STUDIES ON EXCITATION-CONTRACTION COUPLING IN CARDIAC MUSCLE

by

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In Loving Memory of
my Beloved Grandmother,
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ABSTRACT

The purpose of this investigation primarily was to elucidate the pathway of Ca movement during excitation-contraction coupling, and secondarily to identify, if possible, the specific anatomical sites of the Ca responsible for the activation of contraction. Gas perfused kitten hearts were used in this study and served as a useful tool to prevent the mixing of liquid perfusates, and to eliminate the effect of various substances in the vascular space on myocardial contractility. However, in the course of the study it was observed that the process coupling excitation to contraction was different in liquid than in gas perfused hearts.

The effect of Ni and La on Ca distribution and kinetics was determined and associated with the effect of these ions on contractile force. The results show that Ni had no effect on Ca washout kinetics, but abolished contractile force in cardiac muscle, probably by competing with Ca for sites necessary for the activation of the contractile mechanism. La abolished contractile force by selectively blocking the uptake of Ca into a 'trigger Ca' pool which normally causes the release of Ca from a second pool, Ca_{II}, essential for the maintenance of contractile force.

We have proposed a model for the movement of Ca during the excitation-contraction coupling process in liquid perfused hearts. Two

Ca pools are intimately involved in the contractile process, Ca_{II} and 'trigger Ca'. The absence of either 'trigger Ca', or the presence in Ca_{II} of an ion other than Ca, e.g. Ni, inhibits contractile force without affecting electrical activity. The evidence suggests that 'trigger Ca' may in fact be a Ca-carrier complex and may be located on the exterior surface of the sarcolemma. According to the model, Ca in combination with the carrier moves into the cell during depolarization. Once inside the cell, the Ca dissociates from the carrier and activates the release of Ca contained in Ca_{II}. This Ca, perhaps together with 'trigger Ca', then activates the contractile apparatus and initiates contraction. The free Ca in the intracellular space is then accumulated by Ca_{III}, representing perhaps active uptake of Ca by the sarcoplasmic reticulum and/or the mitochondria. The Ca concentration in the vicinity of the contractile proteins is decreased leading to relaxation of the myofibrils. To complete the cycle, Ca probably is pumped out of the cell.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

SECTION I

INTRODUCTION

A. Historical Background

1. Calcium in excitation-contraction coupling.

> "The action of the organ (heart) is so plainly contraction: its function is to propel blood into the arteries."
>
> Harvey in "de Motu Cordis", 1628

It was recognized by Harvey as early as 1628 that contraction is the important function of the heart. Two hundred and fifty years elapsed before Ringer (1882) demonstrated that the maintenance of the ability of the heart to contract depended upon the continuing availability of Ca ions. Since this early observation interest has increased many-fold in the mechanisms which regulate the intracellular distribution and concentration of Ca in muscle cells and by so doing regulate contraction and relaxation of cardiac muscle.

In 1907, Locke and Rosenheim reported that if Ca ions were removed from solutions bathing cardiac muscle preparation, electrical activity persisted long after the mechanical beat became minimal or ceased. This was confirmed by Mines (1913) who showed that even though the contractile activity of heart muscle ceased in Ca-free solution, the surface electrical activity of the tissue remained essentially unchanged. In 1947, Heilbrunn and Wiercinski further established that Ca is the essential link between excitation and contraction by demonstrating that an intracellular injection of Ca is capable of initiating contraction. Further evidence was provided by the experiments of

Niedergerke (1955), and Podolsky and Constantin (1964). Niedergerke (1955, 1956) also showed that the Ca causing contraction exists in the ionized form and the force of contraction is directly related to the amount of ionized Ca in the cell. Weidmann (1959) showed that an increase in the extracellular Ca concentration during depolarization resulted in an enhanced mechanical response in turtle hearts, and from these results concluded that Ca provided the essential link between the electrical and mechanical events in the normal function of cardiac muscle. It is generally agreed at present that Ca is the only ion present in body fluids which is capable of coupling excitation of the cell membrane to the mechanical response in cardiac muscle (Nayler, 1963; Winegrad, 1961; Niedergerke, 1956, 1963a, b). This has also been shown to be the case in skeletal muscle (Sandow, 1952, 1965; Frank, 1958, 1960, 1961a; Luttgau, 1963) and in smooth muscle (Bohr, 1964).

Depolarization of the muscle fibre membrane has been known for many years to be the electrical event responsible for the initiation of the contractile response (Biedermann, 1896, see Frank, 1964a). In 1957, Hodgkin and Keynes showed that electrical stimulation of the squid giant axon caused Ca exchange to increase twenty-fold. Using frog ventricles, Thomas (1960) noted that calcium uptake was increased during potassium-induced contractures. Sleator and coworkers (1964) demonstrated that the amount of Ca which entered the cell was a func-

tion of the duration of the action potential and was correlated with an increase in contractility. Reuter and Beeler (1969), using voltage clamp techniques confirmed that there was a movement of Ca into the cell during depolarization. Similarly, many investigators including Henrotte and coworkers (1960), Winegrad and Shanes (1962), and Niedergerke (1963a, b) have shown that contraction is accompanied by an increase in the uptake of Ca relative to that found in resting preparation, and that the mechanical activity of the heart muscle is correlated with this influx.

In his review on excitation-contraction coupling in skeletal muscle, Sandow (1965) concluded that although excitation of skeletal muscle is undoubtedly accompanied by an enhanced uptake of Ca, these data from Ca flux studies suggest that contraction in skeletal muscle is not activated by Ca ions which enter the cell during excitation. He postulated that an internal release and translocation of ionized Ca almost certainly provides the basis for activation of contraction in skeletal muscle. The Ca is released from the Ca-loaded membranous sacs, the lateral cistenae of the triads, in response to the increased influx of Ca associated with the excitatory stimulus.

In cardiac muscle, however, the studies of Winegrad (1961) and Niedergerke (1963a) indicate that the activation of contraction could directly involve and depend upon the Ca which entered the muscle

during or as the immediate result of excitation. According to Niedergerke (1963a), the Ca which activates contraction in cardiac muscle may initially combine with a carrier, located superficially on the cell membrane (Niedergerke, 1957), and by forming a soluble complex, move across the membrane to be released into the myoplasm in the vicinity of the myofibrils as 'activator Ca'. The contractile process in a particular muscle cell, therefore, can be activated by increasing the intracellular concentrations of Ca, and in cardiac muscle at least a part of this Ca arises from an enhanced influx of Ca from the extracellular space. The Ca which enters the cell during excitation either directly activates the muscle to contract, or triggers the release of Ca from intracellular storage sites and/or superficial membrane sites and these Ca ions then activate the contractile process.

The distribution of Ca within cardiac tissue is not homogenous. It has been suggested by Niedergerke (1963b) and many other investigators (e.g. Grossman and Furchgott, 1964a; Teiger and Farah, 1967; Bailey et al. 1972) that approximately 50-60% of the total Ca contained in the tissue is involved in contraction. However, there is considerable evidence that there are more than the two components for Ca storage in cardiac tissue suggested by these investigators. In their study of guinea pig atria, Winegrad and Shanes (1962) found kinetic evidence for three components of Ca exchange: rapidly exchangeable, slowly

exchangeable, and non-exchangeable. The halftime for the rapidly exchangeable fraction was 4.5 min which was considerably longer than would be expected if the Ca arose from the extracellular space. This suggested that there is an 'intermediary' locus for Ca storage between the extracellular and intracellular compartment. Evidence for such a locus for Ca storage was found by Niedergerke (1957) who showed that in addition to extracellular Ca ions, an appreciable amount of the Ca involved in tension changes in cardiac muscle is superficially located. In his study on frog ventricle strips, he postulated that a certain quantity of Ca is reversibly bound by a 'superficial' layer of tissue and suggested that this component of tissue Ca is responsible for the maintenance of contractile tension. The studies of Langer and coworkers (1964, 1965, 1967) using arterially perfused dog papillary muscle provided a further definition of Ca within the cardiac muscle. Five phases of Ca exchange were defined (phase 0 through 4), and it was suggested that the maintenance of myocardial contractility resided almost exclusively in one fraction, phase 2. Langer concluded that phase 2 Ca was correlated with the 'superficial' Ca of Niedergerke (1957) and the intermediary locus of Winegrad and Shanes (1962). The halftime of exchange of phase 2 was 6.0 min and is made up of about 25% of the total tissue Ca. Similar findings were reported by Shelbourne et al. (1967). Langer (1965) was also able to correlate the decay of contractile tension

with the washout of Ca from phase 2 in Ca-free perfusate. Recently, however, Shine et al. (1971) showed a correlation between the decay of contractile tension with the washout of Ca from phase 1 which was in contrast to previous reports from the same laboratory (Langer, 1965). They concluded that Ca originating in phase 1 supports contractile force while phase 2 represents a storage pool of Ca. Teiger and Farah (1967) demonstrated that only a three compartment system for Ca exchange existed in isolated rabbit atria. They postulated that the Ca compartment which has a halftime of exchange of approximately 2 min (k_2 = 0.329 min^{-1}) was associated with the maintenance of contractility. These investigators presented evidence that several compartments for Ca exist in cardiac muscle and from indirect evidence have related one or possibly two of these compartments to the force of contraction of the heart.

In 1968, Bailey and Dresel showed a direct relationship between cellular Ca stores and isometric contractile force in isolated, gas perfused cat hearts. They demonstrated that the washout of Ca was characteristic of a three compartment system and proposed the following phase assignments for their washout Ca fractions--Ca_I, representing vascular Ca; Ca_{II}, the Ca associated with the maintenance of contractile force in the heart; and Ca_{III}, the Ca tightly bound in tissue sites. They showed that the rate of washout of Ca from Ca_{II} was cor-

related with the monoexponential rate of decay of contractile force, and moreover, that the force of contraction developed by the heart was directly related to the content of Ca_{II}. The halftime of washout of Ca_{II} was between 20-40 sec (Bailey and Dresel, 1968; Bailey and Sures, 1971; Bailey et al. 1972). Thus, the available evidence from exchange and flux studies strongly indicates that there is a single, separable component of myocardial Ca involved in the maintenance of contractile activity of the heart.

2. Ions and muscle function.

Initially, the study of heavy metals and their salts on living tissues were directed mainly to their general toxicity, absorption, distribution and elimination in the tissues and organs of the body since many are found frequently in various food products and some may give rise to industrial poisoning (Drinker et al. 1924). More recently, the study of the effect of heavy metals on the various organs has given some insight into the various mechanisms of cellular function. Of particular interest are the effects of the divalent cations, Zn, Ni, Mn, Co, the trivalent cation, La and various anions, Br, SO_4, and No_3, on the mechanism of excitation-contraction coupling and the role of Ca in skeletal, smooth, and cardiac muscle (e.g. Frank, 1961b, 1962, 1964b; Bianchi, 1968; Sandow, 1965; Nayler, 1964; Nayler and McCulloch, 1960; Daniel, 1964).

It has been known for at least 70 years that the heavy metals in general, are cardiac depressants. As early as 1910, Mines investigated the action of some heavy metals on the heart and found that the salts of the divalent ion, Be, and the trivalent ions of La, Y and Ce, depressed contractile activity in the frog heart. Their effect was similar to that of hydrogen ion and he suggested that both the hydrogen and the trivalent cations produced their effect by altering the permeability of the cell membrane. Of the ions tested, the divalent cation, Be, was less potent than the trivalent cation, La, which was the most potent of all the ions causing cardiac arrest. Salant and Connet (1920) investigated the effect of several heavy metals (Cd, Cu, Fe, Mn, Co, Ni, U, Zn) on the frog heart and found that all these ions depressed contractility to varying degrees. Cd, however, was the most toxic of the ions tested.

In contrast to cardiac muscle, Frank (1962) observed that the addition of divalent cations to Ca-free fluids bathing skeletal muscle preparations restored their ability to contract and to develop tension after K-induced depolarization. He concluded that these divalent cations restored excitation-contraction coupling in skeletal muscle by apparently releasing bound cellular Ca. Isaacson and Sandow (1963) showed that Zn (0.005 - 0.1 mM) potentiated contraction of skeletal muscle.

Nayler (1964) reported that the divalent cations except Sr and

Ba failed to restore the ability of the cardiac muscle to contract or to develop tension in response to K-induced depolarization in Ca-free Tyrode solution. Using radioisotope studies, she concluded that the divalent cations, Zn, Mg, Ni, and Co failed to release bound cellular Ca from cardiac muscle cells. She concluded that these divalent cations release Ca from a superficially located site of the Ca exchange system. Nayler and Anderson (1965) and Ciofalo and Thomas (1965) found that Zn reversely diminished tension in actively contracting cardiac tissue. They proposed that this inhibitory effect of Zn was due to an interference with the excitation-contraction coupling process since cellular electrical activity remained essentially normal. Both groups showed a decrease in Ca content of the tissue following exposure to Zn. Sabatini-Smith and Holland (1969) demonstrated that Mn inhibits contractility and depresses Ca exchange across the mammalian atria. It was concluded from these studies that a part of the Ca involved in contraction moves inward across the membrane or is released from superficial sites to participate in contraction and this is blocked by Mn.

(a) Nickel - Ni.

The early investigations on Ni, dwelt on the question of potential toxic effects in man from using food or drink cooked or stored in Ni or Ni-lined containers (Drinker et al. 1924). These investigators concluded that Ni is not a serious pollutant, and when introduced with

food into the body in small quantities for long periods, it is relatively harmless. More recent investigations have centred on the role of Ni in physiological function. Ni is one of the relatively non-toxic trace metals found in the tissues of man. Ni has not been shown to be essential to life, but, the precise physiological role of Ni is still unknown. It is reported to activate various enzyme systems including arginase (Hellerman and Perkins, 1935), carboxylase (Speck, 1949), acetyl coenzyme A synthetase (Webster, 1965), and trypsin (Sugai, 1944). Ni is present in RNA obtained from various sources (Wacker and Vallee, 1959) and may play a part in maintaining the configuration of the protein molecules of crystalline complexes of ribonuclease (King, 1964). Schroeder et al. (1962) suggested that Ni may be involved in the pigmentation of the skin, but this role for Ni has not been proven.

There are a number of reports of the therapeutic use of Ni between 1850-1900. Nickel sulphate has been considered to be useful for migraine headache and for the relief of amenorrhea (Gerekens, 1883, see Drinker et al. 1924), for neuralgia and sleeplessness in cases where opium failed (Palmer, 1868, see Drinker et al. 1924), for leukorrhea (Broadbent, 1869, see Drinker et al. 1924), and has also been prescribed as an antiseptic for the treatment of parasitic skin diseases. A salt of Ni, the bromide has been used as an antiepileptic. However, Cushny (1918) in his 'Textbook of Pharmacology' did not mention a single thera-

peutic use for Ni. Surprisingly, Henkin and Bradley (1970) reported the usefulness of Ni and Zn in correcting hypogeusia, but this therapeutic role of Ni is questionable. This element may be of clinical interest in the future since it is reported to be greatly elevated in serum from patients immediately after myocardial infarction (D'Alonzo et al. 1963; Sunderman et al. 1970). Its potential role in disease or as a diagnostic tool in myocardial infarct, is still speculative at present.

Richet (1882, see Drinker et al. 1924) noted that Ni arrested contractions of the heart. Voegtlin (1915) suggested that the toxic effect of the lactates of many heavy metals including Ni appeared to be produced by a change in the permeability of the superficial layer of cells of the heart. Nayler (1964) reported, that unlike skeletal muscle, Ni does not release tightly bound cellular Ca from cardiac muscle, and is unable to maintain contraction as in skeletal muscle (Frank, 1962). Nayler observed that Ni released Ca from a superficially located pool of the Ca exchange system. Babskii and Donskih (1965) and Kaufmann and Fleckenstein (1965) investigated the effect of Ni on the electrical and mechanical activity of cardiac tissue. Both groups found that the addition of 2 mM Ni to a normal physiological solution containing Ca can abolish the contractility of cardiac muscle without affecting the electrical excitation process or the resting membrane potential. This effect of Ni on contractility is reversible and may be due to competition

with Ca at some point in the process coupling, excitation to contraction in cardiac muscle.

(b) Lanthanum - La.

La, the most electropositive element of the rare earth group, is considered to be most similar to the alkaline earth elements in its chemical properties (Levy, 1915). This ion has a very high affinity for phosphate and carboxyl groups (Takata et al. 1967; van Breemen, 1968) and bind strongly to phospholipids. It was noted by Hagiwara and Takahashi (1967) that the membrane-binding ability of La is much greater than that of Ca in the barnacle muscle fibre.

It has been predicted that an ion with much greater affinity than Ca for anionic groups will be poorly transported and at the same time make the transport sites unavailable for Ca (van Breemen and van Breemen, 1969). As expected, La completely blocks the flux of ^{45}Ca across artificial phospholipid membranes (van Breemen and van Breemen, 1969). Ca fluxes across mitochondrial membranes (Mela, 1968a, b, 1969) and across the squid axolemma (van Breemen and de Weer, 1970) have also been shown to be blocked by La. Van Breemen (1969), and van Breemen and McNaughton (1970) showed that La blocks ^{45}Ca flux across vascular smooth muscle membranes, thereby abolishes contraction.

