hearts

* Mean $\pm$ S.E.

TABLE VII

The Effect of Different Perfusate Ca Concentrations on the Ca Content of Ca_2 in the CW and CNW Hearts.

Ca_2 CONTENT (mEq/Kg TISSUE WET WEIGHT)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.91 ± 0.15 * (7)	2.15 ± 0.41 (10)	4.64 ± 0.86 (10)	5.60 ± 1.60 (6)
CW	-	1.40 ± 0.40 (6)	3.00 ± 0.42 (5)	4.50 ± 3.90 (7)

Number in parentheses indicates number of hearts

* Mean ± S.E.

with the different Ca concentrations in the CW and CNW hearts. Progressive increases in Ca content which correspond with the increasing perfusate Ca concentrations are evident in both groups of hearts. A comparison between the two groups indicated no significant differences in Ca content. The Ca content reading of 14.50 ± 3.90 at the 10.0 mEq Ca/l in the CW group may be related to the calcium paradox (Zimmerman et al., 1967, 1966) which has been shown to occur after prolonged Ca-free wash in rat hearts (see Discussion). Additional problems were encountered in the analysis of the quantity of Ca taken up by Ca_2 of the CW hearts; consequently there are no reliable data available from the 1.25 mEq Ca/l perfusate of the CW hearts.

B. The Effect of Ouabain in the Ca Perfusates, and its Effect on Contractile Force and Ca Kinetics of Ca_2.

1. Contractile force

a. Maximum contractile force

As with the two groups of control hearts, (CNW and CW), maximum levels of contractile force in the corresponding groups of ouabain-treated hearts (DNW and DW) were measured after each reperfusion of Ca concentration. Similar comparisons were done to determine the rate of restoration of contractile force under the influence of ouabain (Fig. 9b).

Figures 8a and 8b show a comparison between the maximum levels of contractility achieved by the ouabain-treated (DW and DNW) and those attained by the control (CW and CNW) hearts. When compared with the appropriate control groups both the DW and DNW hearts demonstrate a PIE, although this PIE was not statistically significant. The lack of a signi-

ficant effect of ouabain on contractility may be attributed to the great variance in the data because of the wide range of contractile force (5 - 40 grams) consistently observed during the experiments. A comparison of contractile force between the DW and DNW groups reveals no significant differences which may reflect a lack of effect of Ca-free wash prior to each ouabain perfusate (DW) on the contractile force of the kitten heart.

It is of interest that the groups of ouabain-treated hearts follow the same pattern of restoration of contractile force (Figs. 8 a and b) as the two control groups in that the maximum contractile force increases very little at perfusate Ca concentrations above 5.0 mEq Ca/l.

b. Rate of restoration of contractile force.

The rate of restoration of contractile force was computed for both DW and DNW hearts, and the results are shown in Figures 9a and 9b with the control values. Both groups of ouabain treated hearts demonstrated consistently higher rates of restoration of contractile force than their corresponding control groups. Statistical significance $p < 0.05$ is recorded only in comparing the CW hearts with the DW hearts at the 2.5 mEq Ca/l perfusate. This increased rate of return of contractile force in ouabain treated hearts agrees with the findings of Bailey and Sures (1971). Although, there are no significant differences in rate of restoration of contractile force between the DW and DNW groups, the data points of the DW hearts are always above the level of the DNW hearts (Fig. 9b). This finding may suggest an enhanced effect of ouabain in hearts initially depleted of Ca.

Figure 8a: The effect of perfusate Ca concentration on the maximum contractile force achieved in CNW hearts (O----O) and CW hearts (●——●).

Figure 8b: The effect of ouabain on maximum contractile force in DNW hearts (□ ----□) and DW hearts (■——■) when perfused with different perfusate Ca concentrations.

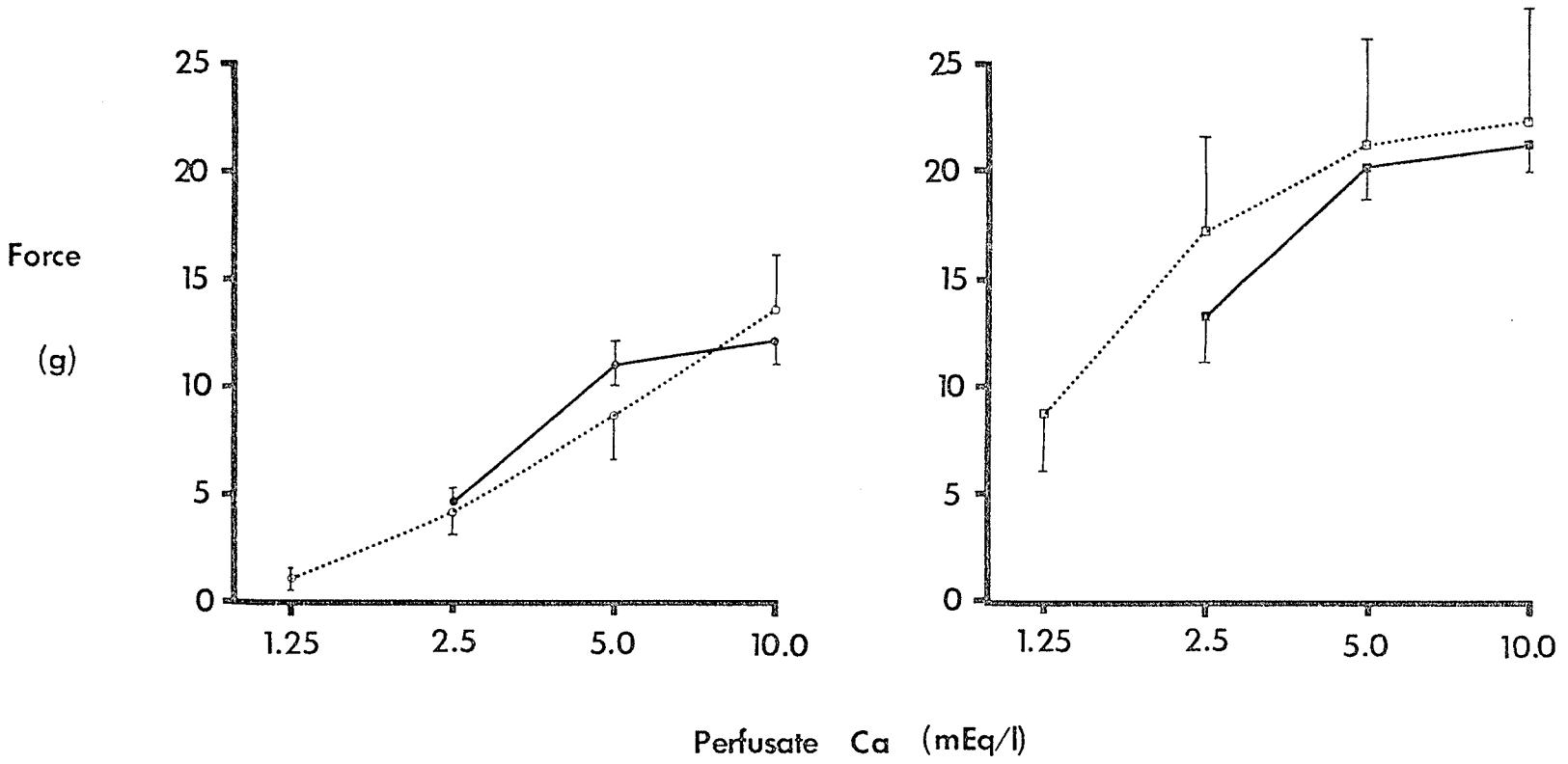
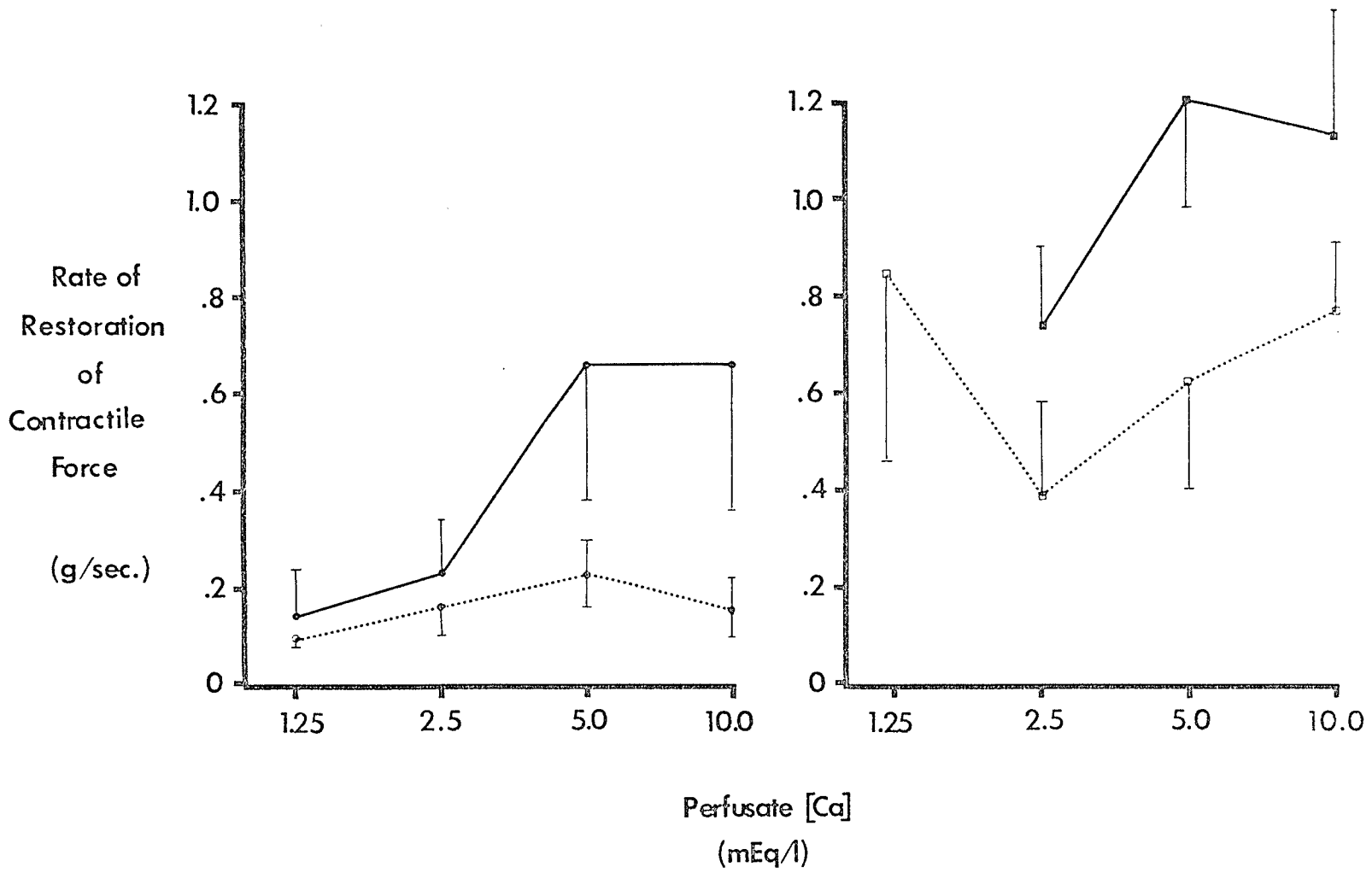