Electron microscopic evidence shows that La does not pene-

trate cell membranes and these electron dense ions can be used as extracellular markers (Revel and Karnorvky, 1967). This suggests a surface located or superficial site of action of La. In accord with this supposition, Weiss and Goodman (1969) demonstrated that La replaces Ca at superficial membrane sites, decreases the mobility of Ca located at less superficial membrane sites and prevents the uptake of ^{45}Ca to various cellular sites in intestinal smooth muscle. They concluded that La apparently alters Ca movements and Ca binding, thereby inhibiting contraction of smooth muscle. Weiss (1970) has also found that La inhibits ^{45}Ca movements and tension responses elicited by 80 mM K but not those induced by 5.0 mM caffeine in frog sartorius muscle. Therefore, La appears to have an action on the superficial surface of the cell membrane.

Such a valuable tool has not escaped the eye of the cardiac physiologists. Palmer and van Breemen (1970) and Sanborn and Langer (1970) reported that La abolishes contractile tension in cardiac muscle preparations. Since electrical activity of the cardiac cell is not affected at the concentrations used, Sanborn and Langer (1970) concluded that La apparently displaces Ca (phase 2) which they identified as the Ca essential for contraction. However, their results were not conclusive since La displaced only part of the Ca in phase 2 when contractile force was abolished.

B. Statement of the Problem.

Despite the differences in the pathways for Ca movement in the three main classes of muscle, smooth, skeletal and cardiac, there is little question that Ca is required in all types of muscle to couple excitation to contraction. Several models to define the role of Ca in this process in cardiac muscle have been proposed. According to the most widely accepted model (Niedergerke, 1963a), Ca from the extracellular space traverses the cell membrane in combination with a carrier, CaR, and is released into the myoplasm as 'activator Ca' during depolarization of the cell. 'Activator Ca' activates the contractile proteins and initiates contraction, after which the Ca is accumulated by a second Ca pool to lower the intracellular Ca concentration and initiate relaxation. Ca is finally expelled from the cell against a significant electrochemical gradient by an active process. Recently, Bailey et al. (1972) have shown evidence supporting this hypothetical model for the movement of Ca during excitation-contraction coupling in cardiac muscle, and have suggested that CaR may be identical to a Ca compartment, Ca_{II}, known to be directly involved in the maintenance of contractile force. The pool which accumulates Ca after contraction may be the pool identified in their experiments as Ca_{III}, which is perhaps the sarcoplasmic reticulum, known to actively accumulate Ca (Martinosi and Feretos, 1964; Weber et al. 1963). According to the modification of Niedergerke's (1963a)

model proposed by Bailey et al. (1972), the pathway for normal Ca movement during the contractile cycle is as follows: 1) Ca is taken up from the vascular space, represented by Ca_I, 2) is stored temporarily in Ca_{II}, which may represent Ca bound to the inner surface of the sarcolemma, 3) released by depolarization of the cell membrane, 4) activates the contractile mechanism, 5) inactivated by active accumulation in Ca_{III}, and 6) actively removed from the cell.

The major objectives of this investigation were first, to determine the exact role of Ca_{II} in the contractile process, and second to elucidate the distribution and pathways of Ca movement before, during and after contraction. As a corollary to these objectives it was hoped to identify the anatomical location of the Ca responsible for activation of contraction in cardiac muscle.

C. Approach to the Problem.

It has been virtually impossible to correlate a specific Ca pool in the heart with the maintenance of contractile force when measurements of tracer Ca flux were made under steady state conditions of contractility. One cannot be certain that a Ca pool is related to the maintenance of contractility unless changes in Ca flux of that pool induced by drugs or other interventions are accompanied by simultaneous and parallel changes in contractile force. However, when the Ca steady state in the heart was disturbed by perfusion with a Ca-free K-H solu-

tion, the washout of a single Ca compartment was found to be directly related to the decay of contractile force (Bailey and Dresel, 1968). Although Ca fluxes measured under Ca-free perfusions are not physiological, this technique has eliminated a factor which interferes with the measurement of tracer Ca flux under steady state conditions of contractility. Back diffusion or back flux of Ca into the tissues is negligible during perfusion with Ca-free media since the diffusion gradient for Ca out of the tissue into the vascular space was infinite and is maintained by constant replenishment of Ca-free fluid. Moreover, by upsetting the Ca steady state of the heart by Ca-free perfusion, Bailey and Sures (1971) have been able to show a direct relationship between Ca uptake and the restoration of contractile force upon reperfusion. Other investigators (e.g. Langer and Brady, 1963; Grossman and Furchgott, 1964b; Niedergerke, 1963a, b; Niedergerke et al. 1969a, b; Tieger and Farah, 1967; Wasserman and Holland, 1971) could only indirectly relate one of the phases of Ca uptake and/or exchange to the maintenance of contractile activity in the heart under steady state conditions.

The gas perfused kitten heart was used as the main experimental preparation in this study because gas perfusion removes Ca and other substances in the vascular space as a contributing factor in the maintenance of contractile force (Krip et al. 1971). Gas perfusion also

eliminates a source of error and interference in the analysis of the Ca washed out with respect to the decay of contractile force during Ca-free washouts. By eliminating the liquid dead space, gas perfusion provides convenient and exact starting and ending points for relating changes in contractile force induced by liquid perfusion with changes in the flux of Ca and other ions.

Ni and La were used to uncouple excitation from contraction because it was believed that these ions would interrupt Ca flux at specific points in the cycle of events coupling excitation to contraction. Ni has been reported to release Ca from superficial binding sites in cardiac muscle (Nayler, 1964). However, Kaufmann and Fleckenstein (1965) suggested that Ni inhibited contractile activity of cardiac muscle by competing with Ca for activation of the contractile process. If on the one hand, Ca_{II} is superficially located, then Ni may interrupt the process coupling excitation to contraction by displacement of Ca in Ca_{II}. On the other hand, Ni may have blocked the activation of the contractile apparatus by competing with Ca after the release of both ions from Ca_{II}. In either eventuality, the information gained by interrupting the cycle of Ca involvement in coupling excitation to contraction with Ni would provide a better understanding of the pathway of Ca movement during contraction and relaxation.

La has been reported to interfere with excitation-contraction

coupling by displacing the Ca known to be directly involved in the maintenance of contractile force in the heart (Sanborn and Langer, 1970). In contrast to the effects of Ni, La may have a more superficial site of action since La abolishes contractile force more rapidly, $T_{1/2}$ = 8 sec (Palmer and van Breemen, 1970) compared to a halftime of approximately 25 sec for Ni. In addition, La is known to block Ca flux across artificial membranes, and smooth and skeletal muscle membranes (van Breemen and van Breemen, 1969; van Breemen and McNaughton, 1970; Weiss, 1970). Thus, La may interrupt the process coupling excitation to contraction by at least two mechanisms. First, by displacing Ca from Ca_{II}, or perhaps from another superficial pool of Ca not detected in washout studies (Bailey and Dresel, 1968; Bailey et al. 1972). Second, it is equally probable that La may have interfered with the inward movement of Ca from the extracellular space into Ca_{II} during depolarization. If La affects contractility by the former mechanism, the use of La would provide an insight into the exact role of Ca_{II} in the contractile process or allow identification of a more labile Ca pool essential to contraction not detected previously. If, on the other hand, La interferes with the inward movement of Ca into Ca_{II}, then it would aid in understanding the role of extracellular Ca in coupling excitation to contraction. In any event, La most likely interferes with contraction at a step earlier in the excitation-contraction coupling process than does Ni, and thus it

will serve as a second valuable tool in clarifying the sequence of events occuring between excitation and the initiation of contraction in cardiac muscle.

In addition, La also may be a useful marker for histological studies to identify the morphological location of the Ca involved directly in the maintenance of contractile force. That is, since La is electron dense and apparently is bound to extracellular sites directly involved in the binding and/or transport of Ca into cells, it may be possible with the aid of electron-micrographs to identify the morphological location of Ca_{II} or perhaps another Ca pool necessary for contraction.

SECTION II

METHODS

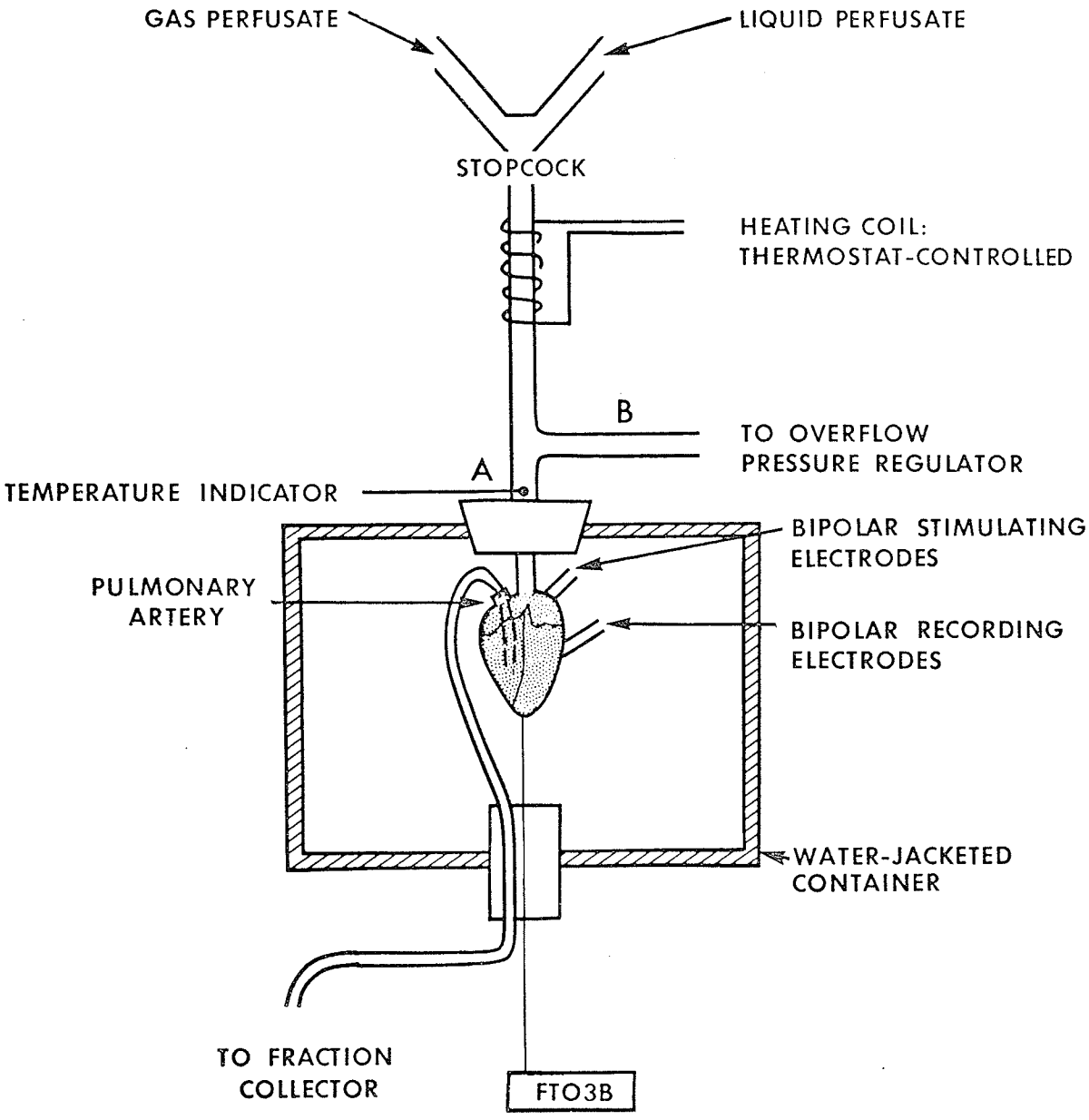
A. Experimental Preparation.

1. The perfusion apparatus.

The apparatus used for gas perfusion in the cat heart has been described by Gabel et al. (1966) and by Bailey and Dresel (1968). A schematic diagram of the perfusion apparatus used in this investigation is shown in Figure 1. The heart was perfused by the Langendorff technique from a cannula with two side arms close to its tip. A thermistor probe (YSI Model 403) extended directly into the perfusate in the cannula tip through one of the side arms, A, and was used to monitor all perfusate temperatures. A thermoregulator (YSI Model 73) controlled the flow of current through a heating coil placed around the glass tube leading to the cannula. The heating coil served only as a final temperature adjustment to maintain the temperature of gaseous perfusates at 37.0 $\pm$ 0.5^{o}C. Perfusion pressures were regulated by immersing a glass tube into a column of water to a depth corresponding to 60 mm Hg. A trap was attached to the side arm B before it was connected to the pressure regulator, to collect liquid perfusate remaining in the cannula at the time of change from liquid to gas perfusion.

Liquid perfusates of different composition were stored in reservoirs pressurized to 60 mm Hg with 95% O_2 - 5% CO_2 or 100% O_2 by exhausting the overflow gases into a column of water to a depth corresponding to 60 mm Hg as described above. The liquid perfusates were

Figure 1: Schematic diagram of the perfusion apparatus. See text for detailed description.


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

warmed to 37.0 ± 0.5°C through spiral condensers before reaching the cannula. The 95% O_2 - 5% CO_2 gas mixture or 100% O_2 for perfusion was preheated in a water jacketed spiral condenser and then humidified in a water jacketed scrubbing bottle containing saline, before reaching the heating coil and cannula.

The perfused heart was enclosed in a water-jacketed plexiglass box. High humidity was maintained in the chamber by keeping the base of the box moist. Temperature within the box was maintained at 37.0 ± 0.5°C by the water jacket. The cannula from the right ventricle and a monofilament line attached to the apex of the heart were passed out of the plexiglass box through the hole at the bottom of the box. The hole was sealed by Plastibase^R (Squibb, New York) which was held in place by a finger obtained from a rubber glove. This afforded an efficient seal and prevented liquid from dripping on to the Grass FTO3B force displacement transducer directly below it. Some damping of contractile force recordings was unavoidable because of the Plastibase^R adhering to the monofilament line.

2. Preparation of the heart.

Kittens of either sex weighing 0.7 - 1.2 kg were obtained commercially. Heparin (1000 u/kg) (Connaught Labs, Toronto, Canada) was injected intramuscularly one hour before the animal was sacrificed. This was to prevent clotting of blood in the coronaries after

the animal was killed. The animal was killed by a blow on the head and the heart was removed and placed in a beaker of cold Krebs-Henseleit solution (Krebs and Henseleit, 1932). Extraneous tissue was removed from the heart and the aorta was attached to the cannula of the perfusion apparatus. A slit was made in the pulmonary artery and a cannula was inserted in the right ventricle through the slit. The effluent from the heart was collected from this cannula. The venae cavae and the pulmonary vein were ligated to prevent loss of perfusate. A small incision was made in the apex of the left ventricle to allow the excess gas and liquid to escape during the perfusion and also as a point of attachment for a stainless steel clip. A length of nylon monofilament line connected the clip to a Grass FTO 3B force displacement transducer. Changes in isometric tension were recorded on a Grass 5D polygraph. Resting tension was adjusted to produce a developed force of contraction which was 50% of maximum as determined by the length-tension relationship. This was found to be approximately 10 g for the cat heart, and thus resting tension was maintained at 10 g for all the experiments. In some experiments two platinum electrodes were attached to the left ventricle to record electrical activity during the various experimental manipulations. The hearts were driven electrically by a Grass S6 stimulator at twice threshold voltage at a frequency of 180 beats/min. In experiments in which only contractile force was recorded, a single

electrode was attached to the right atrium and the stainless steel clip at the apex of the heart served as the indifferent electrode. When electrical activity was also recorded, bipolar stimulating electrodes were attached to the right atrium.

B. Composition of Perfusion Solutions.

1. Modified Krebs-Henseleit solution.

One liquid perfusate used in this study was modified Krebs-Henseleit solution (K-H solution) equilibrated with 95% O_2 - 5% CO_2. After equilibration at 37°C the solution had a pH of 7.4. The modified K-H solution had the following composition:

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

2. Hepes solution.

The liquid perfusate used in all experiments with La was Hepes solution (Sigma Chemicals, St. Louis). It had the following composition:

	mM	g/l
NaCl	140.0	8.17
KCl	4.5	0.33
$MgCl_2 . 6H_2O$	1.2	0.24
$CaCl_2 . 6H_2O$	2.5	0.55
Glucose	11.2	2.00
HEPES (N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid)	3.0	0.72

After all the compounds had dissolved in glass distilled water, the solution was titrated at 37°C with 3M NaOH to a pH of 7.4 $\pm$ 0.05 (Sanborn and Langer, 1970).

Hepes was substituted for $NaHCO_3$ as a buffer to prevent the formation of the insoluble compound $La_2(CO_3)_3$, $MgCl_2$ was used in place of the sulphate to prevent the formation of $La_2(SO_4)_3$, which is also insoluble in water. The pKa of Hepes is within the range for physiological buffering, pKa = 7.55 at 20°C. Good et al. (1966) demonstrated that Hepes is essentially inert as measured by the Hill reaction (ferricyanide reduction by illuminated spinach chloroplasts) and succinate oxidation by bean hypocotyl mitochondria. The perfusate was bubbled with 100% O_2 instead of the mixture of 95% O_2 - 5% CO_2 when K-H solution was used. This was to prevent the formation of the car-

bonate, and moreover, CO_2 is not required to keep the pH at 7.4. After perfusion with Hepes, the hearts were gas perfused with 100% O_2. Ca kinetics measured in hearts perfused with Hepes solution did not differ from K-H perfused hearts (see below).

3. Further modification of perfusates.

a. Ca-free perfusates.

The composition of the Ca-free K-H and Hepes solutions were not altered except that the $CaCl_2 . 6H_2O$ was omitted.

b. Ni-Krebs.

A stock solution of the chloride salt of Ni was prepared and aliquots were added to K-H solution to prepare Ni-Krebs. The concentration of Ni varied between 0.8 - 4 mEq/l.

c. La-Hepes.

La Cl_3 stock solution (500 mM La) was prepared by adding concentrated HCl to 8.15 mg of La_2O_3 (American Potash & Chemical Corporation, Chicago). When all solid materials were dissolved, glass distilled water was added to make up to 100 ml. Aliquots of the stock solution were added to the Hepes solution and pH again adjusted to 7.4 with 1N NaOH. La in the concentrations 5-500 uM were used in this investigation.

The osmolarity of these modified perfusates was between 300-320 mOsm and this did not differ significantly from the osmolarity of

physiological body fluids, 290-320 mOsm (Ruch and Patton, 1965).

All perfusion solutions were prepared fresh each day. Four stock solutions containing a) NaCl + KCl, b) $NaH_2PO_4 . 2H_2O$ + $NaHCO_3$, c) $MgSO_4 . 7H_2O$ and d) $CaCl_2 . 6H_2O$ were prepared as separate solutions and stored in the cold, 4°C. The $NaH_2PO_4 . 2H_2O$ + $NaHCO_3$ solution was bubbled with 100% CO_2 for one hour as described by Krebs and Henseleit (1932). Aliquots of each of the stock solutions were added to make K-H solution. This prevented the formation of microcrystals of insoluble calcium carbonate and calcium phosphate in the K-H solution. Similar stock solutions were also prepared for Hepes solution except that the stock solution containing Hepes was prepared fresh each week. This prevented the possible degradation of Hepes on standing in solution. At present very little is known about the chemical nature of Hepes and its degradation products. We encountered adverse effects on contractility in hearts perfused with a solution made from old stocks of Hepes. However, the supplier (Sigma Chemical Company) was unable to detect any degradation of our Hepes solution by chemical analysis at their plant (Personal Communication).

C. Experimental Procedures.

1. Perfusion procedures.

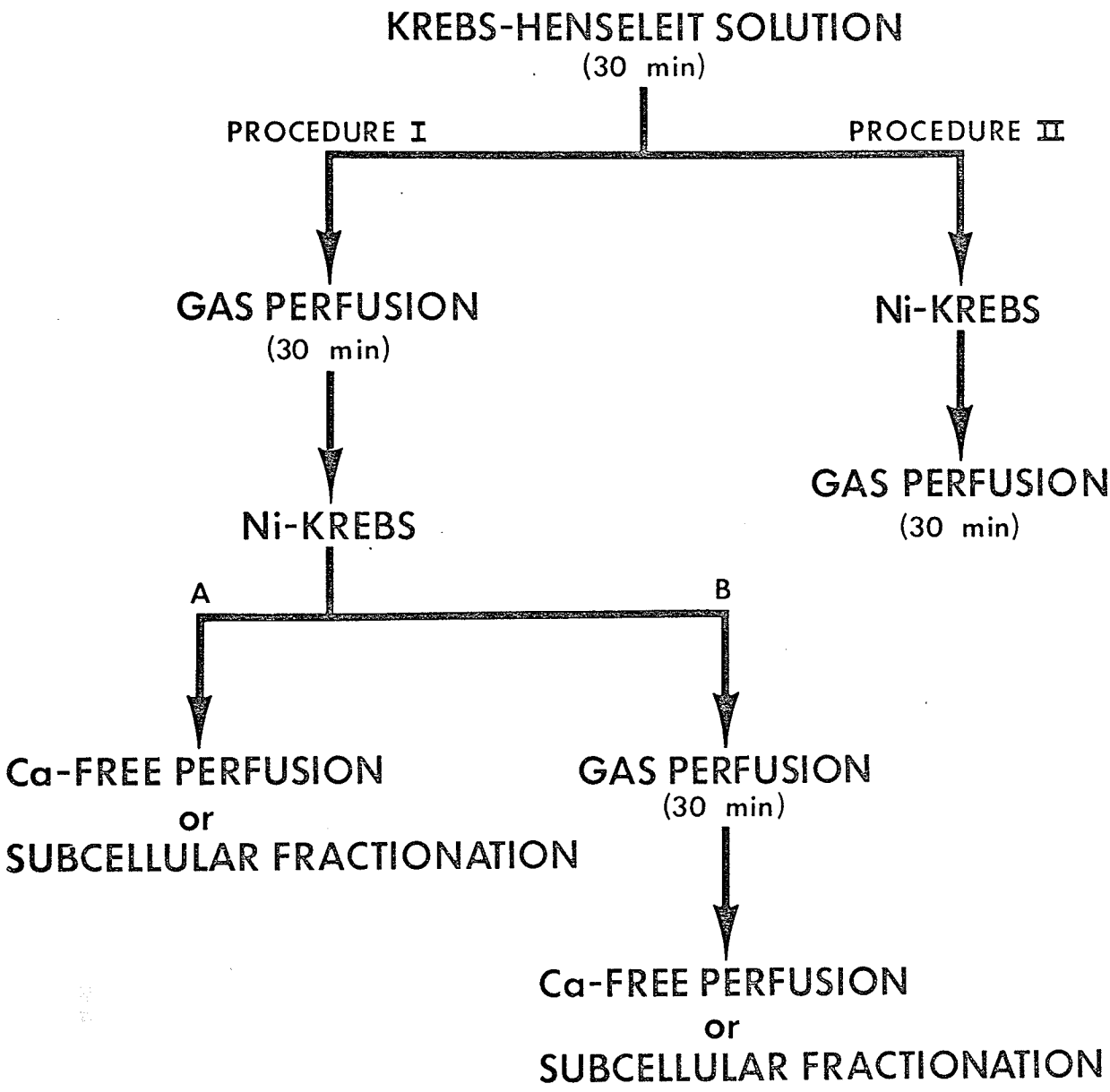
a. Ni-Krebs perfusion.

Thirty-five hearts were cannulated and perfused for 30 min

with K-H solution. As shown in Figure 2, they were then either gas perfused with a water saturated gas mixture of 95% O_2 - 5% CO_2 and reperfused with Ni-Krebs until contractile force had decreased to less than 1 g (Figure 2, Procedure I), or perfused with Ni-Krebs without gas perfusion again until contractile force had decreased to less than 1 g (Figure 2, Procedure II). The Ni-Krebs contained either 4 mEq Ni/l or 1 mEq Ni/l (see Results). Subsequent to the Ni-Krebs perfusion, both groups of hearts were gas perfused for 30 min.

The effect of Ni on the washout of Ca and the subcellular distribution (see C. 3 below) of Ni and Ca were investigated in hearts gas perfused for 30 min and then reperfused with Ni-Krebs (Figure 2, Procedure I).

In one group of hearts, gas perfusion was continued for 30 min after Ni-Krebs perfusion, the hearts were then either washed out with Ca-free, K-H solution or were removed for subcellular fractionation (Figure 2, Procedure IB). A second group of hearts was gas perfused briefly (5-10 sec) to clear the coronary vessels of fluid and then washed out with Ca-free K-H solution or removed for subcellular fractionation (Figure 2, Procedure IA). The hearts were briefly gas perfused (5 sec) in the second group so as to prevent admixture of Ni-Krebs with Ca-free K-H solution. Ouabain (5×10^{-8} g/ml or 0.85 mM) (Fluka, Switzerland) was included in all liquid perfusates in eight experiments.


KREBS-HENSELEIT SOLUTION
(30 min)
PROCEDURE I
PROCEDURE II
GAS PERFUSION
(30 min)
Ni-KREBS
Ni-KREBS
GAS PERFUSION
(30 min)
A
B
Ca-FREE PERFUSION
or
SUBCELLULAR FRACTIONATION
GAS PERFUSION
(30 min)
Ca-FREE PERFUSION
or
SUBCELLULAR FRACTIONATION

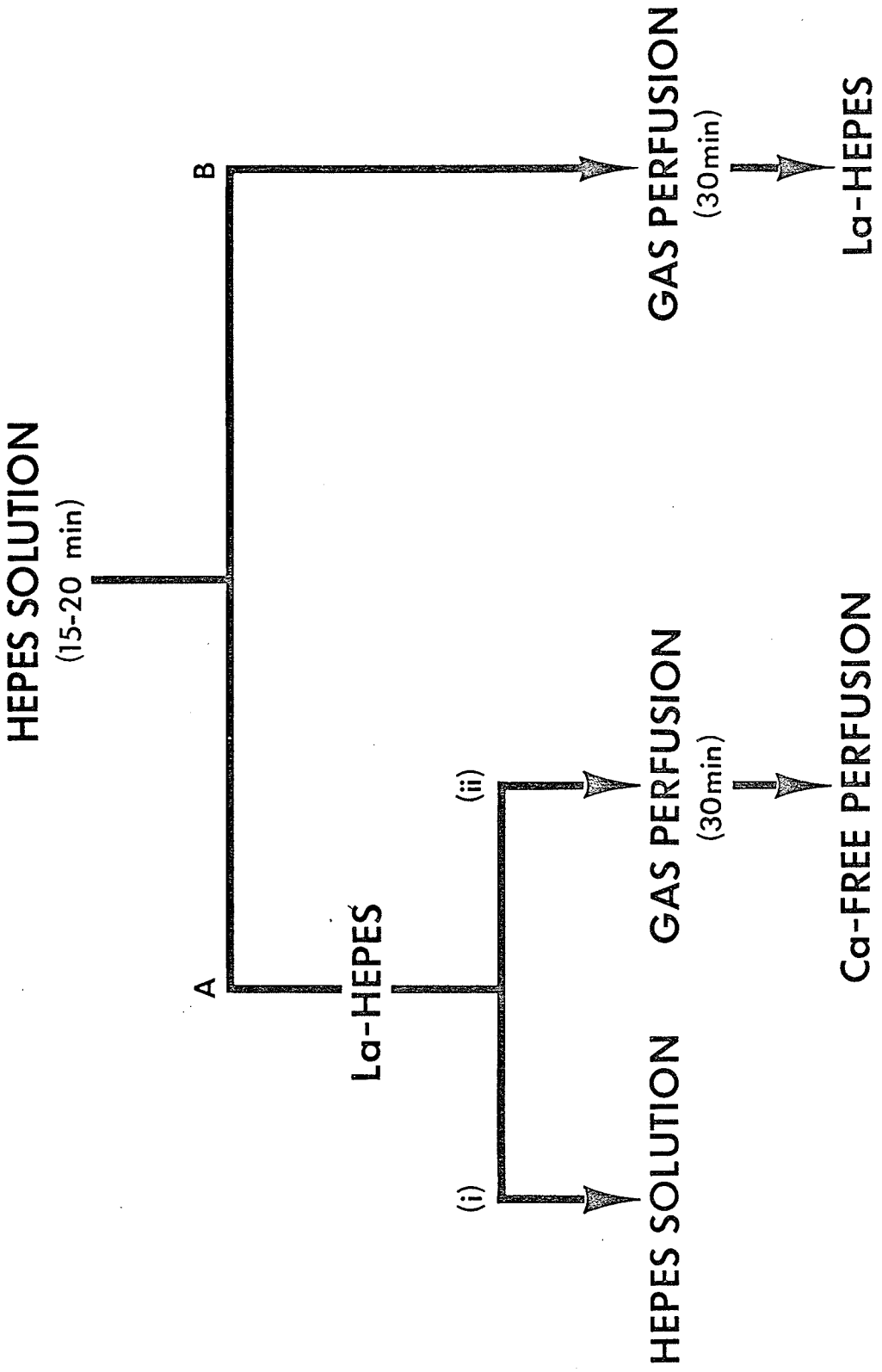
b. Metabolic inhibition and anoxia.

Four hearts were perfused for 30 min with K-H solution and then gas perfused for another 30 min. To study the effect of a metabolic poison 2,4 di-nitrophenol (DNP), and the effect of anoxia on perfused hearts, they were then either reperfused with K-H solution containing 0.1 mM DNP or with K-H solution bubbled with 95% O_2 - 5% CO_2.

c. La-Hepes perfusion.

(i) Preliminary experiments.

To investigate the effect of La, a series of twenty-five experiments was conducted. The hearts were equilibrated by preperfusion with Hepes solution for 15-20 min until contractile force reached a steady level. They were then either gas perfused with 100% O_2 for 30 min and then perfused with La-Hepes containing 500 uM La (Figure 3, Procedure B), or perfused with La-Hepes until contractile force was less than 1 g, gas perfused with 100% O_2 for 30 min and then perfused with Ca-free Hepes solution (Figure 3, Procedure A(ii)). The La-Hepes used in the experiments illustrated in Figure 3 contained 25 to 500 uM La (see Results). A few hearts were perfused with Hepes solution after La-Hepes (Figure 3, Procedure A(i)). An additional two hearts were perfused with La-Hepes containing 5 uM La for 20 min after equilibration for 15 min with Hepes solution.

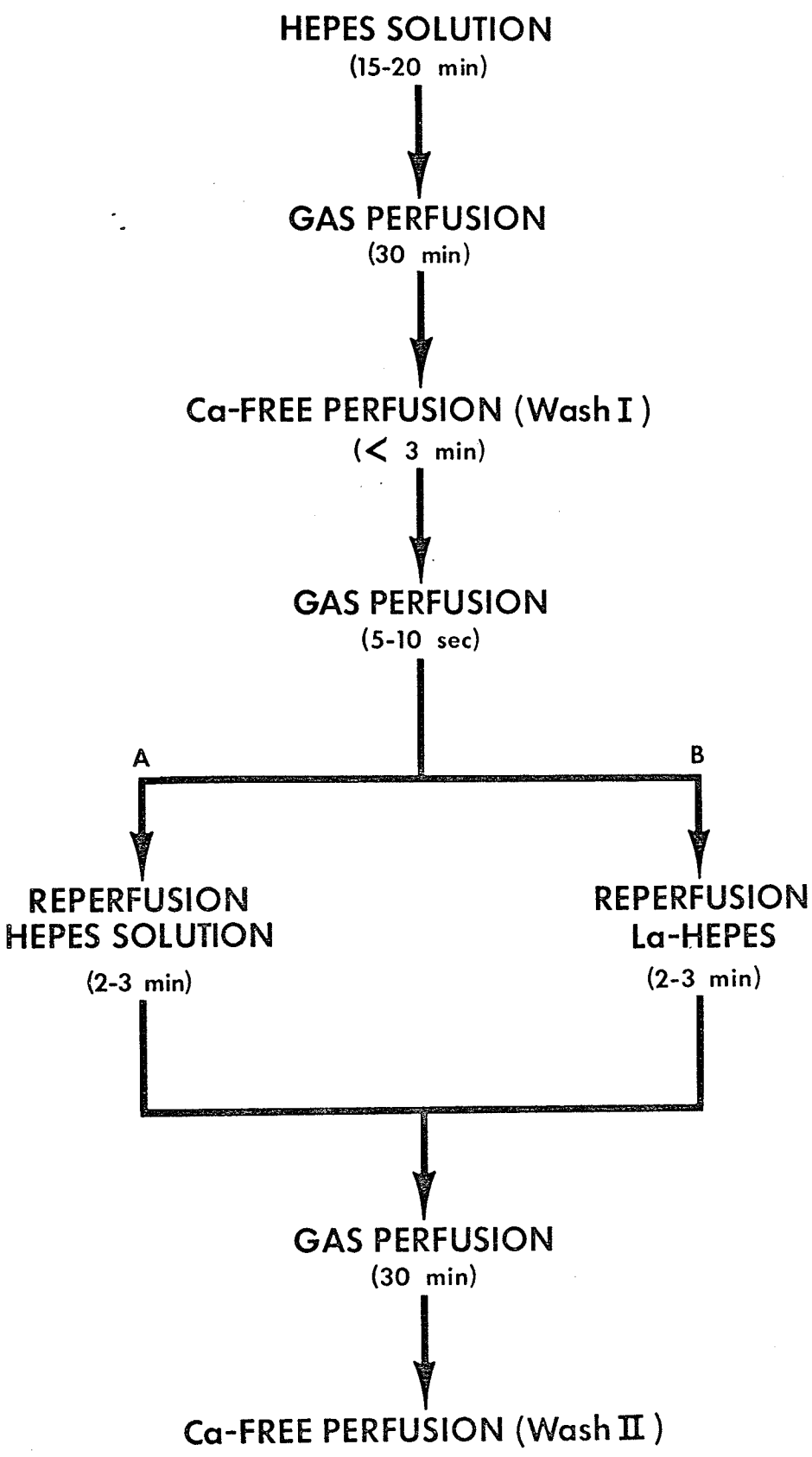

HEPES SOLUTION
(15-20 min)
A
B
La-HEPES
(i)
(ii)
HEPES SOLUTION
GAS PERFUSION
(30min)
Ca-FREE PERFUSION
GAS PERFUSION
(30min)
La-HEPES