Force
(g)
25
20
15
10
5
0
1.25
2.5
5.0
10.0
Perfusate Ca (mEq/l)

Figure 9a: The effect of different perfusate Ca concentrations on the rate of restoration of contractile force in CNW hearts (O----O) and CW hearts (●——●).

Figure 9b: The effect of ouabain on the rate of restoration of contractile force in DNW hearts (□ ----□) and DW hearts (▣——▣) at different perfusate Ca concentrations.


Rate of Restoration of Contractile Force
(g/sec.)
1.2
1.0
.8
.6
.4
.2
0
1.25
2.5
5.0
10.0
Perfusate [Ca]
(mEq/l)

Some inconsistency is noted when each of the ouabain treated groups is compared separately for effects due to Ca concentration. Readings for the DNW group appear to be related directly to the Ca concentration in the perfusate with the exception of the extreme value of 0.85 g/sec at the 1.25 mEq Ca/l perfusate. A survey of the DW hearts (Fig. 9b) shows no consistent trend between rate of restoration of contractile force and perfusate Ca concentration.

2. Kinetics of Ca uptake into Ca_2

As with the control hearts, the three parameters describing the uptake of Ca into Ca_2 were investigated after treatment with ouabain.

a. Half-time

Table 8 presents the data for the half-times of both the control and ouabain exposed hearts. In the DNW group, with the exception of the 10.0 mEq Ca/l perfusate, there is little effect of different Ca perfusate concentration on half-time of Ca uptake. In the DW group, the data are too inconsistent to suggest an influence of the perfusate Ca concentration on half-time. Comparison between the DW and DNW groups at 2.5 mEq/l and 10.0 mEq/l shows prolonged, but not statistically significant half-time for the DW group. As mentioned previously the half-time of the CW hearts at 5.0 mEq Ca/l was significantly ($p < 0.05$) prolonged over its corresponding CNW group.

Bailey and Sures (1971) found an increased rate of Ca uptake in the presence of ouabain, it is not unreasonable to postulate a shorter half-time in the presence of ouabain. A comparison of the half-times of the DW and DNW hearts with their proper controls reveals no significant difference in half-time values.

TABLE VIII

The Effect of Ouabain on the Half-time of Ca Uptake into Ca_2 in Hearts Perfused with Different Ca Concentrations.

HALF-TIME (sec)

Treatment	Perfusate [Ca] (mEq/l) 1.25	2.5	5.0	10.0
CNW	34.9 ± 6.3 * (7)	24.8 ± 2.6 (10)	23.5 ± 2.4 (10)	19.6 ± 1.9 (6)
CW	-	27.4 ± 5.7 (6)	32.8 ± 2.6 † (6)	25.6 ± 2.4 (7)
DNW	24.2 ± 3.9 (7)	24.0 ± 3.5 (8)	23.8 ± 3.0 (8)	16.1 ± 1.8 (4)
DW	-	27.9 ± 4.8 (5)	16.6 ± 2.9 (4)	32.6 ± 6.4 (6)

Number in parentheses indicates number of hearts

* Mean ± S.E.

† Significant at $P < 0.05$ between CW and CNW hearts

b. Rate of Ca accumulation into Ca_2

Data describing the rate of Ca accumulation into Ca_2 are presented in Table 9 and Fig. 10. Each of the four groups of hearts CNW, CW, DNW and DW has one characteristic feature; within each group the rate of Ca accumulation is dependent on perfusate Ca concentration. These data support the increasing Ca content of each group which is also related to perfusate Ca concentration (see Table 10). There are no significant effects on rate of Ca accumulation due to ouabain in DW or DNW hearts. This is in contrast with the findings of Bailey and Sures (1971) who reported a significant increase in rate of Ca accumulation due to ouabain at the 5.0 mEq/l perfusate. Similarly, there is little effect on Ca accumulation attributed to the Ca-free wash in either control or ouabain-perfused hearts.

c. Ca content of Ca_2

Data describing Ca content of Ca_2 are organized in Table 10. An increase in Ca content in Ca_2 which is dependent on the perfusate Ca concentration is recorded in both of the ouabain-treated groups (DW and DNW).

The Ca content of the DW hearts at the 10.0 mEq Ca/l was nearly as large (13.23 mEq/kg) as the Ca content value of its control group. This consistent elevation in Ca content in both washed groups after perfusion with high Ca concentration suggest that the Ca-free wash interpolated between each uptake perfusate in the DW hearts has in some way effected the uptake of Ca. The relation of these findings to the "Ca paradox" will be elaborated in the Discussion. A comparison of the ouabain-treated hearts with their appropriate controls at all perfusate Ca concentrations indicated

Figure 10a: The effect of different perfusate Ca concentrations on the rate of Ca uptake into Ca_2 in CNW hearts O----O and CW hearts ●——●.

Figure 10b: The effect of ouabain on the rate of Ca uptake into Ca_2 in DNW hearts □---- □ and DW hearts ■——■ perfused with differenct Ca concentrations.

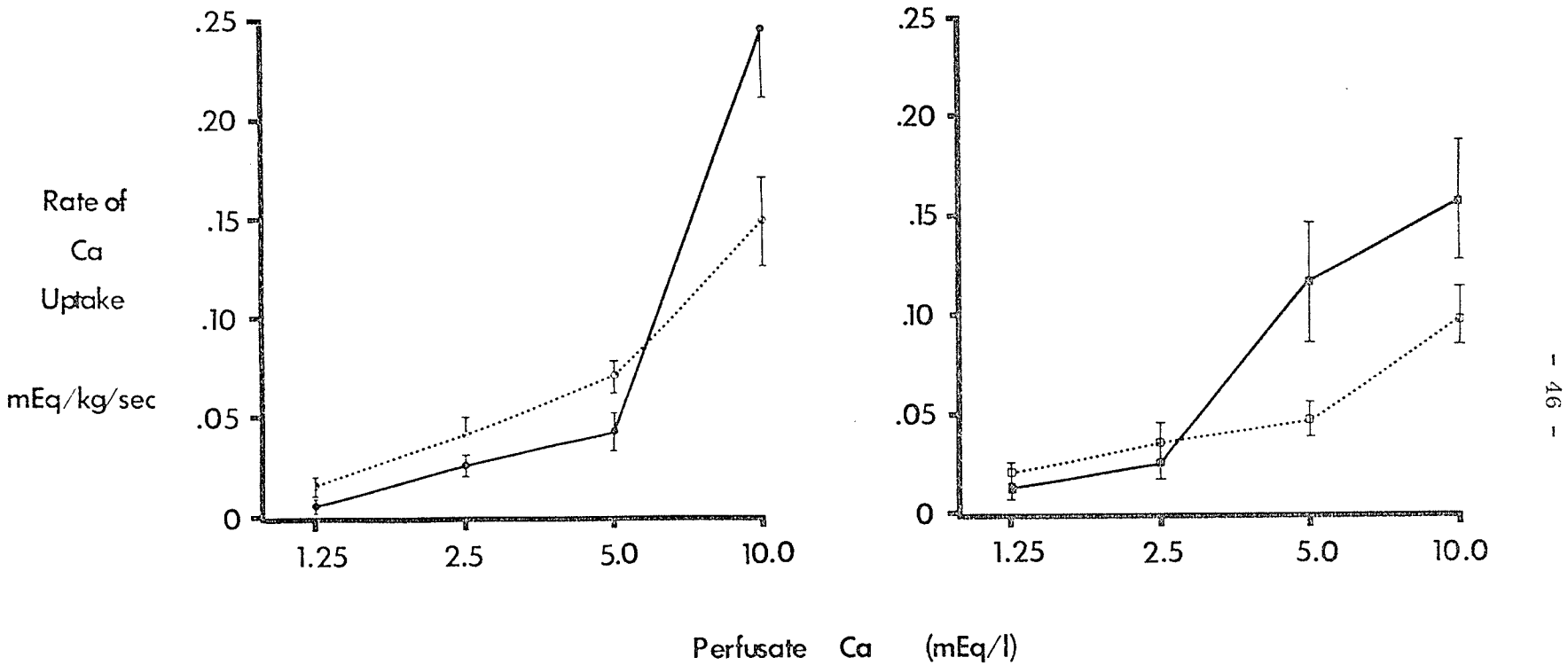

Rate of
Ca
Uptake
mEq/kg/sec
.25
.20
.15
.10
.05
0
1.25
2.5
5.0
10.0
Perfusate Ca (mEq/l)