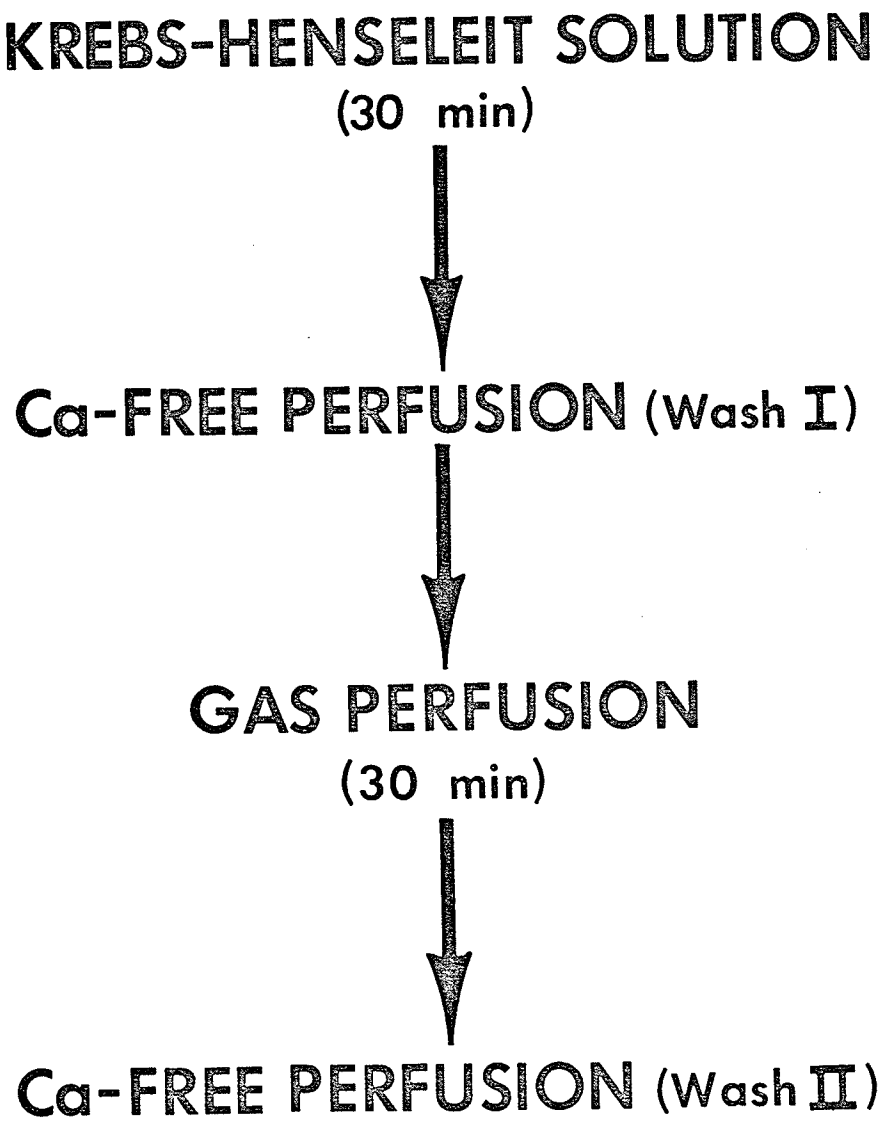
(ii) Ca uptake.

The effect of La on the restoration of contractile force and the uptake of Ca into Ca-depleted hearts was investigated by the method of Bailey and Sures (1971). As shown in Figure 4, ten hearts were perfused for 15-20 min with Hepes solution and then gas perfused for 30 min. They were depleted of Ca by Ca-free perfusion (Wash I) until contractile force had decayed to less than 1 g, briefly gas perfused (5-10 sec) to clear the coronary vessels of fluid and reperfused with perfusate containing 5.0 mEq Ca/l and tracer amounts of ^{45}Ca in the presence (Figure 4, Procedure B) and absence (Figure 4, Procedure A) of La. After Wash I the various Ca pools of the heart still contained Ca, and the Ca taken up could not be differentiated from that present in the heart. Thus, the perfusate contained tracer amounts of ^{45}Ca to enable us to trace the distribution of Ca taken up during the reperfusion. To determine the distribution of Ca taken up by the heart during reperfusion, both groups of hearts (Figure 4, Procedure A and B) were perfused with Ca-free perfusate (Wash II) after 30 min of gas perfusion. Tracer amounts of ^{3}H-Inulin were added to the perfusate in six experiments, three in the presence of La and three in the absence of La. ^{3}H-Inulin was used as an extracellular marker. The uptake and volume of distribution of ^{3}H-Inulin was not affected by La.

d. Comparison of liquid perfusion and gas perfusion.

The perfusion protocol is shown in Figure 5. Five hearts were


HEPES SOLUTION
(15-20 min)
GAS PERFUSION
(30 min)
Ca-FREE PERFUSION (Wash I)
(< 3 min)
GAS PERFUSION
(5-10 sec)
A
B
REPERFUSION
HEPES SOLUTION
(2-3 min)
REPERFUSION
La-HEPES
(2-3 min)
GAS PERFUSION
(30 min)
Ca-FREE PERFUSION (Wash II)


KREBS-HENSELEIT SOLUTION
(30 min)
Ca-FREE PERFUSION (Wash I)
GAS PERFUSION
(30 min)
Ca-FREE PERFUSION (Wash II)

equilibrated with K-H solution for 30 min, then perfused with Ca-free K-H solution (Wash I) until contractile force was less than 1 g. When Ca-free K-H solution was substituted for K-H solution, care was taken to ensure that the cannula was cleared of the K-H solution first, to prevent admixture of fluids. The hearts were then gas perfused for 30 min and again perfused with Ca-free K-H solution (Wash II). In a few experiments, ^{3}H-Inulin was added to K-H solution and the hearts perfused for the final 5-10 min during the 30 min preperfusion with this solution. The rates of decay of contractile force and the washout of Ca in Wash I (liquid perfused hearts) were compared with the rate of decay of contractile force and washout of Ca in hearts which were gas perfused before washout with Ca-free perfusate (gas perfused hearts).

2. Analytical methods.

a. Determination of ion concentrations.

The effluent from the hearts during the Ca-free washes and uptakes was collected at 6 sec intervals on a Model 272 (ISCO) fraction collector (ISCO, Lincoln, Nebraska). The volume of each sample was noted. The flow of liquid perfusates during all phases of the experiments did not differ significantly. The average flow rate of the perfusate was 4.5 ± 0.2 ml/min/g wet weight of heart tissue and did not vary significantly from heart to heart.

At the conclusion of the experiments, the hearts were briefly

perfused with gas to clear the coronaries of fluid. The ventricles were removed from the apparatus and weighed after excess moisture was removed from the tissue by blotting lightly between filter paper. A tissue sample of approximately 1 g was placed in a platinum crucible, dried for 24 hr at 80°C in vacuo. The dry weight was noted. The dried tissue sample was then ashed for 24 hrs at 600°C in a Thermolyne muffle furnace (Dubuque, Iowa). The ashed sample was dissolved in 2 ml of 10N HCl.

Aliquots of the effluent and the ashed samples were diluted with 1% lanthanum in 5% (v/v) HCl for determination of Ca and Ni concentrations. The ion concentrations were read on a Perkin Elmer 303 Atomic Absorption Spectrophotometer (Norwalk, Connecticut).

La concentration could not be determined in this study because the atomic absorption spectrophotometer was not sufficiently sensitive in its present configuration to measure the low concentrations of La used in our experiment (5-500 uM). The radionuclide of La, ^{140}La, was not available from commercial sources in North America.

Radioactivity from ^{3}H-Inulin and ^{45}Ca in the effluent and tissue samples was measured on a Phillips Liquid Scintillation Analyser, Model PW4510/B (Phillips, Netherlands). A 0.1 ml aliquot of the effluent and of the ashed tissue sample was diluted with 9.9 ml of scintillation solution of the following compositions:

Toluene	700 ml/l
Methanol	300 ml/l
PPO (2, 5 - Diphenyloxazole)	8 g/l
POPOP (1, 4- Di[2- (Phenyloxozolyl)] Benzene)	100 mg/l

Quench corrections for ^{45}Ca activity were found to be unnecessary since counting efficiency did not vary significantly from sample to sample.

Contractile force during all perfusion procedures was measured from the records at 6 sec intervals to correspond to the time of collection of each effluent sample.

b. Definition of compartments.

Ca_I, Ca_{II}, and Ca_{III} define the first, second and third Ca compartments in the Ca-free washout curves from gas perfused hearts (Table I). Ca_{II} describes the Ca compartment correlated with the decay of contractile force in the same washout. Ca_{IIA}, Ca_{IIB}, and Ca_{IIC} define the second Ca compartment obtained in washout curves when the washout differs significantly ($P < 0.05$) from the decay of contractile force and also is significantly different ($P < 0.05$) from the washout of Ca_{III}.

Ca_1 and Ca_2 define the Ca compartments obtained from the

TABLE I

Definition of Symbols

Symbols	Definition
Ca_n	The n^{th} compartment, where n = I, II, or III for washout data or 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 n^{th} compartment (mEq/l).
$[Ca]_{nt=0}$	Ca concentration for 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).

uptake of Ca in Ca-depleted hearts. The Ca compartments described by the washout, Ca_I and Ca_{II} and by the uptake, Ca_1 and Ca_2 may neither represent the same kinetic pool nor have the same anatomical location in the myocardium.

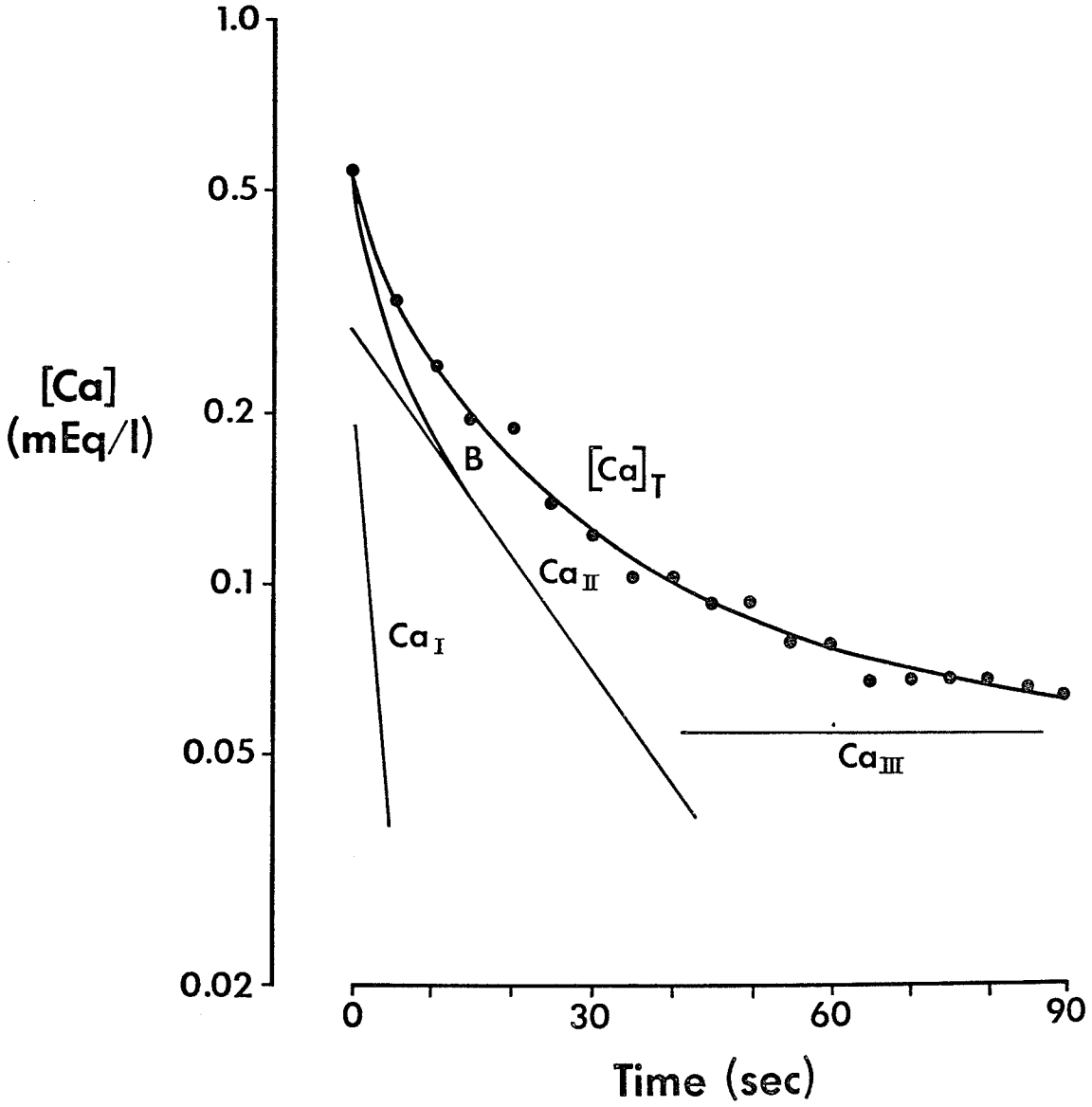
c. Graphical analysis of washout data.

(i) Ca

The logarithm of Ca concentrations measured in the effluent during Ca-free washouts was plotted as a function of time in seconds. Graphical compartmental analysis of the Ca washout data was done as outlined by Riggs (1963). Figure 6 shows the resolution of a typical Ca washout curve. Briefly the procedure was as follows: First, a value for $[Ca]_{III}$ was estimated from the asymptote of the curve, $[Ca]_T$. Second, $[Ca]_{III}$ was subtracted from the curve $[Ca]_T$ and the resulting curve B was plotted on the same piece of logarithmic graph paper. Third, the straight line portion, Ca_{II}, or the asymptote of the resulting curve B was extended to the origin (t=0). The value at zero time represents Ca arising from pool II or $[Ca]_{IIt=0}$ in the first sample. Finally, the straight line, $[Ca]_{IIt=0}\exp(-k_{II}t)$, was subtracted from curve B to yield Ca_I, a straight line described by $[Ca]_{It=0}\exp(-k_I t)$. The symbols used are defined in Table I and the curve follows the general equation reported by Bailey and Dresel (1968).

$$[Ca]_T = [Ca]_{It=0}\exp(-k_I t) + [Ca]_{IIt=0}\exp(-k_{II} t) + [Ca]_{III} \qquad (1).$$

Figure 6: Graphical analysis of typical washout curve. Typical washout curve, $[Ca]_T$, from a heart perfused with Ca-free Krebs-Henseleit solution. The three compartments were resolved by graphical analysis. Ca_I, Ca_{II}, and Ca_{III} were described in text.
The filled circles (●) indicate the Ca concentration measured in the effluent.


1.0
0.5
0.2
0.1
0.05
0.02
[Ca]
(mEq/l)
B
[Ca]T
CaII
CaI
CaIII
0
30
60
90
Time (sec)

In this way, the Ca washout curve was resolved into three components, Ca_I which has been shown to represent the Ca adhering to the perfusion apparatus and the walls of the coronary vasculature; Ca_{II}, the Ca involved in the maintenance of contractile force in the heart; and Ca_{III}, assumed to represent tightly bound tissue Ca (Bailey and Dresel, 1968). During the brief 2-3 min washout, Ca_{III} was assumed to contribute a constant amount of Ca to the washout since the slope was not significantly different ($P > 0.05$) from zero (Bailey and Dresel, 1968).

Compartment analysis was initially done by eye and subtracted, but later analyses were done with the aid of a computer program written by Mr. S. Vivian. In contrast to the usual manual procedure, compartmental analysis using the computer program is a method where the element of personal bias has been minimized. Briefly the logic behind this procedure was as follows: The least squares best fit line for the last five data points was calculated and then each subsequent data point was tested. If four subsequent data points were above the best fit line and if both the 3rd and 4th points deviated from the line by more than one standard deviation, another best fit line was drawn through the four data points and the slopes of the two lines compared. At this point, there were two alternatives: If the slopes were not significantly different ($P > 0.05$) then a new best fit line through all of the data points (9) examined became a tentative compartment and subsequent

data points tested in the same way. However, if the slopes were significantly different ($P < 0.05$) then the second best fit line became another tentative compartment and subsequent data points tested as above until all data points in the washout were analyzed. The results obtained by the computer analysis did not differ from the manual method for washout curves. However, this was not the case when Ca uptake curves were analyzed by both methods (see below).

The halftimes, $T_{1/2}$, for the washout of Ca from each compartment are related to the slope of each curve by equation 2.