TABLE IX

The Effect of Ouabain on the Rate of Ca Uptake into Ca_2 in Hearts Perfused with Different Ca Concentrations. (Control Values also Included)

RATE OF Ca ACCUMULATION (mEq/Kg/sec)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.015 ± 0.010 * (5)	0.047 ± 0.020 (7)	0.068 ± 0.010 (7)	0.147 ± 0.030 (6)
CW	0.004 ± 0.002 (2)	0.026 ± 0.004 (5)	0.043 ± 0.010 (5)	0.249 ± 0.100 (5)
DNW	0.02 ± 0.003 (4)	0.038 ± 0.010 (6)	0.044 ± 0.010 (6)	0.098 ± 0.010 (6)
DW	0.015 ± 0.002 (2)	0.027 ± 0.010 (4)	0.118 ± 0.030 (5)	0.151 ± 0.040 (5)

Number in parentheses indicates number of hearts

* Mean ± S.E.

TABLE X

The Effect of Ouabain on the Ca Content of Ca_2 in Hearts Perfused with Different Ca Concentrations. (Control Values also Included)

Ca CONTENT OF Ca_2 (mEq/Kg TISSUE WET WEIGHT)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.91 ± 0.15 * (7)	2.15 ± 0.41 (10)	4.64 ± 0.86 (10)	5.60 ± 1.60 (6)
CW	-	1.40 ± 0.40 (6)	3.00 ± 0.42 (5)	14.50 ± 3.90 (7)
DNW	1.318 ± 0.30 (7)	2.00 ± 0.27 (8)	2.81 ± 0.62 (8)	3.22 ± 1.31 (3)
DW	-	1.02 ± 0.60 (6)	4.19 ± 1.26 (4)	13.23 ± 3.28 (6)

Number in parentheses indicates number of hearts

* Mean ± S.E.

no significant increase in the Ca content of Ca_2 in the presence of ouabain.

d. Ca content derived from a one-compartment analysis

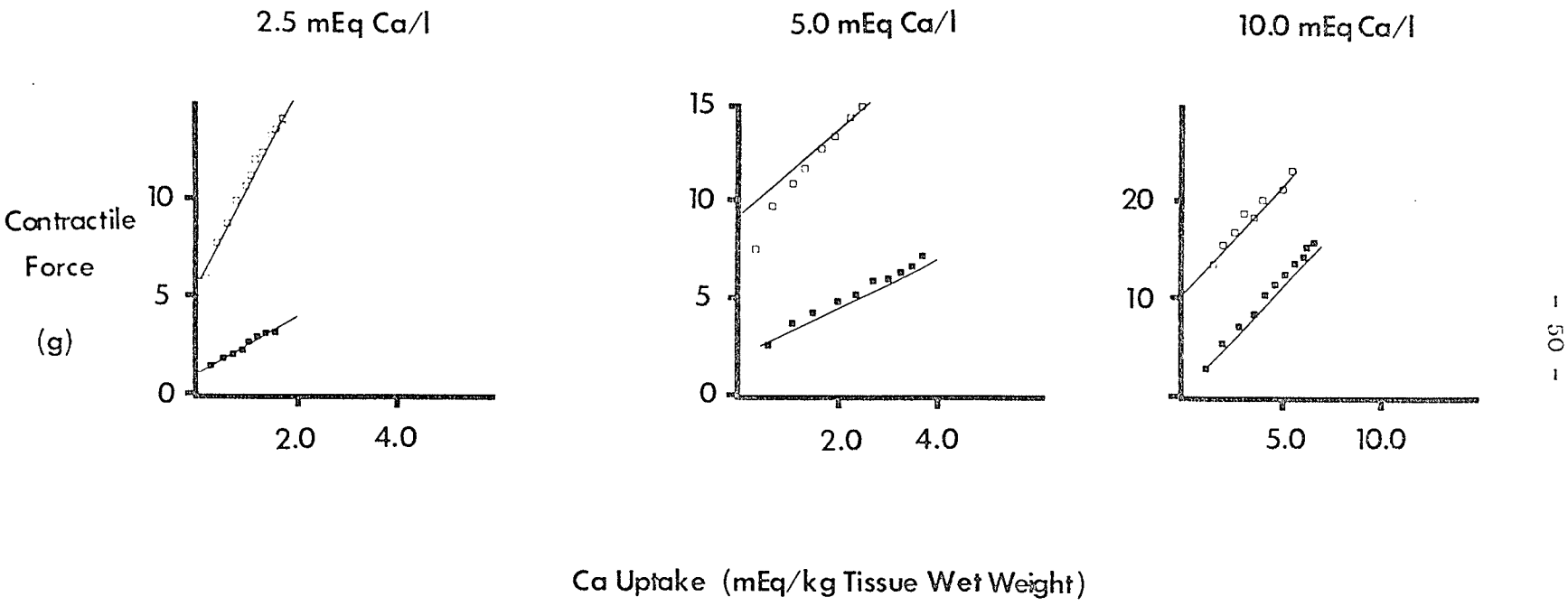
While no significant differences were recorded between the Ca content of Ca_2 in ouabain-treated and control hearts, in the present investigation, Bailey and Sures (1971)noted a significant difference in Ca content using a monoexponential analysis of the Ca uptake. Present data for Ca content of DW and CW hearts at 5.0 mEq Ca/l were re-analyzed using a one-compartment analysis. No significant difference was recorded between the Ca content in ouabain-treated hearts, 3.48 $\pm$ 0.60 mEq/kg, and control hearts, 2.43 $\pm$ 0.65 mEq/kg. However, both values compare favourably with the Ca content readings of 2.01 $\pm$ 0.19 mEq/kg for control hearts and 2.96 $\pm$ 0.26 mEq/kg for ouabain-treated hearts as reported by Bailey and Sures, (1971).

3. Correlation of Contractile Force with Ca uptake

Coefficients of correlation comparing Ca uptake with contractile force were determined for all four groups of hearts CW, CNW, DW and DNW, at perfusate Ca concentrations of 2.5, 5.0 and 10.0 mEq Ca/l. With one exception, all of these correlation analyses had a coefficient of correlation of 0.90 or greater. These findings are in agreement with the coefficient of correlation value of 0.93 for hearts perfused with 5.0 mEq Ca/l, determined by Bailey and Sures, (1971).

A comparison using analysis of covariance (Ancova) of the three DNW vs CNW correlation graphs (Fig. 11), indicated a significant ($P < 0.05$) displacement of all three DNW lines above the corresponding control lines, due to the treatment effect of ouabain. However, this significance must be

Figure 11: A comparison of the correlation coefficients (Ca uptake of Ca_2 vs contractile force) of DNW hearts (□----□) and CNW hearts (O----O) at three perfusate Ca concentration values.


2.5 mEq Ca/l
5.0 mEq Ca/l
10.0 mEq Ca/l
Contractile
Force
(g)
10
5
0
15
10
5
0
20
10
2.0
4.0
2.0
4.0
5.0
10.0
Ca Uptake (mEq/kg Tissue Wet Weight)

interpreted with some caution. For the Ancova analysis to be entirely valid, the slope of both lines in question should be parallel. As there was no other suitable analysis available to compare one coefficient of correlation with another, it was felt that this method would at least yield some indication of the influences of ouabain on the combination of Ca uptake and contractile force. The same analysis was used to compare the CW and DW correlation coefficients and no significant differences were found which could be attributed to ouabain treatment.

C. The Effect of Perfusate Ca Concentration and Ouabain on the Ca Kinetics of Ca_{Tr}

1. Ca uptake kinetics into Ca_{Tr}

Considering the data for Ca_{Tr}, there is an absence of information for both the DW and CW groups. A common feature of both of these groups was the Ca-free wash which may be responsible for the inability to detect Ca_{Tr}. However, at present, there is no further evidence to support this speculation.

A complete description of the Ca kinetics of Ca_{Tr} should also include some analysis of the contractile force recordings. However, Ong (Ph.D. Dissertation) has demonstrated that there is no linear relationship between the Ca uptake into Ca_{Tr} and the restoration of contractile force. Therefore, only the three parameters of Ca kinetics previously discussed will be described for the CNW and DNW groups.

a. Half-time

Half-times for Ca uptake to approach steady state into Ca_{Tr} are shown in Table 11. Values at three perfusate concentrations (1.25, 2.5 and 5.0 mEq Ca/l) for both groups of hearts did not differ significantly

TABLE XI

The Effect of Ouabain on the Half-time of Ca Uptake into Ca_{Tr} in DNW Hearts, plus the Control Values of CNW Hearts.

HALF-TIME (sec)

Treatment	Perfusate [Ca] (mEq/l) 1.25	2.5	5.0	10.0
CNW	4.8 ± 0.5 * (4)	5.4 ± 0.9 (7)	3.5 ± 0.4 (2)	-
DNW	6.9 ± 1.3 (5)	3.4 ± 1.1 (3)	6.5 ± 1.7 (3)	

Number in parentheses indicates number of hearts

* Mean ± S.E.

from the previously reported value of 5.6 seconds (Ong, Ph.D. Dissertation). Thus, there appears to be little effect of perfusate Ca concentration, or ouabain on the half-time values of Ca_{Tr}.

b. Ca content

The results of increasing Ca perfusates and ouabain on the Ca content of Ca_{Tr} are outlined in Table 12. There is a sequential increase in Ca content which corresponds with the increasing perfusate Ca concentrations in both the DNW and CNW groups. A further comparison between these two groups revealed no significant effect of ouabain on the Ca content of Ca_{Tr}.

c. Rate of Ca accumulation

The rate of Ca accumulation in Ca_{Tr} by the CNW and DNW hearts is shown in Table 13 and Fig. 12. Both of these groups exhibited an increase in the rate of Ca accumulation that was dependent on perfusate Ca concentration. This progressive increase in accumulation corresponds with the sequential elevation of Ca content in CNW and DNW hearts as the perfusate Ca was increased. Of greater importance to the present experimental objectives is the finding of a consistent increase in the rate of Ca accumulation into Ca_{Tr} in the ouabain-treated hearts (DNW) at each perfusate Ca concentration. Lack of statistical significance is explained by the inclusion of some apparent outlier data points which increased the measure of variance in the analysis substantially.

TABLE XII

The Effect of Ouabain on the Ca Content of Ca_{Tr} in DNW Hearts, plus the Control Values of CNW Hearts.

Ca CONTENT OF Ca_{Tr} (mEq/Kg TISSUE WET WEIGHT)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.19 ± 0.10 * (4)	0.33 ± 0.06 (7)	1.69 ± 1.21 (2)	-
DNW	0.50 ± 0.34 (5)	0.64 ± 0.17 (3)	0.94 ± 0.32 (3)	

Number in parentheses indicates number of hearts

* Mean ± S.E.

Figure 12: The effect of ouabain on the rate of Ca accumulation into Ca_{Tr} in DNW hearts (□ ----□) compared with CNW hearts (O----O) at three perfusate Ca concentrations.

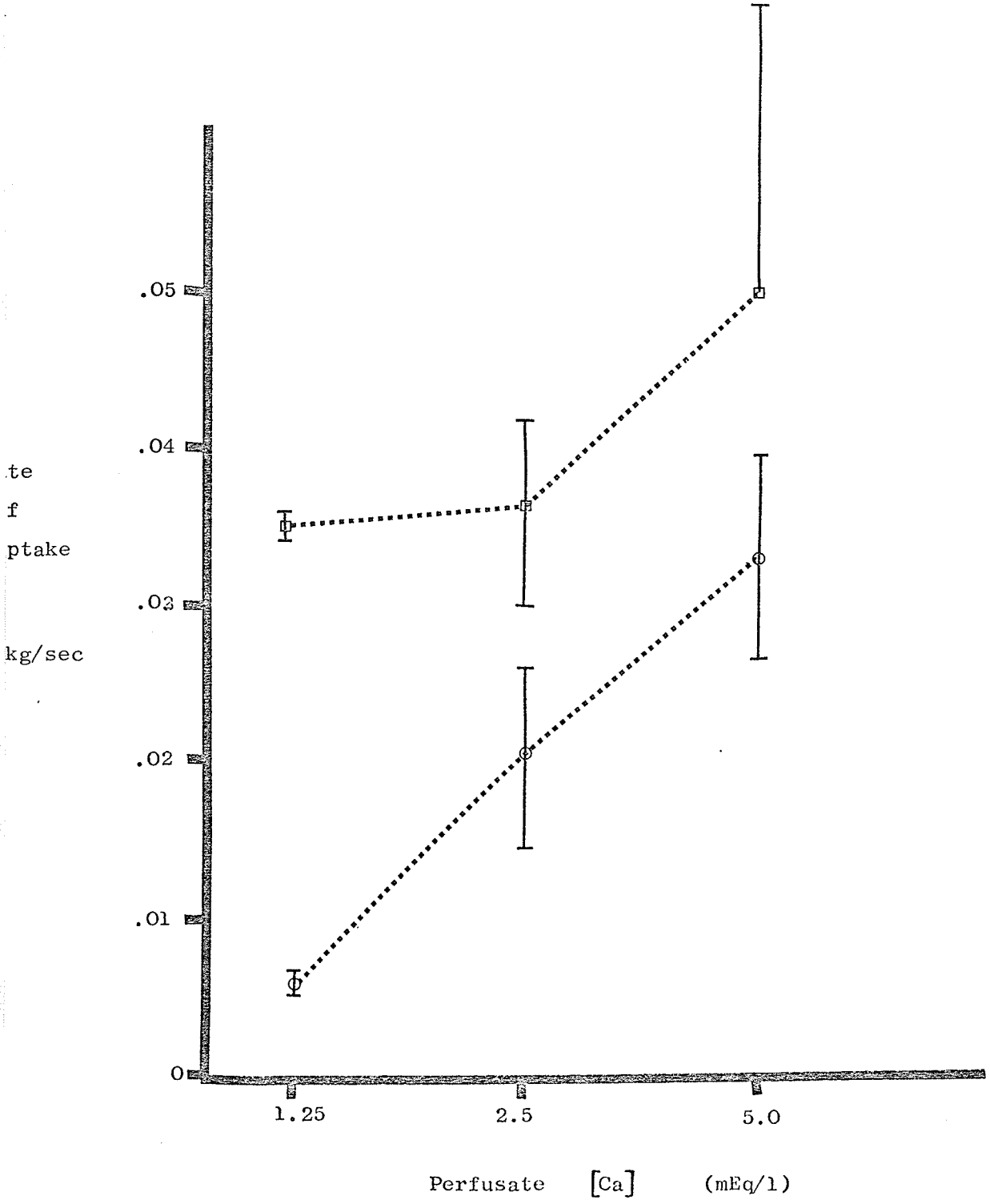

.05
.04
.03
.02
.01
0
te
f
ptake
kg/sec
1.25
2.5
5.0
Perfusate [Ca] (mEq/l)

TABLE XIII

The Effect of Ouabain on the Rate of Ca Accumulation into Ca_{Tr} in DNW Hearts, plus the control values in CNW Hearts.

RATE OF Ca ACCUMULATION (mEq/Kg/sec)

	Perfusate [Ca] (mEq/l)			
Treatment	1.25	2.5	5.0	10.0
CNW	0.006 ± 0.001 * (4)	0.020 ± 0.006 (7)	0.033 ± 0.007 (2)	-
DNW	0.035 ± 0.01 (5)	0.036 ± 0.006 (3)	0.050 ± 0.020 (3)	

Number in parentheses indicates number of hearts

* Mean ± S.E.

SECTION IV

DISCUSSION

A. Preliminary Considerations

1. The gas-perfused kitten heart

The merits and disadvantages of the gas-perfused heart have been discussed in a number of previous papers (e.g., Gabel et al. 1966, Bailey and Dresel, 1968). The favourable aspects of this preparation include the elimination of the vascular component of the perfusate, plus a convenient method of changing from one perfusate to another. The major drawback is found in the non-physiological conditions forced upon the heart and the attempt to extrapolate such data to the organ in vivo.

2. Ca-free wash and the "Ca paradox"

The Ca-free wash is also non-physiological, however, it does serve to eliminate back-flux of Ca during a Ca-free wash. A variety of side effects referred to as the "Calcium paradox" (Zimmerman et al.1966, Bielecki, 1969), have been associated with Ca-free wash in rat hearts. Very simply, these side effects involve the deterioration of electrical and mechanical activity of the rat heart, plus some cell destruction. Ong, (Ph.D. Dissertation) has questioned the presence of the Ca paradox in the kitten heart; because the duration of her Ca-free wash was much briefer than the period of time suggested by Zimmerman et al., (1967), to cause the "Ca paradox". In addition, Bielecki (1969), has suggested a minimum requirement of 3 minutes of Ca-free wash to cause the "Ca paradox". However, the total duration of Ca-free washes in the present study averaged between six and eight minutes. Therefore, despite the qualifications of Ong (Ph.D. Dissertation), it is believed that the "Ca paradox" is

responsible for some of the unexpected findings in the current preparation. For example, unusually high Ca content readings at the 10.0 mEq Ca/l.

3. Analytical procedures - employment of best-fit Ca uptake curves

With regard to the Ca uptake data, the employment of best-fit Ca uptake curves requires some justification. When the experimental uptake data points were subtracted from the experimental asymptote value and plotted as washout curves, it was noticed that increased error margins were generated as the uptake values approached the asymptote. This was due to the expanded scale of the semi-log paper in the lower portion of the plotted washout curve. That is, small deviations from a smooth curve as the uptake approaches the asymptote became disproportionately larger when the difference (asymptote - Ca_T portion of the uptake curve) was plotted on the expanded scale as a washout curve. Therefore, to minimize this generated error, it was decided to introduce best-fit curves and fix the asymptotes at 0.05 units above the highest Ca concentration reading.