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

(See Table I for definition of symbols.)

The amount of Ca washed out of each compartment was calculated from the product of the effluent volumes and $[Ca]_n$ summed over the N samples. It was expressed in terms of tissue wet weight as follows:

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

(See Table I for definition of symbols.)

(ii) Ni

The logarithm of Ni concentrations measured in the Ca-free K-H solution was plotted as a function of time and graphical compart-

mental analysis was done in the same manner as for Ca described above. The Ni content for each compartment was calculated by equation 3. The halftimes, $T_{1/2}$, were obtained from the slope of the line for each compartment by equation 2.

d. Graphical analysis of contractile force.

The logarithm of contractile force measured during perfusion with Ca-free, and Ni and La containing solutions was plotted against time. The halftime, $T_{1/2}$, for the decay was determined as above.

e. Graphical analysis of Ca uptake data.

The logarithm of Ca concentrations measured in the effluent during the reperfusion of Ca-depleted hearts with Hepes solution was plotted as a function of time. Each value of Ca concentration was subtracted from the asymptote, that is, the concentration of Ca in the perfusate, and the resultant data points plotted semi-logarithmically against time. The curves were analyzed by the computer program used for the washout data and by the manual method. Unlike the washout data, compartmental analysis by the manual method (Bailey and Sures, 1971) yielded only a monoexponential function for the approach to the steady state of Ca flux during the reperfusion, while the non-biased computer analysis yielded a double exponential function.

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

$$[Ca]_T = 5.0 - [Ca]_{1t=0}\exp(-k_1 t) - [Ca]_{2t=0}\exp(-k_2 t) \qquad (4)$$

(The symbols used are defined in Table I.)

The unknowns in equation 4, $[Ca]_{1t=0}$, $[Ca]_{2t=0}$, k_1 and k_2 were solved by computer analysis in the same way as for washout data since the resultant data points from $5.0 - [Ca]_T$ is described by the double exponential function, $[Ca]_{1t=0}\exp(-k_1 t) + [Ca]_{2t=0}\exp(-k_2 t)$. Since the Ca concentration in the effluent was equal to the Ca concentration in the perfusate when Ca influx into the heart was equal to the Ca efflux from the heart, the slopes, k_1 and k_2 then estimate the rate of approach to a steady state of Ca flux into Ca_1 and Ca_2 respectively. That is, k_1 is the rate constant for the approach to the steady state of Ca uptake in Ca_1 and similarly k_2 is the rate constant for uptake in Ca_2. The slopes of the lines thus can be interpreted as the rates of filling of two pools in the heart. The mathematical relationship between $T_{1/2}$ and the rate constants is defined by equation 2.

The Ca taken up or the quantity of Ca extracted from perfusate by each compartment was calculated by equation 3. The quantity of Ca taken up by each compartment during the first 6 sec of reperfusion was obtained by multiplying $[Ca]_{nt=0}$ by the volume of the first sample and dividing by the heart weight. Then, the quantity of Ca extracted by each compartment at any subsequent time was obtained by multiplying $[Ca]_{nt=0}\exp(-k_n t)$ by the volume of that sample divided by the heart weight.

The Ca accumulated by each compartment was then obtained by adding the quantity of Ca taken up in the first 6 sec to the quantity of Ca taken up in the next 6 sec and subsequent quantities added. Thus, a cumulative graph of Ca extraction by each compartment as a function of time was drawn (Figure 24). The total quantity of Ca extracted from the perfusate was the quantity of Ca extracted by both Ca_1 and Ca_2.

f. Analysis of ^{45}Ca washout and uptake data.

In experiments where ^{45}Ca was used, the ^{45}Ca concentration in the effluent from the heart was plotted logarithmically and analyzed in the same way as the Ca data.

To enable comparison of ^{45}Ca content in the various compartments and from experiment to experiment, all measured ^{45}Ca concentrations were adjusted arbitrarily to 1×10^8 cpm/l based on the concentration of ^{45}Ca in the perfusate. The correction factor was calculated as follows:

$$\text{Correction Factor} = (1 \times 10^8 \text{ cpm/l}) / \text{measured } ^{45}Ca \text{ concentration in the perfusate (cpm/l).} \quad (5)$$

Each measurement of ^{45}Ca concentration was then multiplied by the correction factor. This procedure was equivalent to expressing radioactivity in each compartment as a percentage of the radioactivity in the perfusate, but has the added advantage that direct comparisons of ^{45}Ca concentration and specific activity can be made between experiments.

g. Determination of extracellular space.

^{3}H- Inulin was used as a marker for determinations of the extracellular space. The uptake and volume of distribution of ^{3}H- Inulin into Ca-depleted hearts was investigated and compared to the uptake and volume of distribution of Ca and ^{45}Ca. During uptake, the effluent from the heart was collected at the usual 6 sec interval. The volume of each sample was noted and the radioactivity determined in each sample as outlined above.

Since both ^{45}Ca and ^{3}H- Inulin was employed in the experiment, dual channel counting was employed. The overflow of ^{45}Ca into the tritium channel was determined by counting a standard ^{45}Ca preparation and the overflow corrected for every sample. The overflow of radioactivity from tritium labelled compound into the ^{45}Ca channel was less than 1% and this was considered negligible.

The k and $T_{1/2}$ for the uptake of the ^{3}H- Inulin was calculated in the same manner as for the uptake of Ca. The total quantity of ^{3}H- Inulin extracted by the heart was determined and expressed as cpm/kg tissue water. Tissue water was determined by subtracting the dry weight of the ventricles from the wet weight. To calculate the extracellular space, the quantity of ^{3}H- Inulin extracted by the heart from the perfusate was divided by the concentration of ^{3}H- Inulin in the perfusate and expressed as a percent of total tissue water.

$$\text{Extracellular space} = \frac{\sum_{i=1}^{N} ([^3H]_p - [^3H]_e)_i \cdot V_i}{W \cdot T_{H_2O}} \cdot \frac{1}{[^3H]_p} \qquad (6)$$

Where $[^3H]_p$ = perfusate concentration of ^{3}H-Inulin (cpm/l)

$[^3H]_e$ = effluent concentration of ^{3}H-Inulin (cpm/l)

V_i = volume of i^{th} sample (ml)

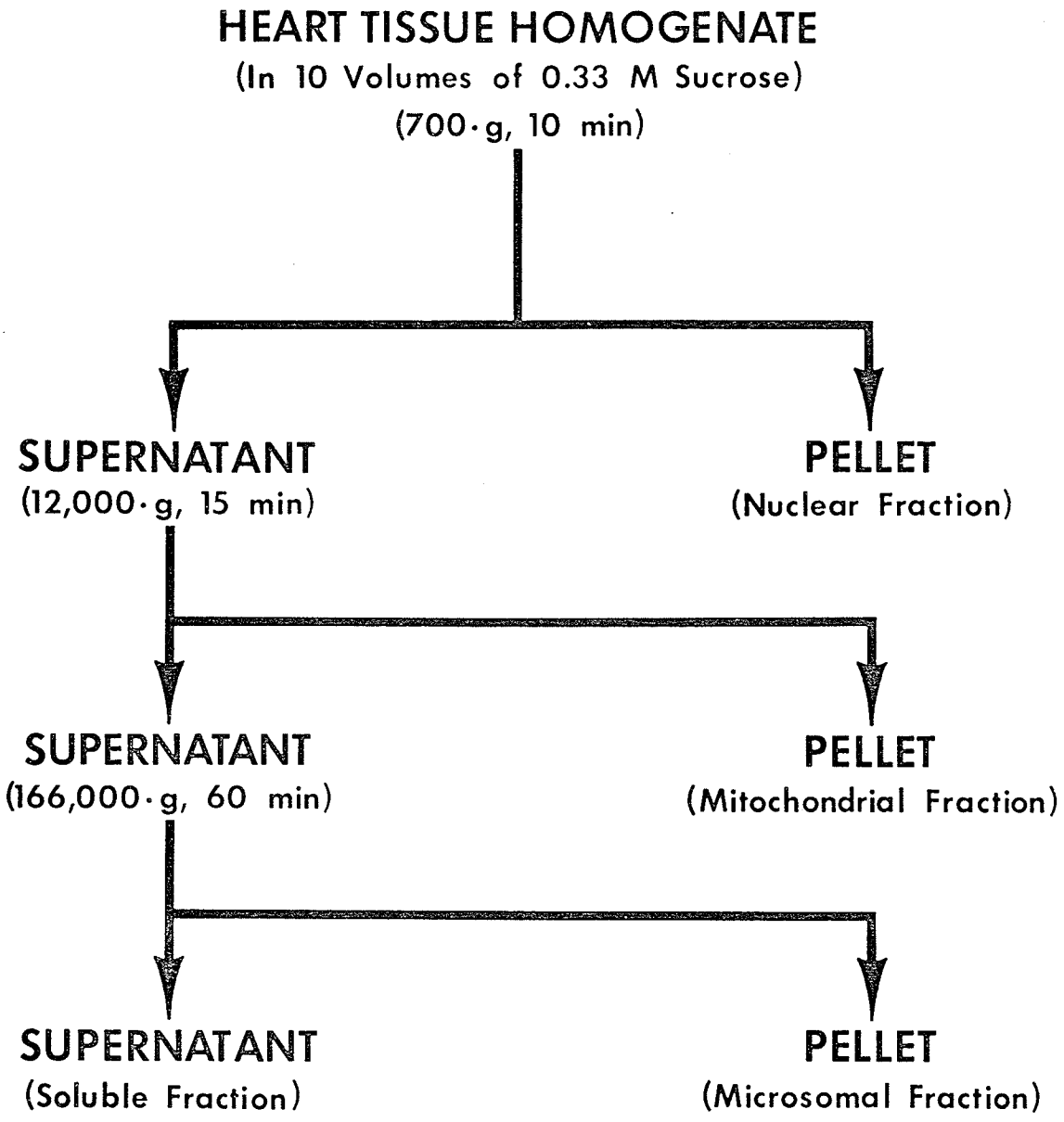
W = tissue wet weight (g)

T_{H_2O} = tissue water as percent tissue wet weight

3. Subcellular fractionation.

Hearts were perfused by Procedure IA and B (Figure 2), removed from the cannula, and blotted on filter paper to remove excess moisture. The ventricular tissue was weighed and subcellular fractionation was carried out as described by Dutta et al. (1968). The flow diagram of the procedure is shown in Figure 7. The tissue was minced in an ice bath and homogenized in 10 volumes of cold (4°C) 0.33 M sucrose solution by 15 gentle strokes of a glass Potter-Elvehjem homogenizer tube fitted with a motor driven Teflon pestle. The homogenate was spun in a Servall refrigerated centrifuge (Head Type SS-1) at 700 x g (2500 rpm) for 10 min to remove nuclei, myofibrils and other cell debris. The sediment represented the nuclear fraction. The supernatant was centrifuged at 12,000 x g (12,000 rpm) for 15 min at 4°C. The resultant pellet was the mitochondrial fraction. The supernatant

Figure 7: Flow chart for subcellular fractionation of kitten heart to determine the distribution of Ca and Ni. See text for details.


HEART TISSUE HOMOGENATE
(In 10 Volumes of 0.33 M Sucrose)
(700·g, 10 min)
SUPERNATANT
(12,000·g, 15 min)
PELLET
(Nuclear Fraction)
SUPERNATANT
(166,000·g, 60 min)
PELLET
(Mitochondrial Fraction)
SUPERNATANT
(Soluble Fraction)
PELLET
(Microsomal Fraction)

was centrifuged in an International Preparative Ultracentrifuge (Model B-60) at 166,000 x g (45,000 rpm) for 60 min at 4°C yielding a supernatant and pellet. The pellet was the microsomal fraction, and the supernatant was the soluble fraction. Both the mitochondria pellet and microsomal pellet were resuspended in 2 ml of distilled water and the nuclear fraction pellet was resuspended in 8 ml distilled water. The Ca and Ni concentrations of each subcellular fraction and the final supernatant (soluble fraction) were measured on the Perkin Elmer Atomic Absorption Spectrophotometer (Model 303) after dilution with 5% lanthanum in 1% HCl (see above). In order to normalize the ion concentrations for the purpose of comparison the protein content in an aliquot of each subcellular fraction was measured and the Ni and Ca contents were calculated on the basis of per mg protein. The protein concentration in these fractions was determined by the method of Lowry et al. (1951), modified by the addition of 5% deoxycholate to each sample to solubilize the protein. Bovine serum albumin was used as the standard.

4. Electron microscope studies.

Kitten hearts were perfused for 30 min with K-H solution, then gas perfused with a water saturated gas mixture of 95% O_2 - 5% CO_2 for another 30 min or subsequent to the gas perfusion were perfused with a Ca-free perfusate until contractile force was less than 1 g. The hearts were then quickly removed from the perfusion apparatus and a

thin slice of muscle excised from the inter-ventricular septum. The slice of muscle was cut into 1 mm cubes and left for half an hour in 2% glutaraldehyde in cold 0.1N sodium cacodylate. The pH of this solution was adjusted to 7.25 with concentrated HCl. The tissue samples were then washed with 0.1N sodium cacodylate-HCl buffer containing 7% sucrose, pH 7.25 at 4°C for one hour, then post-fixed for another hour in Zettqvist's OsO_4 (Sjostrand, 1967). They were then stained en bloc in 2% aqueous uranyl acetate for 25 min in dehydrated alcohol and embedded in Epon.

Thin sections were cut with a Riechert OmU_2 ultramicrotome, mounted on uncoated copper grids and stained with Reynold's lead citrate (Sjostrand, 1967). The specimens were examined with a Hitachi HS 8 electron microscope operating at 50 kv and with a 50 or 70 u aperture in the objective. Sections were also prepared from hearts which were only perfused for 30 min with K-H solution.

To study the distribution of electron dense La ions before and after gas perfusion, the hearts were perfused with Hepes solution (pH 7.4) for 30 min. They were then either perfused with La-Hepes (500 uM La) for 30 sec or perfused with water saturated 100% O_2 for 30 min and then with the La-Hepes (500 uM La) for 3 min. The hearts were quickly removed and slices fixed and stained for examination under the electron microscope as outlined above. It should be empha-

sized that La was not added to either the fixing or the staining solutions.

5. Isolated kitten atria.

Kittens of either sex weighing 0.7 - 1.2 kg were killed by a blow on the head, the hearts were excised immediately and placed in cold K-H solution (4°C). The left atrium was dissected free and divided into two equal parts. Each half of the atrium was attached at one end to an electrode holder and at the other to a Grass FTO 3B force displacement transducer. Each half of the atrium was suspended in an organ bath containing 15 ml of K-H solution continuously bubbled with 5% CO_2 - 95% O_2. The bath temperature was maintained at 37°C and the atrial preparations were allowed to equilibrate for one hour. The resting length of the preparation was adjusted to produce a force of contraction which was 50% of the maximum force as determined by the length-tension relationship. The force of contraction was recorded on a Grass 5D polygraph. The atrial preparations were stimulated at a rate of 2/sec by twice threshold voltage delivered by two platinum field electrodes (2 mm x 10 mm) on either side of the muscle.

After one hour of equilibration in K-H solution, the preparation was bathed in a K-H solution containing 1 mEq Ca/l. Ca was then added in increments of either 1 or 2 mEq Ca/l to determine the cumulative dose-response curve to Ca of the preparation. The atria were then washed several times with K-H solution. After 30 min recovery

in normal K-H solution, a second cumulative dose-response curve to Ca was determined as above, but in the presence of 0.8 mEq Ni/l. In four atrial preparations, the second cumulative dose-response curve was determined in the presence of 0.8 mEq Ni/l and 5×10^{-8} g/ml (0.85 mM) ouabain. Time controls showed that the first cumulative dose-response curve to Ca did not differ from the second dose-response curve to Ca ($P > 0.05$).

D. Statistical Analyses.

The maximum height, the slope of the probit transformed regression line (Goldstein, 1964) and the ED_{50} extracted from the line between the control and the test dose-response curves were analyzed statistically using a paired t-test (Dixon and Massey, 1957).

Correlation analysis (Steel and Torrie, 1960) between the half-times for the decay of contractile force and the washout of Ca from each compartment was done as outlined by Bailey and Dresel (1968). A similar analysis was conducted on the linear relationship between the rate of Ca uptake by Ca_2 and the rate at which contractile force returned during reperfusion of Ca-depleted hearts.

Tests of significance between means of the various parameters of ion kinetics were evaluated by Student's unpaired t-test (Dixon and Massey, 1957). In all analysis a probability of 0.05 was preselected as the criterion of statistical significance.

SECTION III

RESULTS

A. Characteristics of Liquid and Gas Perfused Hearts.

1. The effect of gas perfusion and Ca-free perfusion on the ultrastructure of cat heart muscle.

Plate I shows a photograph of an electron micrograph of cardiac muscle taken from a cat heart perfused with K-H solution for 30 min, followed by 30 min perfusion with a water saturated gas mixture of 95% O_2 - 5% CO_2 and then washed out with Ca-free K-H solution for 3 min. The morphology of the muscle cell was normal with no signs of necrosis or damage. The mitochondria were intact and there were no obvious signs of swelling. The endothelial cells bordering the capillaries were normal, and the pinocytotic vesicles and the cell junctions were intact. Sympathetic nerve endings remained intact. The perfusion procedures employed did not cause any overt damage to the muscle nor the nervous tissue.

2. Ca-free perfusions after liquid and gas perfusion.

Figure 8 shows a tracing of contractile force taken from a typical experiment following the procedure described in Figure 5. Perfusion of the heart with Ca-free K-H solution immediately after perfusion with K-H solution (Wash I, Figure 5 and 8), caused a monoexponential decay of contractile force to 1 g or less. The decay of contractile force was significantly more rapid ($P < .05$) than the decay of contractile force caused by Ca-free perfusion after gas perfusion. The mean halftime for the decay of contractile force during Ca-free washout

Plate I: Electronmicrograph of a cardiac muscle fibre (M), longitudinally sectioned, a capillary (cap), and a sympathetic nerve axon (Ax), from a heart gas perfused and then perfused with Ca-free solution. Note that the mitochondria (Mit) are not swollen. x 28,600.

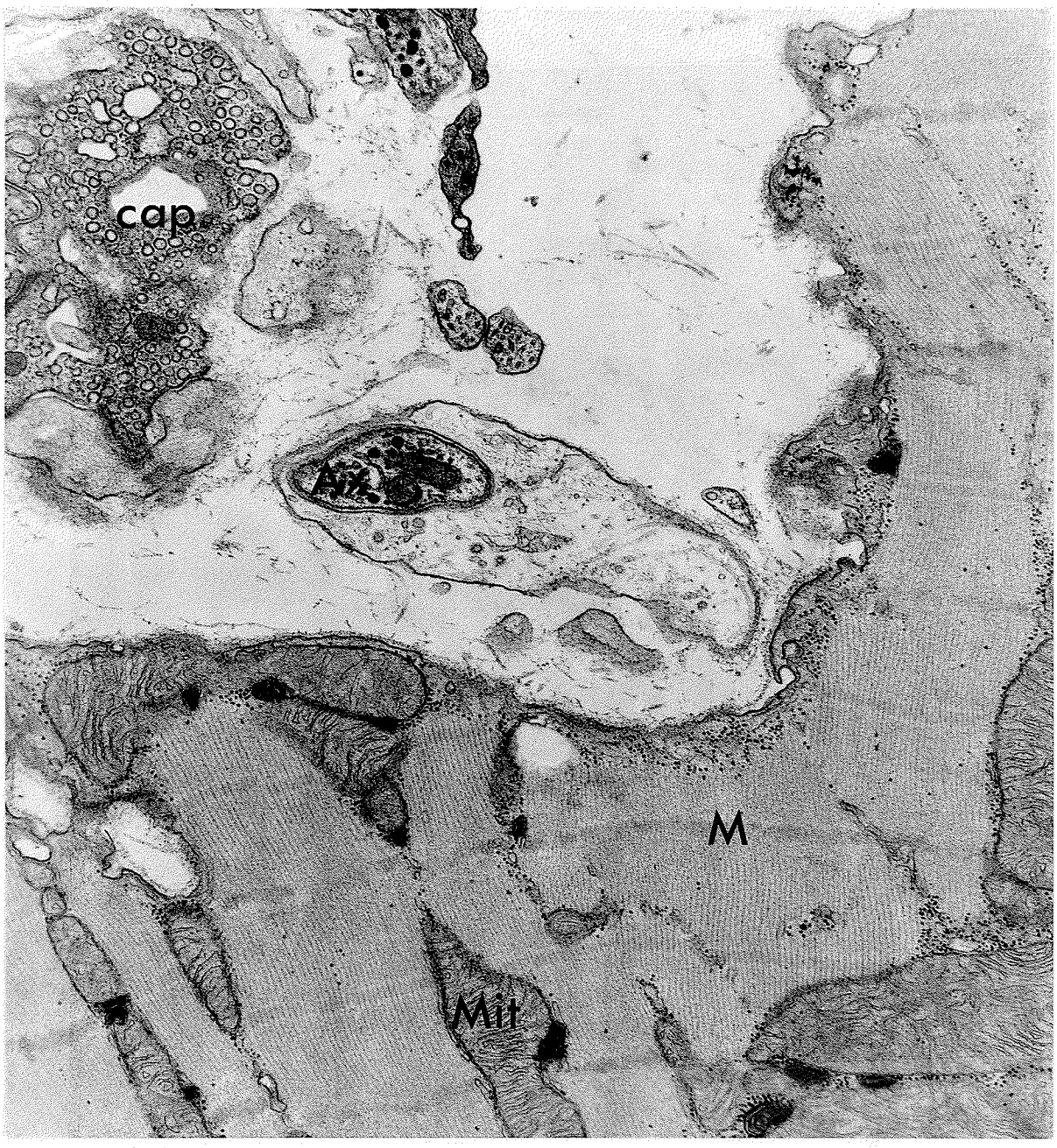
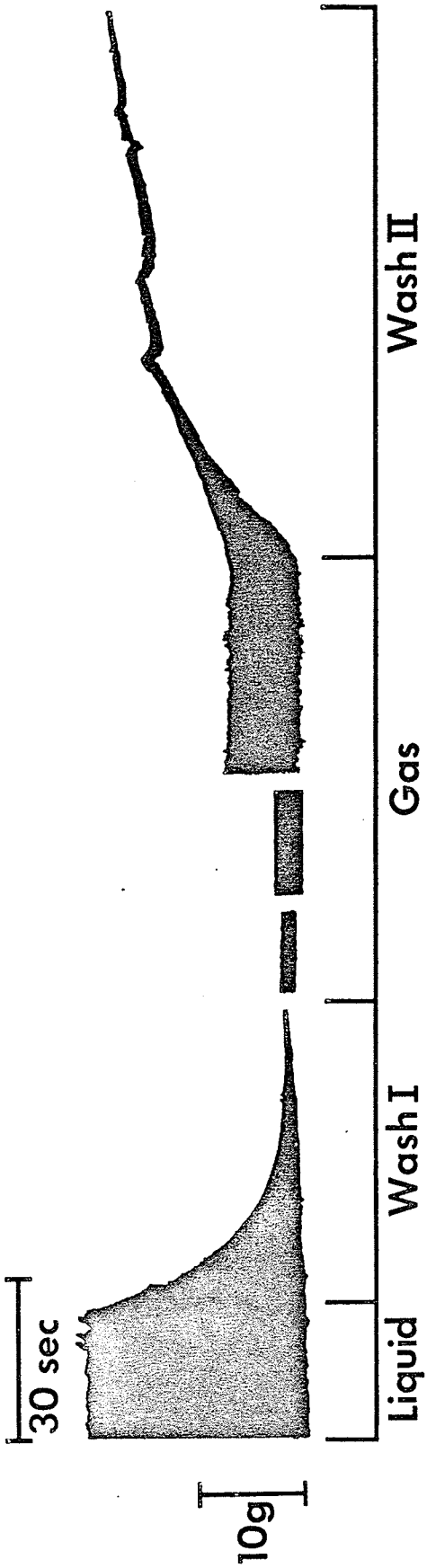
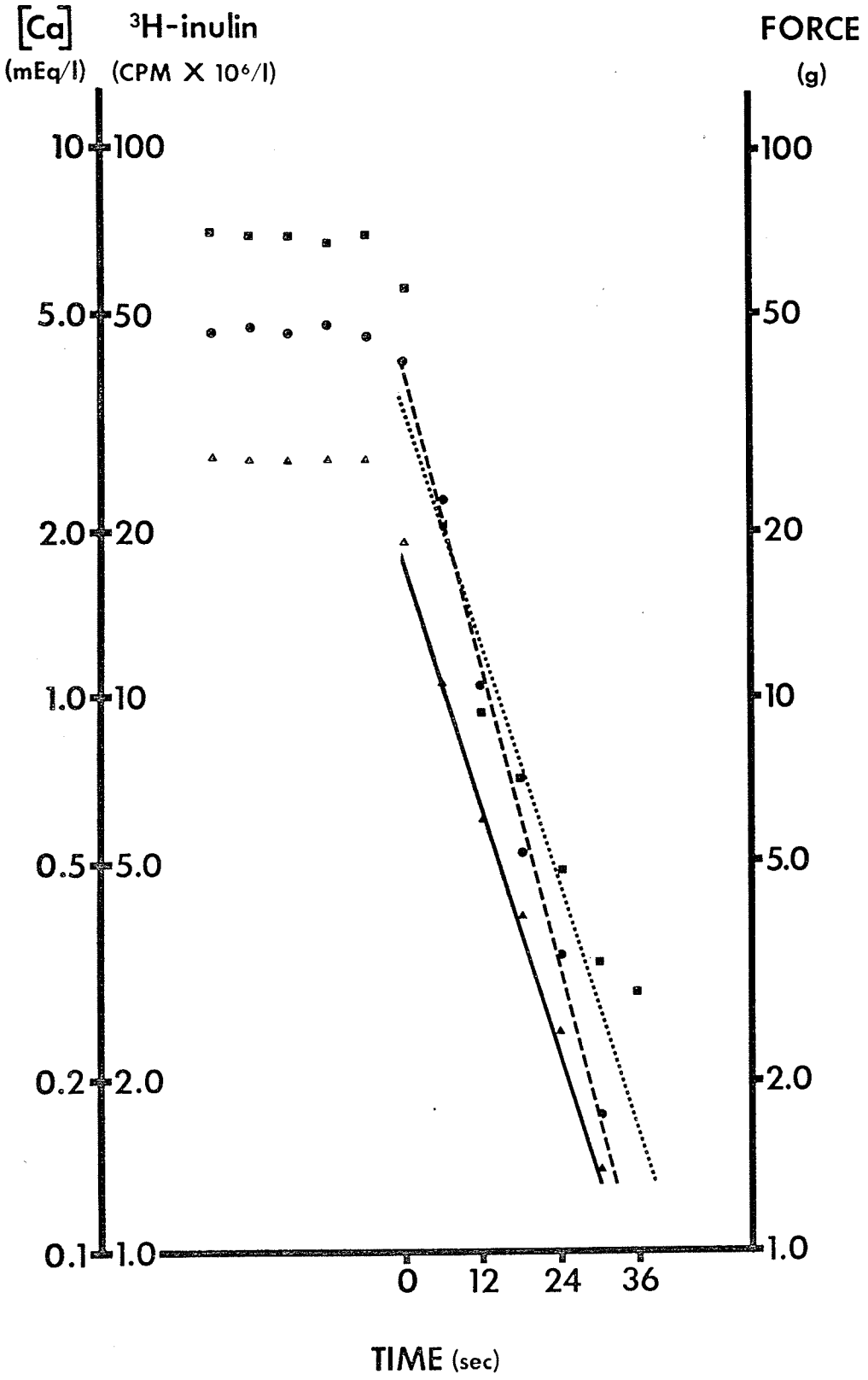

cap
Aix.
M
Mit

Figure 8: The effect of Ca-free perfusion on contractile force in a liquid perfused heart. Note that the tracings are interrupted and that the time scale refers to the contractile force tracings only.


30 sec
10g
Liquid
Wash I
Gas
Wash II

after gas perfusion was 21.6 ± 3.9 sec while the mean halftime for the decay of contractile force in hearts washed out without gas perfusion was 9.3 ± 0.6 sec. The logarithm of the concentrations of Ca and ^{3}H-Inulin measured in a typical experiment in Wash I (Figure 5) are plotted as a function of time in Figure 9. The points describing the logarithms of Ca and ^{3}H-Inulin concentrations were fitted with individual least squares best fit straight lines. The mean halftimes of the washout were 9.6 ± 0.7 sec and 9.0 ± 0.5 sec respectively. They were not significantly different ($P > .05$) from the halftime for the decay of contractile force during Wash I (Figure 9). These results suggest that extracellular Ca and perhaps superficially bound Ca was removed during Wash I and the removal of that Ca was correlated with the decay of contractile force. However, comparison of Ca and ^{3}H-Inulin washout kinetics may be invalid and the interpretation of these results may be misleading (see Discussion). The total amount of Ca removed by Wash I (Figure 5) was 4.05 ± 0.14 mEq/kg tissue wet weight. The mean tissue water calculated from twenty-five hearts was 79.8 ± 4.3% of tissue wet weight. If the Ca was distributed homogeneously in total tissue water, then the mean Ca concentration would be 5.06 mEq Ca/l of tissue water. The mean volume of distribution of ^{3}H-Inulin was 27.5 ± 2.6% of total tissue water. Thus, the concentration of Ca removed by Wash I if distributed homogeneously in the interstitial space

Figure 9: Typical washout curves of Ca, ^{3}H-Inulin and contractile force in Wash I. The effect of Wash I on the decay of contractile force, and the washout of Ca and ^{3}H-Inulin. The concentrations of Ca and ^{3}H-Inulin measured in the effluent are indicated by the filled circles (●), and the filled squares (■) respectively. The filled triangles (▲) indicate contractile force measured during Wash I. The least squares best fit line for the variables during Wash I are shown by the dashed line (Ca), the dotted line (^{3}H-Inulin) and the solid line (contractile force). Zero time is the first sample collected after the initiation of Wash I.


[Ca]
(mEq/l)
³H-inulin
(CPM X 10⁶/l)
FORCE
(g)
10
5.0
2.0
1.0
0.5
0.2
0.1
100
50
20
10
5.0
2.0
1.0
0
12
24
36
TIME (sec)

would be 18 mEq Ca/l interstitial water. However, the validity of these calculations is questionable since a large error in the total Ca washed out may have been induced by mixing of the K-H solution with the Ca-free solution. This was inevitable even though great care was taken to prevent admixture of the two perfusion fluids.

During 30 min of gas perfusion subsequent to Wash I, contractile force gradually returned (Figure 8) to about 30% of the value obtained during liquid preperfusion or to approximately 60% of that measured during gas perfusion (Table II). Ca-free perfusion (Wash II, Figure 5 and 8) following this period of gas perfusion caused a contracture. There was a marked increase in resting tension as contractile force decreased when Ca was being removed by the Ca-free perfusate. The decay of contractile force during Wash II followed a monoexponential function and the halftime of decay was 14.8 ± 1.3 sec (Table III). This was significantly slower ($P < .05$) than the decay of contractile force during Wash I, 9.3 ± 0.6 sec. The Ca washout curve (Wash II, Figure 5) was analyzed graphically and yielded a two compartment system instead of the usual three compartment Ca washout (Figure 10 and 6, respectively). The first compartment, Ca_I, was washed out very rapidly, with a halftime of 3.2 ± 0.5 sec, and the second compartment, Ca_{IIA}, was washed out with a halftime of 53.2 ± 9.6 sec (Table III). The decay of contractile force was significantly different ($P < .05$)

TABLE II

Contractile Force Measured After 30 min Liquid and 30 min Gas Perfusion Showing the Restoration of Contractile Force During Gas Perfusion after Wash I

Treatment	Contractile Force (g)
Liquid Perfusion (10)	18.6 ± 3.1[a]
Gas Perfusion before Wash I (11)	11.6 ± 0.8[b]
Gas Perfusion after Wash I (5)	6.8 ± 0.4[b]

Number in parenthesis indicates the number of hearts.

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

[b] Significant difference ($P < .05$) from contractile force during liquid perfusion.

TABLE III

The Halftimes for the Washout of Ca and the Decay of Contractile Force in Wash II (Figure 5)

Compartment	$T_{1/2}$ (sec)
Ca_I (5)	3.2 ± 0.5[a, b]
Ca_{IIA} (5)	53.2 ± 9.6[b]
Contractile Force (5)	14.8 ± 1.3

Number of parenthesis indicates the number of hearts.