B. Effect of Ouabain, Ca-Free Wash and Ca Perfusate Concentrations on the Ca Kinetics and Contractile Force of Ca_{Tr} and Ca_2

1. Ca kinetics of Ca_{Tr}

Ong, (Ph.D. Dissertation), has demonstrated no linear correlation between the restoration of contractile force and the rate of Ca accumulation into Ca_{Tr}. Therefore, rather than deal with contractile force, discussion will emphasize the Ca kinetics of Ca_{Tr} of which there are three noteworthy aspects. firstly, an increase in rate of Ca accumulation into Ca_{Tr} in the presence of ouabain, secondly, little change in Ca content of Ca_{Tr} in the presence of ouabain, and finally, the absence of data for the

Ca kinetics of Ca_{Tr} in the presence of a Ca-free wash in both DW and CW hearts.

a. Increased rate of Ca accumulation into Ca_{Tr}

The rate of Ca accumulation into Ca_{Tr} was consistently elevated at each perfusate Ca concentration in the DNW hearts compared with the CNW hearts. Most of the differences were on the verge of being statistically significant at $P < 0.05$ level. The importance of this finding is appreciated when the current data are compared with the findings of Bailey and Sures (1971). From their research, it appears that the PIE due to ouabain occurs in the presence of an increased Ca uptake into Ca_2. This discrepancy is difficult to reconcile, however, it may be due to different analytical techniques of Ca uptake employed by Bailey and Sures (1971), as opposed to the mathematical analysis of the present investigations. With reference to the investigations of Bailey and Sures (1971), the Ca uptake analysis was based on a monoexponential uptake curve which did not distinguish between Ca_{Tr} and Ca_2. In contrast, the current analytical techniques utilized a less biased graphical analysis of the Ca uptake curve which was capable of delineating two Ca compartments (Ca_{Tr} and Ca_2). Therefore, it is not improbable that the increase in rate of Ca accumulation is occurring in Ca_{Tr}, and it may be only because of the nature of the previous analytical method that Ca uptake into Ca_{Tr} was not identified by Bailey and Sures (1971).

It was hoped that the re-analysis of our Ca content data at the 5.0 mEq Ca/l as a monoexponential curve (Methods 3b and Results 2d) might show a significant difference between the ouabain-treated and control

hearts. Had that been the case, it would have made a more convincing argument to suggest that the significance attributed to ouabain by Bailey and Sures (1971), was actually an artifact due to the monoexponential analysis. Having recorded no significant difference in our re-analysis, we can only speculate that the single compartment analysis was at fault.

b. Constant levels of Ca content in Ca_{Tr} in the presence of ouabain

A secondary finding related to the increased rate of Ca accumulation into Ca_{Tr} in the presence of ouabain is the consistency of the Ca content readings. This may be explained through the following scheme: Bailey and Dresel (1968), have demonstrated a logarithmic relationship between Ca content of Ca_2 and contractile force under normal conditions. This implies that a constant portion of Ca is released from Ca_2 to complete the E-C coupling circuit. In addition, Ong (Ph.D. Dissertation), has described a "trigger" pool of Ca which is necessary to initiate release of Ca from Ca_2. To produce a PIE, it is logical that more Ca must be released from Ca_2 to complete the E-C coupling process and account for the extra strength of contraction. There are three possible mechanisms for this to occur, firstly, an increase in Ca_2 content with no accompanying effect on Ca_{Tr}, secondly, no change in Ca_2 but an increase in Ca_{Tr} or finally a combination of the two. Therefore, more Ca should be released from Ca_{Tr} to initiate the release of the additional Ca from Ca_2 which is necessary for the PIE. Under these conditions, the Ca content of Ca_2 and Ca_{Tr} may or may not remain unchanged even in the presence of an increased turnover of Ca in Ca_{Tr}. This would produce an apparent increase in the

rate of Ca uptake into Ca_{Tr} and an increased efflux from Ca_{Tr} without affecting the Ca content of this pool. Such a scheme is reminiscent of the early experiments of Holland and Sekul (1959).

c. Absence of Ca_{Tr} kinetics data in the presence of a Ca-free wash

In both the CW and DW hearts there was a conspicuous absence of a contribution to Ca uptake by Ca_{Tr}. In the light of current investigations, we can only speculate as to the reason for this lack of Ca_{Tr} kinetic data. The simplest possibility may be that we are dealing with a one compartment uptake system as described by Bailey and Sures (1971). However, if one considers the Ca uptake kinetics data obtained by Ong (Ph.D. Dissertation), plus most of the data from the present project, it appears that we are dealing with a Ca uptake system comprised of at least two compartments.

One possible explanation is the shortcoming of the present technique; especially the collection of the effluent samples. Important changes may be occurring in the Ca concentration of the effluent over shorter periods of time than the six second collection interval, which might otherwise be analyzed as Ca_{Tr} kinetic data in the 1.25 Ca perfusates. Another source of error may be the accuracy of the Ca concentration measurements in the effluent. Even with the utilization of best-fit Ca uptake curves, there may still have been enough error present to obliterate that portion of the slope which corresponds to Ca_{Tr}. This is especially true when one considers that the best-fit Ca uptake curves were drawn with the use of french curves which do not entirely eliminate the element of human bias.

Perhaps the most logical explanation for lack of Ca_{Tr} kinetics data in the washed hearts involves the possible back-flux of Ca from a partially depleted Ca compartment after the Ca-free wash. The Ca-free wash was sustained only until contractile force decayed to one gram or less which means that substantial stores of Ca may have remained in Ca_2. With this in mind, during the first few seconds of Ca reperfusion in the CW and DW heart, the concentration gradient for Ca may be such that some of the intracellular Ca may have back-fluxed out of the tissue and contributed enough Ca to the Ca uptake to mask the Ca_{Tr} component of the Ca uptake curve.

2. Contractile force and kinetics of Ca_2

a. Contractile force related to Ca_2

i. Maximum levels of contractile force

A consistent trend in all four groups of hearts (CNW, CW, DNW, DW) was a relative levelling of contractile force recordings at perfusate Ca concentrations above 5.0 mEq/l, despite a continued increase in the accumulation of Ca even at the 10.0 mEq Ca/l perfusate. Dresel, (personal communication) has suggested that the Ca concentration at which maximal contractile force is achieved is in part dependent on the nature of the bathing solution. In Hepes solution, maximal contractile force is achieved at a Ca concentration of 10.0 mEq/l while the same Ca concentration in K-H solution may cause a transient increase in contractile force which tends to diminish with prolonged perfusion. It has also been reported that Ca at concentrations of 7.5 mEq/l and greater, in K-H solution, tend to precipitate and form micro-crystals of Ca (Dresel, personal communica-

tion). In addition, Young (1968), has suggested that Ca phosphate micro-crystals may obstruct the coronary vessels of the heart, which could result in tissue damage and a disruption of the contractile process.

This background allows for at least three possible explanations for the lack of increase in contractile force at perfusate Ca concentrations above 5.0 mEq Ca/l, in the presence of a sustained Ca uptake. First, a portion of accumulated Ca may be involved in the E-C coupling process. However, some of it may also be going directly from the perfusate solution into the intracellular storage compartment Ca_{III} (Ong and Bailey, 1972), or it may become involved with organelles such as mitochondria. This would explain the continued uptake of Ca after maximal contractility had been achieved. Second, in view of the fact that micro-crystals of Ca will block the coronaries and disrupt contractile force, plus the fact the 10.0 mEq Ca/l is within the range at which Ca will form the micro-crystals in K-H solution, it is not unreasonable to suggest that the levelling of contractile force was due to the structural disruption attributed to micro-crystals of Ca in the 10.0 mEq Ca/l perfusate solution. Finally, Ca in the form of micro-crystals is not available for the E-C coupling process.

ii. The rate of restoration of contractile force

The rate of restoration of contractile force was greater in both groups of ouabain-treated hearts (DW, DNW) than in the corresponding control hearts (CW, CNW). In the presence of a PIE, this observation was not unexpected. However, the increase in rate of return of contractile force should be explained in terms of the current Ca kinetics data and in view

of the two-compartment model (Ca_{Tr} trigger pool, and Ca_2 maintenance pool) proposed by Ong and Bailey (1972). Keeping in mind the logarithmic relationship between Ca content and contractile force, Bailey and Dresel (1968), which requires a proportional release of Ca from Ca_2 to support the contractile response, an increase in the rate of rise of contractile force should be sustained by a concomitant increase in the exchange of Ca between Ca_2 and the CM. With reference to the 'trigger pool' model of Ong and Bailey (1972), an increase in the exchange of Ca between Ca_2 and the CM need not require an increase in the content or rate of accumulation of Ca into Ca_2. The logical requirement is an increase in the stimulation of Ca_2 to promote the exchange of intracellular Ca with the CM. Such stimulation may well be supplied by an increased exchange of Ca between Ca_{Tr} and Ca_2 which the current data appear to support. Data for the Ca kinetics of Ca_{Tr} indicate an increased uptake of Ca yet a constant Ca content, which implies an increase in the efflux of Ca from Ca_{Tr}. According to the plan of Ong and Bailey (1972), this should yield an increased exchange of Ca between Ca_{Tr} and Ca_2 with a subsequent increased stimulation of release of Ca from Ca_2 which would interact with the CM and complete the E-C coupling for the PIE. This explanation satisfies the increased rate of contractile force restoration in the presence of ouabain based on the Ca kinetic data of this study.

b. Ca kinetics of Ca_2

i. Half-time of Ca_2

There are two noteworthy observations in the data for the half-time of Ca_2 in control and ouabain-treated hearts. First, the half-time values

for Ca_2 at most of the perfusate concentrations in both CW and CNW hearts are significantly less ($P < 0.05$) than the value of 46 seconds reported at the 5.0 mEq Ca/l by Ong, (Ph.D. Dissertation). Secondly, there appears to be very little influence of ouabain on the half-time of Ca_2 in either of the ouabain-treated groups.