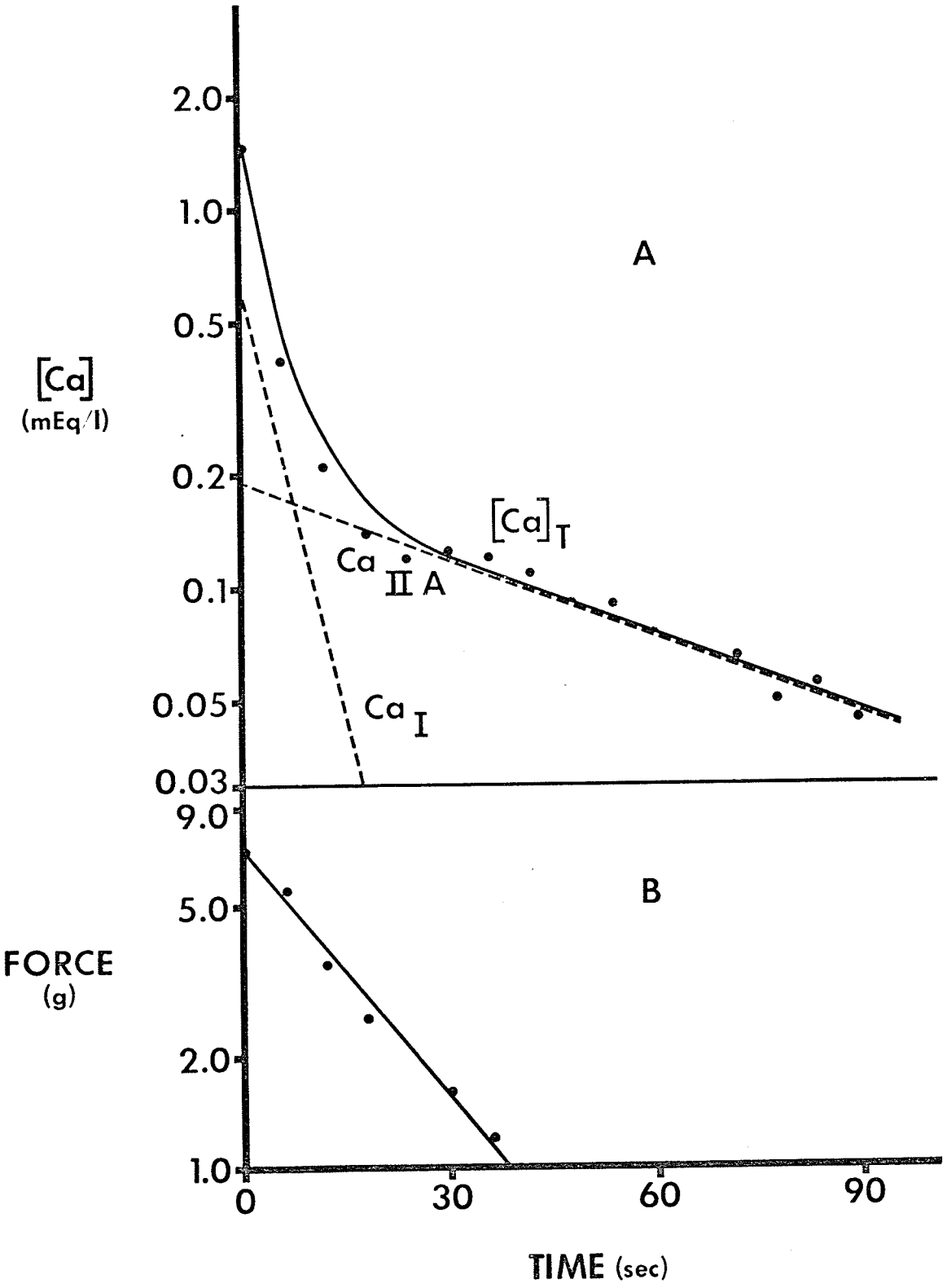
[a] Mean $\pm$ S. E.

[b] Significant difference ($P < .05$) from $T_{1/2}$ of decay of contractile force.

Figure 10: Typical Ca washout curve and decay of contractile force in Wash II.

Panel A: Washout of Ca in Wash II. The solid line $[Ca]_T$, is the least squares best fit for the concentrations of Ca (●) measured in the serial samples and the dashed lines, the two compartments resolved by graphical analysis. Ca_I and Ca_{IIA} are defined in the text.

Panel B: Decay of contractile force in Wash II in the same heart.


2.0
1.0
0.5
0.2
0.1
0.05
0.03
[Ca]
(mEq/l)
A
[Ca]T
Ca II A
Ca I
9.0
5.0
2.0
1.0
FORCE
(g)
B
0
30
60
90
TIME (sec)

from the washout of either compartment. The development of contracture could have changed the washout characteristics and caused a dissociation of the washout of Ca from the decay of contractile force or vice versa. The cause of this contracture is unknown and was not investigated.

B. Uncoupling of Excitation from Contraction by Ni.

1. Interaction between Ni and ouabain.

The change of contractile force in isolated kitten atria in response to increasing concentrations of Ca from 1-20 mEq/l in the absence and presence of 0.8 mEq Ni/l is shown in Figure 11A. Ni shifted the Ca dose-response curve significantly to the right but did not cause significant change in the slope of the curves. Fifty percent of maximal contractile force was developed by atria bathed in 2.43 $\pm$ 0.15 mEq Ca/l in the absence of Ni. However, in the presence of 0.8 mEq Ni/l, 3.57 $\pm$ 0.15 mEq Ca/l was required to produce 50% maximal contractile force ($P < .05$). There was no statistical difference in the maximum response ($P > .05$). Ouabain prevented the shift in the Ca dose-response curve caused in the presence of 0.8 mEq Ni/l (Figure 11B).

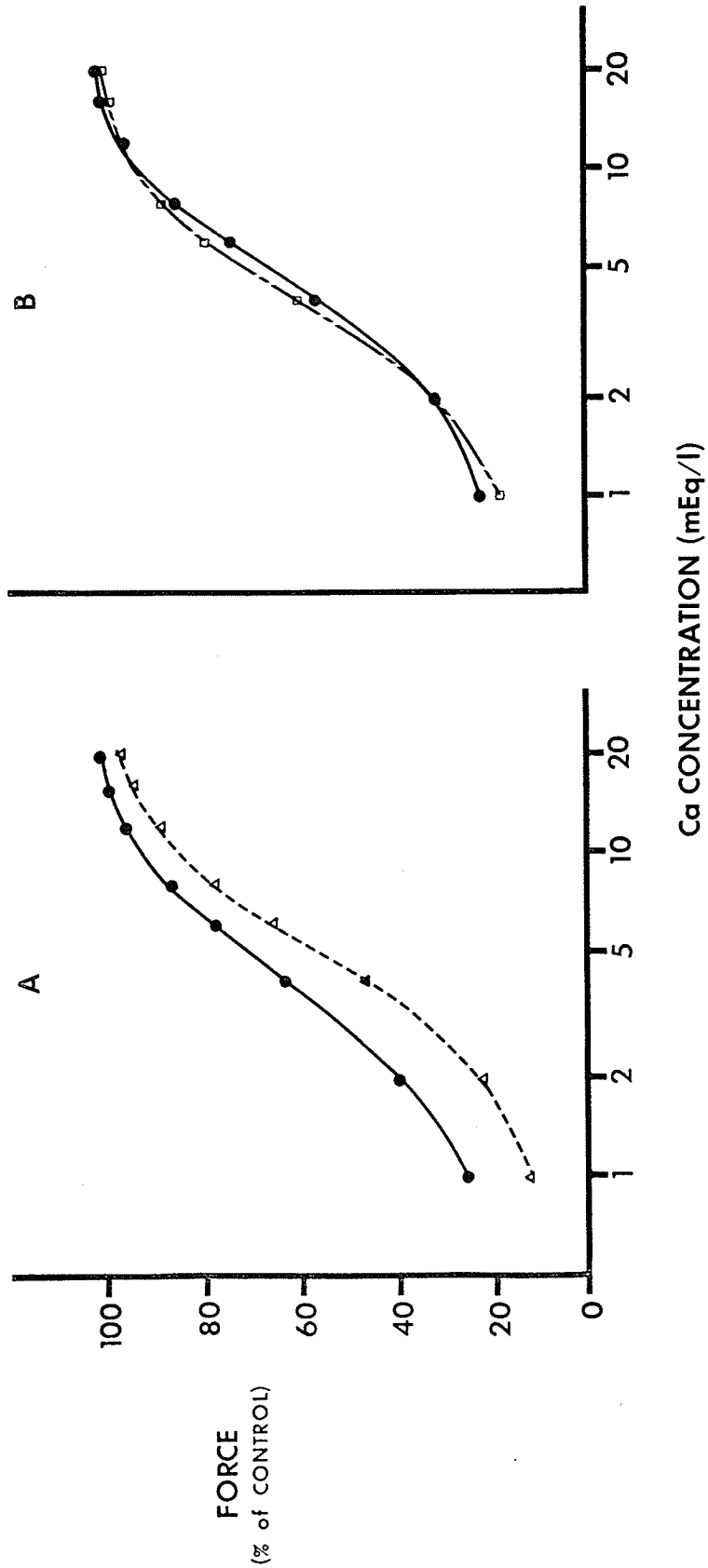
2. The effect of Ni on contractile force.

Contractile force decreased when the 95% O_2 - 5% CO_2 gas mixture was substituted for the liquid perfusion medium in cat hearts

Figure 11: The effect of Ni and ouabain on the relationship between Ca concentration and contractile force in isolated kitten atria.

Panel A: Contractile force is plotted as a percentage of control against logarithm of Ca concentration in the bath. Untreated (●——●), 0.8 mEq Ni/l added (△– – –△). The data points indicated by the filled circles (●) and open triangles (△) are the means of 4 experiments.

Panel B: Untreated (●——●), 0.8 mEq Ni/l and 5×10^{-8} g/ml ouabain added (□– – –□). The data points indicated by the filled circles (●) and open squares (□) are the means of 4 experiments.


A
B
FORCE
(% of CONTROL)
100
80
60
40
20
0
1
2
5
10
20
Ca CONCENTRATION (mEq/l)

(Table IV). This decrease in contractile force has been attributed to the removal of Ca from the vascular space by gas perfusion (Krip et al. 1971). Perfusion with K-H solution containing 4 mEq Ni/l (Ni-Krebs) caused a brief increase in resting length followed by a gradual decrease in diastolic length which was then maintained for the duration of the experiment (Figure 12). However, the decline of contractile force during Ni-Krebs perfusion was always the dominant factor while the decrease in the resting length was relatively minor. Ni-Krebs perfusion caused contractile force to decay in a monoexponential fashion to 0.2 ± 0.1 g (Table IV), but did not affect the electrical activity in the ventricles (Figure 12). The mean halftime for the decay of contractile force during Ni-Krebs perfusion, 23.4 ± 2.4 sec, did not differ significantly from that measured during the subsequent washout with Ca-free K-H solution, 21.0 ± 3.5 sec (Table V). During the 30 min of gas perfusion following Ni-perfusion, contractile force returned gradually to approximately 60% of that measured on gas perfusion (Table IV).

The effect of perfusion with Ni-Krebs containing 4 mEq Ni/l was the same whether the hearts were perfused with the Ni-Krebs immediately after perfusion with K-H solution (Figure 2, Procedure II; Figure 13A) or after a period of gas perfusion (Figure 2, Procedure I; Figure 12). A lower concentration of Ni (1.0 mEq/l) in the Ni-Krebs caused an initial monoexponential decrease in contractile force but

TABLE IV

The Effect of Ouabain on Contractile Force in Hearts Perfused with Ni

Perfusion	Contractile Force (g)	
	Untreated	Ouabain (5×10^{-8} g/ml)
Krebs-Henseleit Perfusion	14.8 ± 1.0[a]	21.7 ± 2.2[b]
	(11)	(8)
Gas Perfusion	11.6 ± 0.8	12.8 ± 2.0
	(11)	(8)
Ni-Perfusion		
Gas Perfusion 5 sec	0.2 ± 0.1	2.4 ± 0.4[b]
	(11)	(8)
Gas Perfusion 30 min	7.2 ± 0.8	12.2 ± 2.4[b]
	(6)	(5)

Number in parenthesis indicates the number of hearts.

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

[b] Significantly greater than untreated ($P < .05$).

TABLE V

The Effect of Ouabain on the Halftimes for the Washout of Ni_{II}, Ca_{II} and Contractile Force

	$T_{1/2}$ (secs)		
	Ca_{II}	Ni_{II}	Force
UNTREATED			
Ni Perfusion			
Gas Perfusion-5 sec (5)	21.7 ± 1.9[a]	23.2 ± 1.4	-
Gas Perfusion-30 min (6)	20.7 ± 1.2	21.2 ± 1.3	21.0 ± 3.5
OUABAIN (5×10^{-8} g/ml)			
Ni Perfusion			
Gas Perfusion-5 sec (3)	19.3 ± 1.4	19.4 ± 1.7	-
Gas Perfusion-30 min (5)	20.9 ± 1.3	21.6 ± 1.3	22.0 ± 2.4

Number in parenthesis indicates the number of hearts.

[a] Mean ± S. E.

Figure 12: The effect of gas perfusion, Ni-Krebs perfusion and Ca-free Wash on electrical activity and contractile force. Electrical activity and contractile force recorded in a typical experiment to illustrate the perfusion protocol and show the effect of gas perfusion, Ni-Krebs perfusion and Ca-free Wash on electrical activity (upper trace) and contractile force (lower trace). Note that the tracings are interrupted and the time scales refer to the tracings only. Note also the difference in the time scale for each measurement.

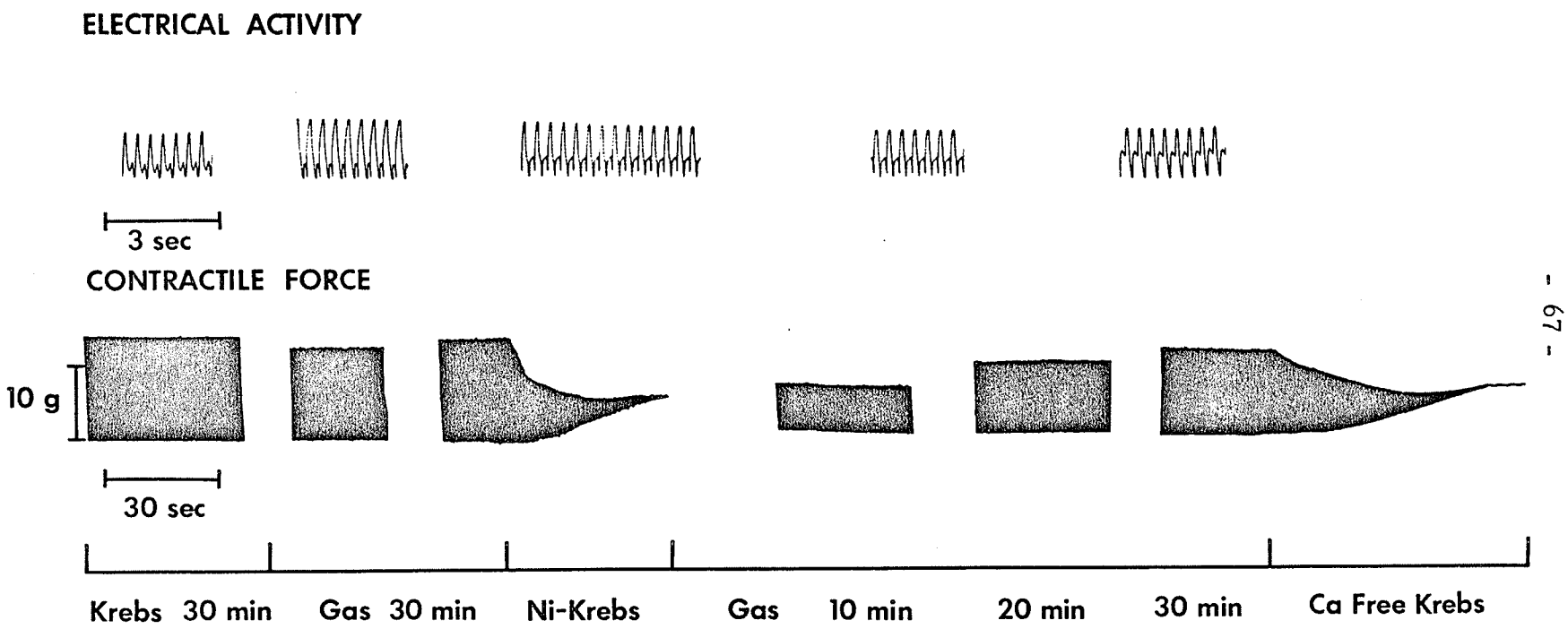
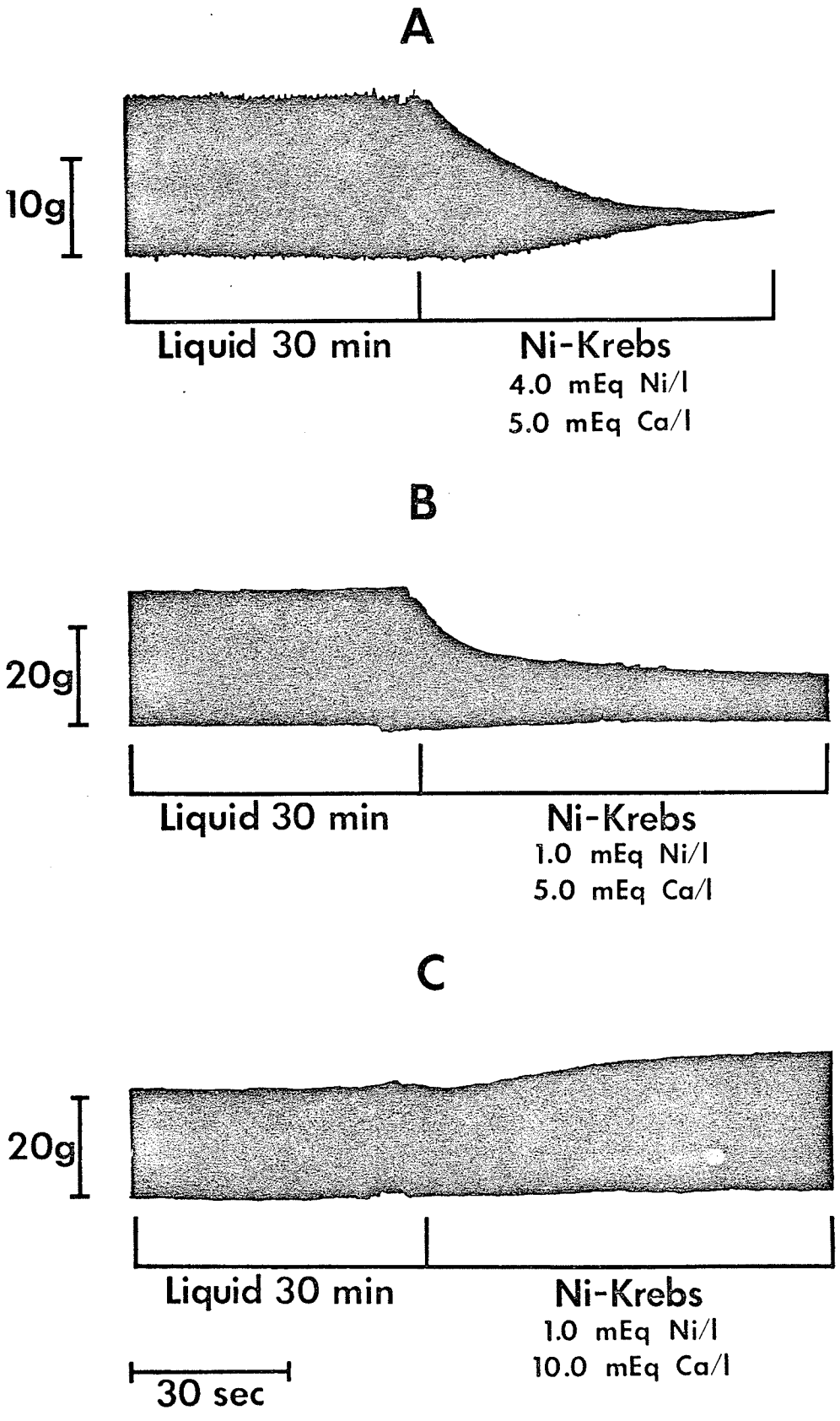