Explanation for the initial observation regarding a discrepancy between present half-time values and those reported by Ong, (Ph.D. Dissertation), may be dependent on pH changes in the perfusate medium once it enters the heart. The only major difference in technique between Ong's preparation and the present one is the use of Hepes solution for a buffer rather than the present use of K-H solution. Studies are available (Bielecki, 1969; Lorkovie, 1966), which suggest that in an acidic bathing medium the transport of Ca across the membrane of the cardiac muscle cell is impeded, in all likelihood due to some competitive mechanism between Ca and H ions for the transport system available. Ong (Personal Communication) has determined that the pH of K-H solution decreased to 6.3 after perfusion through the heart. A similar acidification may occur in Hepes solution as outlined below.

Hepes solution is a weak acid with a pKa of 7.4 and does not require continuous equilibration with 5% CO_2 to maintain a pH of 7.4. However, in 3 mM Hepes as employed by Ong and Bailey, (1972), the buffering effect is small. Thus, it is entirely possible that Hepes solution may become more acidic in composition after being perfused through the heart, and so contribute more free H ions to the perfusate medium. If such is the case, then the prolonged half-time values reported by Ong (Ph.D. Dissertation),

may be due to an increased $[H^+]$ in the Hepes solution which could compete with Ca for entry into the cell.

The absence of any effect of ouabain on the half-time values for Ca_2 is in accord with the findings of Bailey and Sures (1971). This finding also corresponds with the lack of effect of ouabain on the other Ca kinetic parameters (Ca content, and rate of accumulation of Ca into Ca_2). Any interpretation of these unchanging half-time values in terms of permeability change or carrier transport is purely speculative. Because the half-time for the extraction of Ca from the perfusate represents a rate constant for the approach of Ca uptake to a steady state, Bailey and Sures (1971), it may be indicative of changes in permeability or Ca-carrier mobility. Unfortunately, the half-time value does not afford a distinction between these two transport systems and so, on the basis of unchanging half-time values one may only suggest that ouabain did not effect the permeability and/or the mobility of Ca carriers for Ca_2.

ii. Ca content of Ca_2

Common to all four groups of hearts (CW, CNW, DW, and DNW) was the dependence of the Ca content of Ca_2 on the perfusate Ca concentration. Neither the Ca-free wash, nor ouabain exerted any significant effects on the Ca content of Ca_2, which is contrary to the research of Bailey and Sures (1971), who reported an increase in Ca content in ouabain-treated hearts.

The absence of effect of ouabain on the Ca content of Ca_2 may be explained by the scheme already postulated for the Ca kinetics of the system. On the assumption that there is an elevated efflux of Ca from Ca_{Tr},

this Ca can stimulate the release of intracellular stores of Ca from Ca_2 without being taken up by Ca_2. On the other hand, if some of the Ca from Ca_{Tr} is accumulated by Ca_2, it should be offset by a concomitant Ca efflux from Ca_2 to support the E-C coupling of the PIE. With either alternative, in the presence of ouabain, the Ca content of Ca_2 need not rise to support the PIE.

One of the more puzzling findings of the Ca content data are the two extreme Ca content values reported for both groups of washed hearts (CW and DW) at the 10.0 mEq Ca/l perfusate. These Ca content values which are greater than the original perfusate concentration were repeatedly observed, and so cannot be treated as outlier data points. The simplest explanation is the "Ca Paradox", Zimmerman et al., (1966, 1967); Bielecki, (1969), mentioned earlier in the Discussion, because these extreme Ca content values occurred only after repeated Ca-free washes. It seems logical that alterations in the integrity of the membrane structure, attributed to the "Ca Paradox", may allow huge quantities of Ca to enter the cell from the Ca perfusate, thus explaining such elevated Ca content readings at the 10.0 mEq Ca/l perfusates.

iii. Rate of Ca accumulation into Ca_2

Similar to the increase in Ca content of Ca_2 (within each group of hearts) which was dependent on perfusate Ca concentration; the rate of Ca accumulation into Ca_2 exhibited a similar dependent trend. Neither ouabain nor the Ca-free wash exerted any significant influence on the rate of Ca accumulation into Ca_2 in any of the groups of hearts.

This progressive rise in Ca accumulation corresponding with an increase in perfusate Ca concentration supports the data for the other two parameters of Ca_2 kinetics (No effect of perfusate Ca on half-time yet a progressive increase in Ca content). The unchanging half-time values, which represent a rate constant for the approach of Ca uptake to steady state, may indicate no change in either cell permeability or Ca carrier mobility in relation to the perfusate Ca concentrations. Otherwise, an increase in either one of the modes of transport could explain the increased Ca content values. This leaves the increase in rate of Ca accumulation as the logical alternative to explain the elevated Ca content values. If there is no change in either permeability or Ca carrier mobility, then these increasing rates of Ca accumulation at increasing perfusate Ca concentrations may suggest that Ca transport into cardiac tissue is at least partially dependent on simple diffusion which is in turn governed by the Ca concentration of the bathing medium.

Figures 10a and 10b indicate a dramatic increase in the rate of Ca accumulation at all four of the 10.0 perfusate Ca concentrations in comparison with the uptake rates at lower Ca concentrations. This is especially noticeable in both groups of washed hearts (CW and DW). Such extreme values require an attempt at explanation. In view of the findings of Dresel (Personal Communication) that Ca tends to precipitate out of the K-H solution at concentrations of approximately 7.5 mEq Ca/l, it is not unlikely that this precipitation of Ca may be accompanied by some destruction or alteration of the cell membrane which in turn could allow an increased Ca influx at the 10.0 mEq/l Ca perfusate. Because this phenomenon

was also observed in hearts without the Ca-free wash, the "Ca Paradox" is not considered to be a major factor in this augmented rate of Ca influx at 10.0 mEq/l Ca perfusate.

Lack of effect of ouabain on the rate of Ca accumulation into Ca_2 may be explained with reference to the scheme proposed in the section entitled 'Rate of Restoration of Contractile Force'(Discussion 2a ii). As long as there is an increase in Ca uptake into Ca_{Tr}, which yields an increased stimulation of the intracellular Ca exchange between Ca_2 and the CM, there is no necessity for an elevation of the rate of Ca accumulation into Ca_2 in the presence of ouabain.

C. Interpretations of the Influence of Ouabain on DW and DNW Hearts

1. Was there significant effect of ouabain on contractility

The influence of ouabain on contractile force and Ca kinetics in the present study is open to question. Despite a number of trends in the data which favoured the ouabain-treated hearts at all perfusate Ca concentrations, most of these differences did not reach statistical significance. On this basis one might suggest that ouabain did not influence either the contractile force or the Ca kinetics to an appreciable extent. On the other hand, the next two sections are speculations based on the viewpoint that ouabain induced an appreciable increase in the level of contractile force, as well as influencing the Ca kinetics; especially the rate of Ca accumulation into Ca_{Tr}.

2. Explanation of Ca kinetics involved with the PIE in terms of a direct or indirect effect of Ca

Speculation about the Ca kinetics associated with the PIE will be

considered from two points of view:

a. An indirect action of Ca influx on the E-C coupling process

b. A direct influence of incoming Ca on the E-C coupling process

Prior to this a brief qualification of the data is necessary.

In the presence of ouabain, Bailey and Sures (1971), recorded an increased rate of Ca accumulation and content in Ca_2. Ong and Bailey (1972), demonstrated that the Ca_2 pool was responsible for the maintenance of contractility in the kitten heart. Therefore, in the current project, it was somewhat unexpected to observe an increased rate of Ca accumulation into Ca_{Tr} in ouabain-treated hearts, while the kinetic parameters of Ca_2 exhibited no consistent changes under the influence of ouabain. Speculation about this phenomenon will consider both the indirect and direct action of influxing Ca on the E-C coupling process in the heart.

a. Indirect effect of Ca on E-C coupling

A model for the indirect involvement of Ca in the E-C coupling process is based on the concept of incoming Ca initiating the release of intracellular Ca from a compartment. The Ca so released then completes the circuit for E-C coupling by interacting with the CM. All of the models described in the introduction: Niedergerke, (1963a); Langer, (1964, 1965, 1967); Winegrad and Shanes, (1962); Teiger and Farah, (1967); Ong and Bailey, (1972); Ong and Bailey, (1973); are examples of an indirect action of Ca in the E-C coupling circuit.

An interpretation of the present data in terms of the above hypothesis requires reference to the scheme proposed in the section entitled "Rate of Restoration of Contractile Force". An increase in the rate of

Ca uptake into Ca_{Tr} accompanied by a constant Ca content implies an augmented rate of efflux of Ca from Ca_{Tr} in the presence of ouabain. Two options are available for the Ca released from Ca_{Tr}:

It may stimulate the exchange of Ca between Ca_2 and the CM without actually being accumulated by Ca_2, and thereby support the PIE, or a portion of the Ca released from Ca_{Tr} may react with structures other than Ca_2, such as a Ca storage compartment (possibly sarcoplasmic reticulum) or other subcellular organelles such as the mitochondria.

In either instance, the rate of Ca uptake into Ca_2 need not be elevated in the presence of ouabain. The constant Ca content of Ca_2 in the presence of ouabain is also justified on the basis of the logarithmic relationship (Bailey and Dresel, 1968), which requires a release of Ca from Ca_2 in proportion to contractile force. However, both of these speculations are contingent on an increased uptake of Ca into Ca_{Tr} as indicated by our data.

b. Direct effect of Ca on E-C coupling

With regard to the direct action of incoming Ca on the E-C coupling mechanism, several investigations - Wood et al., (1969); Tritthart, (1973); Reuter and Beeler, (1969) - have offered evidence to support this possibility. Through the use of voltage clamp experiments and measurements of Ca influx during depolarization of the muscle cell, it has been suggested that at least some portion of the influx of Ca stimulated the CM directly. In light of the present data, the constant Ca content values of both Ca pools in the presence of ouabain might be explained by the following scheme. A portion of the Ca which enters with membrane depolarization is a trans-

ient Ca flux which interacts briefly with both Ca pools (although not accumulated) prior to its stimulation of the CM. However, a direct action of the incoming Ca implies no interference with the pathway of Ca between the time of influx and the activation of the CM, thus questioning the validity of this suggestion as it pertains to a compartamental model.

Therefore, in light of the speculations presented thus far, it is entirely possible that Ca may mediate the process of E-C coupling through both direct and indirect pathways. However, an indirect pathway involving intracellular Ca compartments is a more adequate explanation of the data in this study.

3. Speculation about the effect of ouabain on Ca carriers

Bailey and Sures (1971), have suggested that ouabain enhances the influx of Ca in cardiac tissue by stimulating additional Ca carriers in the membrane which are inactive under normal conditions, rather than through some action on the membrane permeability or the mobility of normal Ca carriers. In addition, evidence in support of the presence of Ca carriers in cardiac tissue is reported by Reuter and Seitz (1968); and Glitsch et al., (1970).

The effect of ouabain on Ca flux in the present study is open to conjecture because the only positive influence of ouabain appears to have been an increase in the rate of Ca accumulation into Ca_{Tr}. In addition, the lack of effect of ouabain on half-time values suggests that neither Ca permeability nor the mobility of available Ca carriers was altered. On the basis of these findings we can only speculate that ouabain influenced the number of Ca carriers, rather than their mobility. This proposal

becomes more justifiable in light of the following evidence which suggests that Ca uptake into Ca_{Tr} may be carrier-mediated.

Van Breemen and Van Breemen (1969), employing an artificial phosphilipid membrane have demonstrated the presence of facilitated Ca transport which is blocked by La and therefore may be carrier-mediated. Ong (Ph.D. Dissertation), has reported that La will block the uptake of Ca into Ca_{Tr}, the trigger compartment, while having no effect on Ca_2 uptake. Considering that both of these investigations have indicated a common block in Ca transport due to La, then the transport of Ca into Ca_{Tr} may also be facilitated and dependent on Ca carriers. If such is the case, then an increased rate of Ca accumulation into Ca_{Tr} may be due to the influence of ouabain on the proposed carrier-mediated uptake of Ca into Ca_{Tr}, namely by increasing the number of carriers, rather than their mobility.

With regard to the rate of Ca accumulation into Ca_2, present data are insufficient to allow any proposed explanation for the lack of effect of ouabain, except the obvious suggestion that ouabain may not influence the kinetics of Ca_2.

D. Consideration of the Correlation Data

1. Qualification of the method

Statistically, a correlation test between two variables must be viewed with some degree of reservation. This is explained by the possibility that a high value for the co-efficient of correlation may indicate a direct relationship between the two variables, or it may suggest that they are related only through a third factor which is common to both of them (Vivian, Personal Communication). Unfortunately, there is no

analytical procedure to distinguish between these two possibilities. With these qualifications in mind, the correlation data will be considered.

2. A comparison of correlation co-efficients in the CNW and DNW hearts

An analysis of covariance (Ancova) was employed to determine if there was a significant displacement of the ouabain-treated correlation co-efficient regression line above the corresponding control. Statistical values from this Ancova test indicated that there was a significant ($p < 0.05$) displacement of the ouabain line above the controls at all three perfusate concentrations. This significance must be interpreted cautiously, because in order for such an analysis to be entirely valid the slopes of the lines under comparison should be parallel. This was not always the case in the present group of comparisons.

Despite this qualification, if one attaches some significance to these correlation findings, then further speculation about the PIE and E-C coupling is warranted. Had there been a concomitant increase in the rate of Ca accumulation into Ca_2 with the PIE, one would have expected a shift of the ouabain line above, and to the right of the control line, to account for the added Ca uptake. Comparison of the present data indicates a displacement of the ouabain line directly above the control, with little shifting towards the right which suggests an increase in contractile force but little change in Ca uptake into Ca_2. In the presence of ouabain, this may be explained by two different approaches. First, the ouabain-induced PIE is attributed to an increase in the sensitivity of the CM to apparently normal levels of Ca or second, ouabin elicited the PIE by increasing the exchange of intracellular stores of Ca (probably

between Ca_2 and the CM), so that there is more Ca available to complete the increased E-C coupling necessary to support the PIE.

Repke and Katz (1972), studying Ca binding and uptake in cardiac microsomes have suggested on the basis of kinetic analysis that the PIE is not achieved by an increased sensitivity of the CM to Ca. Therefore, in view of the data presented for ouabain-treated hearts, (an increase in Ca accumulation into Ca_{Tr}, reasonably constant Ca kinetics for Ca_2, and the correlation findings) the second alternative involving an increased exchange of intracellular Ca stores appears to be a more valid explanation of the PIE in the presence of unchanged Ca uptake into Ca_2. A summary of the findings are presented in the proposed model of operation.

E. Proposed Model of E-C Coupling in the Presence of Ouabain

Current findings are interpreted through the following model of E-C coupling in the presence of ouabain: Ouabain stimulates an increase in the rate of uptake of perfusate Ca into Ca_{Tr}. Because of the unchanging Ca content of Ca_{Tr}, this influx is probably accompanied by an equally elevated rate of efflux of Ca from Ca_{Tr}. This increased efflux of Ca from Ca_{Tr} is responsible for stimulating an increased exchange of intracellular Ca between Ca_2 and the CM, while this Ca from Ca_{Tr} need not be accumulated by Ca_2. The elevated Ca exchange between Ca_2 and CM completes the augmented E-C coupling necessary to maintain the PIE. Evidence from Ca_2 kinetics suggest that the Ca from Ca_{Tr} is not taken up by Ca_2 after the stimulation process. With this in mind, after the Ca from Ca_{Tr} has stimulated the exchange of Ca between Ca_2 and the CM, it may be taken up by a storage pool (sarcoplasmic reticulum). However, on the basis of present investigations this remains speculative.

F. Conclusion

The present study suggests two effects of ouabain as it relates to the PIE.

1. Ouabain is associated with an increased accumulation of extracellular Ca into Ca_{Tr}.

2. To accommodate the augmented E-C coupling which supports the PIE, there is an increase in the exchange of intracellular Ca, probably between Ca_2 and the CM, rather than an elevated Ca influx into Ca_2.

3. The influence of ouabain on the increased influx of Ca into Ca_{Tr} by stimulation of normally inactive Ca carriers remains speculative. Because of unforseen short comings in the computer analysis and due to the relative grossness of the preparation (i.e. an entire organ and all of its uncontrolled variables), we were unable to employ the Scatchard Format to determine wheter or not ouabain was activating additional carriers.

SECTION V

BIBLIOGRAPHY

BAILEY, L.E. and Dresel, P.E. Correlation of contractile force with a calcium pool in the isolated cat heart. J. Gen. Physiol. 52: 969-982, 1968.

BAILEY, L.E. and Harvey, S.C. Effect of ouabain on cardiac Ca^{45} kinetics measured by indicator dilution. Amer. J. Physiol. 216: 123-129, 1969.

BAILEY, L.E. and Krip, G. The effect of ouabain on the distribution of calcium in the cat heart. The Pharmacologist, 10: 186, 1968.

BAILEY, L.E. and Krip, G. The effect of ouabain on the distribution of calcium in the cat heart. Arch. Int. Pharmacodyn. Ther. 196: 1, 1972.

BAILEY, L.E. and Ong, S.D. Uncoupling of excitation from contraction by nickel in cardiac muscle. Am. J. Physiol. 224(5): 1092-1098, 1973.

BAILEY, L.E., Ong, S.D. and Queen, G. The uptake and redistribution of Ca^{45} in the gas-perfused cat heart (Abstract). Fed. Proc. 29: 351, 1970.

BAILEY, L.E. and Sures, H.A. The effect of ouabain on the washout and uptake of calcium in the isolated cat heart. J. Pharmacol. Exp. Ther. 178: 259-270, 1971.

BIELECKI, K. The influence of changes in pH of the perfusion fluid on the occurrence at the calcium paradox on the isolated rat heart. Cardiovasc. Res. 3: 268-271, 1969.

CAPRIO, A. and Farah, A. The effect of the ionic milieu on the response of rabbit cardiac muscle to ouabain. J. Pharmacol. Exp. Ther. 155: 403-414, 1967.

DIXON, W.J. and Massey, F.S. Introduction to statistical analysis. McGraw-Hill Book Company, New York, 1957.

DRESEL, P.E. Personal Communication

GABEL, L.P., Bihler, I. and Dresel, P.E. Contractility, metabolism, and pharmacological reactions of isolated gas-perfused hearts. Circ. Res. 19: 891-902, 1966.

GERSMEYER, G. and Holland, W.C. Influence of ouabain on contractile force, resting tension, Ca^{45} entry and tissue Ca content in rat atria. Circ. Res. 12: 620, 1963.

GLITSCH, H.G., Reuter, H. and Scholz, H. The effect of internal Na concentration on calcium fluxes in isolated guinea-pig auricle. J. Physiol. 209: 25-43, 1970.

GLYNN, I.M. The action of cardiac glycosides on ion movement. Pharmacol. Rev. 16: 381-407, 1964.

GOVIER, W.C. and Holland, W.C. The relationship between atrial contractions and the effect of ouabain on contractile strength and calcium exchange in rabbit atria. J. Pharmacol. Exp. Ther. 148: 284-289, 1965.

GROSMAN, A. and Furchgott, R.F. The effects of frequency of stimulation and calcium concentration on Ca^{45} exchange and contractility on the isolated guinea-pig auricle. J. Pharmacol. Exp. Ther. 143: 120-130, 1964.

GROSMAN, A. and Furchgott, R.F. The effects of external Ca concentration on the distribution and exchange of calcium in resting and beating guinea-pig auricles. J. Pharmacol. Exp. Ther. 143: 107-119, 1964a.

HOLLAND, W.C. Effect of heart rate and ouabain on action of calcium on atrial contractions. Amer. J. Physiol. 211: 1214, 1966.

HOLLAND, W.C. and Sekul, A.A. Effect of ouabain on Ca^{45} and Cl^{36} exchange in isolated rabbit atria. Amer. J. Physiol. 197: 757-760, 1959.

KLAUS, W. Vergleichende Untersuchungen über die Wirkung verschiedener Digitoxigenin derivate auf die Kontraktionskraft und den Ca-Austausch isolierter Merschweinchenvorhöfe Naunyn-Schmiedebergs. Arch. Pharmakol. 246: 226-239, 1963.

KLAUS, W. and Kuschinsky, G. On the effect of digitoxigenin on the cellular calcium exchange in heart muscle tissue. Arch. exp. Path. Pharmakol. 224: 237-253, 1962.

KOSHLAND, D.E. The molecular basis for enzyme regulation in "The Enzymes" Vol. 1, P. 342-397. Ed. by P.D. Boyer. Academic Press, London, 1970.

KREBS, H.A. and Henseleit, K. Untersuchungen Uber die Harn-Stoff-Bildung im Tierkorper. Z. Physiol. Chem. 210: 33-66, 1932.

KRIP, G., Ostrow, B. and Bailey, L.E. The effect of gas perfusion on myocardial contractility and the inotropic effect of ouabain. Arch. Int. Pharmacodyn. Ther. 190: 322-329, 1971.

LANGER, G.A. Kinetic studies of calcium distribution in ventricular muscle of the dog. Circ. Res. 15: 393-405, 1964.

LANGER, G.A. Calcium exchange in dog ventricular muscle: relation to frequency of contraction and maintenance of contractility. Circ. Res. 17: 78-89, 1965.

LANGER, G.A. Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. Physiol. Rev. 48: 708-757, 1968.

LANGER, G.A. The intrinsic control of myocardial contraction - ionic factors. New Eng. J. Med. 285(19): 1065, 1971.

LANGER, G.A. and Brady, A.J. Calcium flux in the mammalian ventricular myocardium. J. Gen. Physiol. 46: 703-719, 1963.

LANGER, G.A. and Serena, S.D. The relationship of calcium exchange to changes in length, tension, and work in heart muscle. Amer. J. Physiol. 213: 1125-1130, 1967.

LANGER, G.A. and Serena, S.D. Effects of strophanthidin upon contraction and ionic exchange in rabbit ventricular myocardium: relation to control of active state. J. Mol. Cell. Cardiol. 1: 65-90, 1970.

LEE, K.S. and Klaus, W. The subcellular basis for the mechanism of inotropic action of cardiac glycosides. Pharmacol. Rev. 23: 193-250, 1971.

LOEWI, O. Uber den Zusammenhang zwischen Digitalis und K lzium Wirkung. Archiv. fur Exper. Path. und Pharmakol. 82: 10, 1918.

LORKOVIC, H. Influence of changes in pH on the mechanical activity of cardiac muscle. Circ. Res. 19: 711-719, 1966.

LULLMAN, H. and Holland, W.C. Influence of ouabain on an exchangeable calcium fraction, contractile force and resting tension of guinea-pig atria. J. Pharmacol. Exp. Ther. 137: 186-192, 1962.

NIEDERGERKE, R. The rate of action of calcium ions on the heart. J. Physiol. (London) 138: 506-515, 1957.

NIEDERGERKE. R. Movements of Ca in beating ventricles of the frog heart. J. Physiol. (London) 167: 515-550, 1963.

NIEDERGERKE, R. Movements of calcium in beating ventricles of the frog heart. J. Physiol. 167: 551-580, 1963.

NIEDERGERKE, R., Page, S. and Talbot, M.S. Calcium fluxes in the frog heart ventricles. Pflügers Arch. 306: 357-360, 1969.

ONG, S.D. Ph.D. Dissertation - Excitation - Contraction Coupling, 1972.

ONG, S.D. Personal Communication.

ONG, S.D. and Bailey, L.E. Two functionally distinct calcium pools in the excitation - contraction coupling process. Experientia 8: 1446, 1972.

REPKE, D.I. and Katz, A.M. Calcium binding and calcium uptake by cardiac microsomes: a kinetic analysis. J. Mol. Cell. Cardiol. 4: 401-416, 1972.

REPKE, K. Effect of digitalis on membrane adenosine triphosphatase of cardiac muscle. In 'Drugs and Enzymes', Proceedings of 2nd International Pharmacological Meeting. pp. 65-87 Pergamon Press, New York, 1965.

REUTER, H. and Beeler, G.W. Calcium current and the activation of contraction in ventricular myocardial fibers. Science (Washington) 163: 399-401, 1969.

REUTER, H. and Seitz, N. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. J. Physiol. 195: 451-470, 1968.

RIGGS, D.S. The mathematical approach to physiological problems. The Williams and Wilkins Company, Baltimore, 1963.

RINGER, S. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. Lond. 4: 29-42, 1882.

RUCH, T.C. and Patton, H.D. Physiology and Biophysics, p. 888. W.B. Saunders Company, Philadelphia and London, 1965.

SAARI, J.T. and Johnson, J.A. Decay of calcium content and contractile force in the rabbit heart. Amer. J. Physiol. 221: 1572-1575, 1971.

SABATINI-SMITH, S. and Holland W. Effects of temperature and ouabain on the rate of action of calcium on atrial contractions. J. Pharmacol. Exp. Ther. 158: 22-27, 1967.

SANDOW, A. Excitation-Contraction coupling in muscular response. Yale J. Biol. Med. 25: 176-201, 1952.

SEKUL, A.A. and Holland, W.C. Cl^{36} and Ca^{45} exchange in atrial fibrillation. Amer. J. Physiol. 197: 752-756, 1959.

SHELBOURNE, J.C., Serena, S.D. and Langer, G.A. The rate-tension staircase in rabbit ventricular muscle: relation to ionic change. Amer. J. Physiol. 213: 1115-1124, 1967.

STEEL, R.G.D. and Torrie, J.H. Principles and procedures of statistics. Ch. 10 - McGraw-Hill Book Company, Inc. 1960.

TEIGER, D.G. and Farah, A. Calcium movements in resting and stimulated rabbit atria. J. Pharmacol. Exp. Ther. 157: 8, 1967.

THOMAS, L.J., Jr. Ouabain contracture of frog heart: Ca^{45} movements and effect of EDTA. Amer. J. Physiol. 199: 146-150, 1960.

TRITTHART, H., Fleckenstein, A., Doering, H.J. and Byon, K.Y. BAY a 1040: A highly potent Ca^{++}-ant-agonistic inhibitor of excitation-contraction coupling in the mammalian ventricular myocardium. ARZNEIM FORSCH 22(1): 22-33. Illus. 1972 (Engl. summ.)

VAN BREEMEN, D. and Van Breemen, C. Calcium exchange diffusion in a porous phospholipid ion-exchange membrane. Nature (London) 223: 898-900, 30 Aug. 1969. Cit. no. 4276045.

VIVIAN, S.R. Personal Communication.

WINEGRAD, S. The possible role of Ca in excitation-contraction coupling of heart muscle. Circulation, 24: 523-529, 1961.

WINEGRAD, S. and Shanes, A.M. Ca flux and contractility in guinea-pig atria. J. Gen. Physiol. 4: 371-394, Jan. 1962.

WOOD, E.H., Heppner, R.L. and Weidmann, S. Inotropic effects of electric currents. I. Positive and negative effects of constant electric currents or current pulses applied during cardiac action potentials. II. Hypotheses: calcium movements, excitation-contraction coupling and inotropic effects. Circ. Res. 24: 409-445, Mar. 1969. Cit. no. 3501614.

YOUNG, D.A.B. Factors controlling the washout curve of the interstitial space of the isolated, perfused rat heart. J. Physiol. (London) 196: 747-759, 1968.

ZIMMERMAN, A.N.E., Daem, S.W., Hulsmann, W.C., Snijder, J., Wisse, E. and Durrer, D. Morphological changes of heart muscle caused by successive perfusion with calcium-free and calcium-containing solutions (Ca-paradox). Cardiovasc. Res. 1: 201-209, 1967.

ZIMMERMAN, A.N.E. and Hulsmann, W.C. Paradoxical influence of calcium ions on the permeability of the cell membranes of the rat heart. Nature (London) 211: 646-647, 1966.