ELECTRICAL ACTIVITY
3 sec
CONTRACTILE FORCE
10 g
30 sec
Krebs 30 min
Gas 30 min
Ni-Krebs
Gas 10 min
20 min
30 min
Ca Free Krebs

Figure 13: The effect of various concentrations of Ni and Ca on contractile force in liquid perfused hearts.
Panel A: The effect of Ni-Krebs (4 mEq/l) on contractile force in liquid perfused hearts.
Panel B: The effect of 1 mEq Ni/l in K-H solution on contractile force in liquid perfused hearts.
Panel C: The effect of 10 mEq Ca/l on the inhibition of contractile force produced by 1 mEq Ni/l.


A
10g
Liquid 30 min
Ni-Krebs
4.0 mEq Ni/l
5.0 mEq Ca/l
B
20g
Liquid 30 min
Ni-Krebs
1.0 mEq Ni/l
5.0 mEq Ca/l
C
20g
Liquid 30 min
Ni-Krebs
1.0 mEq Ni/l
10.0 mEq Ca/l
30 sec

contractile force was not completely inhibited. Contractile force reached a steady level after the initial decrease (Figure 13B). Ten mEq Ca/l overcame the inhibition of contractile force caused by 1 mEq Ni/l (Figure 13C).

A significant positive inotropic response was produced by liquid perfusion with ouabain. However, this increase in force disappeared immediately after gas perfusion was started (Table IV). Like the decrease of contractile force seen with the initiation of gas perfusion, the loss of the positive inotropic effect has been attributed to the absence of Ca in the vascular space (Krip et al. 1971; Bailey and Sures, 1971). In the ouabain treated hearts, contractile force could not be reduced below 2.4 $\pm$ 0.6 g by perfusion with 4 mEq Ni/l even though Ni-Krebs perfusion was continued for 30 min (Table IV). This was significantly greater ($P < .05$) than that measured during Ni-Krebs perfusion in the untreated hearts. Contractile force gradually increased during gas perfusion and returned to 12.2 $\pm$ 2.4 g, after 30 min. This was not different ($P > .05$) from the force measured prior to Ni-Krebs perfusion, 12.8 $\pm$ 2.0 g, but was significantly greater ($P < .05$) than that measured in the untreated hearts (Table IV). However, the level of contractile force, although greater than that measured in the untreated hearts after Ni-Krebs perfusion, was significantly less ($P < .05$) than that measured during liquid perfusion in the ouabain treated hearts.

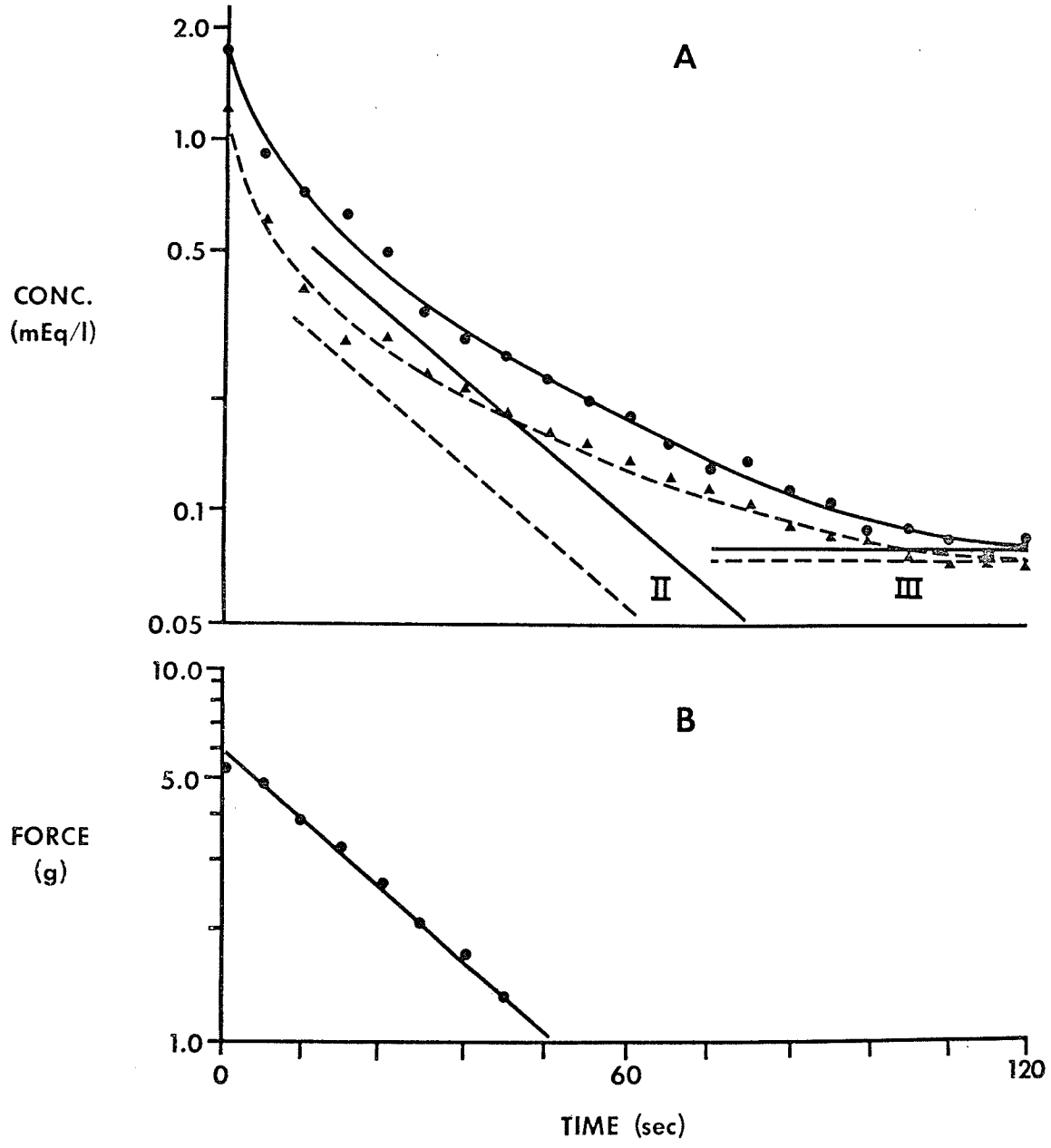
3. The effect of Ni on the kinetics and tissue content of Ca.

Figure 14A shows typical Ca and Ni washout curves obtained in the Ca- and Ni-free washout measured 30 min after perfusion with Ni-Krebs. Graphical analysis of the Ca washout curve yielded three compartments and the kinetic parameters of the three Ca compartments were not different than those reported by Bailey and Dresel (1968) and Bailey et al. (1972). Ca compartment 1, Ca_I (omitted from Figure 14A for clarity), represents Ca adhering to the perfusion apparatus and the coronary vasculature (Bailey and Dresel, 1968). The third compartment, Ca_{III}, has a halftime greater than 300 sec and has been assumed for the purposes of the analysis to contribute a constant amount of Ca to the total washout curve during the 90 to 150 sec Ca-free perfusion. The Ca in the second compartment, Ca_{II}, was washed out at a rate identical to the decay of contractile force. Furthermore, the tension developed by the heart had been shown to be related to the Ca content in this pool (Bailey and Dresel, 1968). For these reasons, Ca_{II} is believed to be directly involved in the maintenance of contractile force. The Ca washout curves obtained from hearts washed out 5 sec after Ni-Krebs perfusion were similar to the curves shown in Figure 14A. The mean halftime for the washout of Ca_{II} from untreated hearts gas perfused for 30 min was 20.7 ± 1.2 sec and that from untreated hearts with 5 sec gas perfusion was 21.7 ± 1.9 sec (Table V). The

Figure 14: Typical Ca and Ni washout curves and decay of contractile force in the same heart.

Panel A: Typical washout curves for Ca (●——●) and for Ni (▲- - -▲) in the same heart after exposure to 4.0 mEq Ni/l and 30 min of gas perfusion. II and III indicated the second and third Ca and Ni compartments respectively. The first Ca and Ni compartments were omitted for clarity.

Panel B: Decay of contractile force in the same heart.


A
2.0
1.0
0.5
0.1
0.05
CONC.
(mEq/l)
II
III
B
10.0
5.0
1.0
FORCE
(g)
0
60
120
TIME (sec)

halftimes for Ca_{II} washout were not significantly different ($P > .05$) from one another, nor did they differ ($P > .05$) from the halftime for the decay of contractile force (Table V, Figure 14). The halftimes did not differ ($P > .05$) from those obtained from hearts not perfused with Ni, $T_{1/2} = 21.6 \pm 3.9$. These data show that Ni perfusion did not affect the kinetics of washout of Ca_{II} even though contractile force was abolished by Ni-Krebs perfusion and was absent 5 sec after Ni-Krebs perfusion.

The effect of Ni on Ca uptake and/or exchange into the myocardium from the vascular compartment was investigated by adding tracer quantities of ^{45}Ca to the Ni-Krebs solution. The amount of ^{45}Ca taken up by the heart during Ni-Krebs perfusion was $50.47 \pm 9.43 \times 10^6$ cpm/kg wet weight of tissue and the quantity of ^{45}Ca taken up during K-H perfusion was $45.95 \pm 4.88 \times 10^6$ cpm/kg. Hence, the quantity of ^{45}Ca taken up, a measure of Ca exchange, was the same ($P > .05$) irrespective of the presence of Ni in the perfusion fluid. It should be noted that the quantity of ^{45}Ca exchanged by hearts not depleted of Ca is less ($P < .05$) than the amount taken up into Ca depleted hearts (see below).

Table VI shows that the Ca_{II} content of untreated Ni-Krebs perfused hearts washed out after 5 sec of gas perfusion was not significantly different ($P > .05$) from the Ca_{II} content of the control hearts or from

TABLE VI

The Effect of Ouabain on Ca_{II} and Ni_{II} Content and Residual Tissue Ni and Ca Content

	COMPARTMENT II CONTENT (mEq/kg Tissue Wet Wt)		RESIDUAL TISSUE CONTENT (mEq/kg Tissue Wet Wt)	
	Ca	Ni	Ca	Ni
UNTREATED				
Normal Perfusion				
Gas Perfusion-30 min (7)	1.31 ± 0.18[a]	-	0.75 ± 0.10	-
UNTREATED				
Ni-Perfusion				
Gas Perfusion-5 sec (6)	1.09 ± 0.39	1.77 ± 0.08	0.53 ± 0.06[d]	0.56 ± 0.07
Gas Perfusion-30 min (6)	1.26 ± 0.06	0.80 ± 0.07[b]	0.45 ± 0.02[d]	1.03 ± 0.07[b]
OUABAIN ($5x10^{-8}$ g/ml)				
Ni-Perfusion				
Gas Perfusion-5 sec (3)	1.74 ± 0.27[c]	1.55 ± 0.10	0.65 ± 0.02	0.45 ± 0.08
Gas Perfusion-30 min (5)	1.86 ± 0.14[c]	1.06 ± 0.19	0.29 ± 0.06	0.86 ± 0.09

Number in parenthesis indicates the number of hearts.

[a] Mean ± S. E.

[b] Significant difference ($P < .05$) between hearts washed out 5 sec and 30 min after Ni-perfusion.

[c] Significant difference ($P < .05$) between untreated and ouabain treated hearts.

[d] Significant difference ($P < .05$) between normal perfusion and Ni perfusion.

hearts washed out 30 min after the conclusion of Ni-Krebs perfusion. Thus, Ni-Krebs perfusion did not affect the total amount of Ca contained in Ca_{II}. Treatment with ouabain yielded an increased Ca_{II} content after perfusion with 4 mEq Ni/l over that measured in untreated hearts ($P < .05$) (Table VI). The Ca_{II} content was 1.74 ± 0.27 mEq/kg and 1.86 ± 0.14 mEq/kg in hearts treated with ouabain 5 sec and 30 min after the conclusion of Ni-Krebs perfusion, respectively. These values were not significantly different ($P > .05$) from each other but were significantly greater ($P < .05$) than the Ca_{II} content in the untreated hearts (Table VI).

There was a significant decrease ($P < .05$) in the residual tissue Ca content after Ni-Krebs perfusion. The residual tissue Ca content of the heart was the same in hearts washed out 5 sec and 30 min after the termination of Ni-Krebs perfusion (Table VI). The residual tissue Ca content of ouabain treated hearts 30 min after Ni-Krebs perfusion was significantly reduced ($P < .05$) when compared with untreated hearts. However, in hearts washed out 5 sec after Ni-Krebs perfusion, residual Ca did not differ significantly ($P > .05$) in the ouabain treated and untreated hearts (Table VI).

4. Ni washout kinetics and tissue contents.

Graphical analysis of the Ni washout curve shown in Figure 14A yielded three compartments similar to those of the Ca washout curves.

The mean halftime for the washout of Ni_I, Ni_{II}, and Ni_{III} were < 10 sec, 21.2 ± 1.3 sec, and >300 sec respectively and were not significantly different ($P > .05$) from the washout of Ca. There was no difference ($P > .05$) in the kinetics of Ca and Ni washout curves obtained from ouabain treated and untreated hearts (Table V).

Ni_{II} content 5 sec after the conclusion of Ni-Krebs perfusion was significantly greater ($P < .05$) than when the hearts were washed out 30 min after the termination of Ni-Krebs perfusion. Therefore, these data show that the partial restoration of contractile force during the 30 min gas perfusion following Ni-Krebs perfusion was associated with a decrease in the content of Ni contained in Ni_{II}. Ni_{II} content in the ouabain treated hearts was not significantly different ($P > .05$) from untreated hearts (Table VI).

The residual Ni content measured in the ventricles after Ca-free washout was greater ($P < .05$) in untreated hearts gas perfused for 30 min than in hearts after 5 sec of gas perfusion (Table VI). The residual Ni content was not affected by ouabain treatment (Table VI).

5. Distribution of Ca and Ni in subcellular fractions of cardiac muscle.

The distribution of Ca and Ni in various subcellular components of kitten hearts gas perfused for either 5 sec or 30 min after Ni-Krebs perfusion is given in Table VII. The mitochondrial fraction contained

TABLE VII

Ca and Ni Content of Subcellular Fractions Obtained From Isolated Kitten Hearts, Gas Perfused for Either 5 sec or 30 min

Subcellular Fractions	Ca n Eq/mg Protein		Ni n Eq/mg Protein	
	Gas Perfusion 5 sec (4)	Gas Perfusion 30 min (4)	Gas Perfusion 5 sec (4)	Gas Perfusion 30 min (4)
Nuclear Fraction	23.4 ± 3.2[a]	28.8 ± 1.6	8.8 ± 1.4	9.4 ± 1.5
Mitochondria	91.4 ± 5.2	96.0 ± 9.2	9.9 ± 1.3	5.9 ± 0.9[b]
Microsomes	25.0 ± 1.0	23.6 ± 1.2	17.0 ± 2.0	18.2 ± 0.7
Supernatant (Soluble Fraction)	3.1 ± 0.3	4.0 ± 0.7	18.0 ± 1.2	16.4 ± 1.8

Number in parenthesis indicates the number of hearts.

[a] Mean ± S. E.

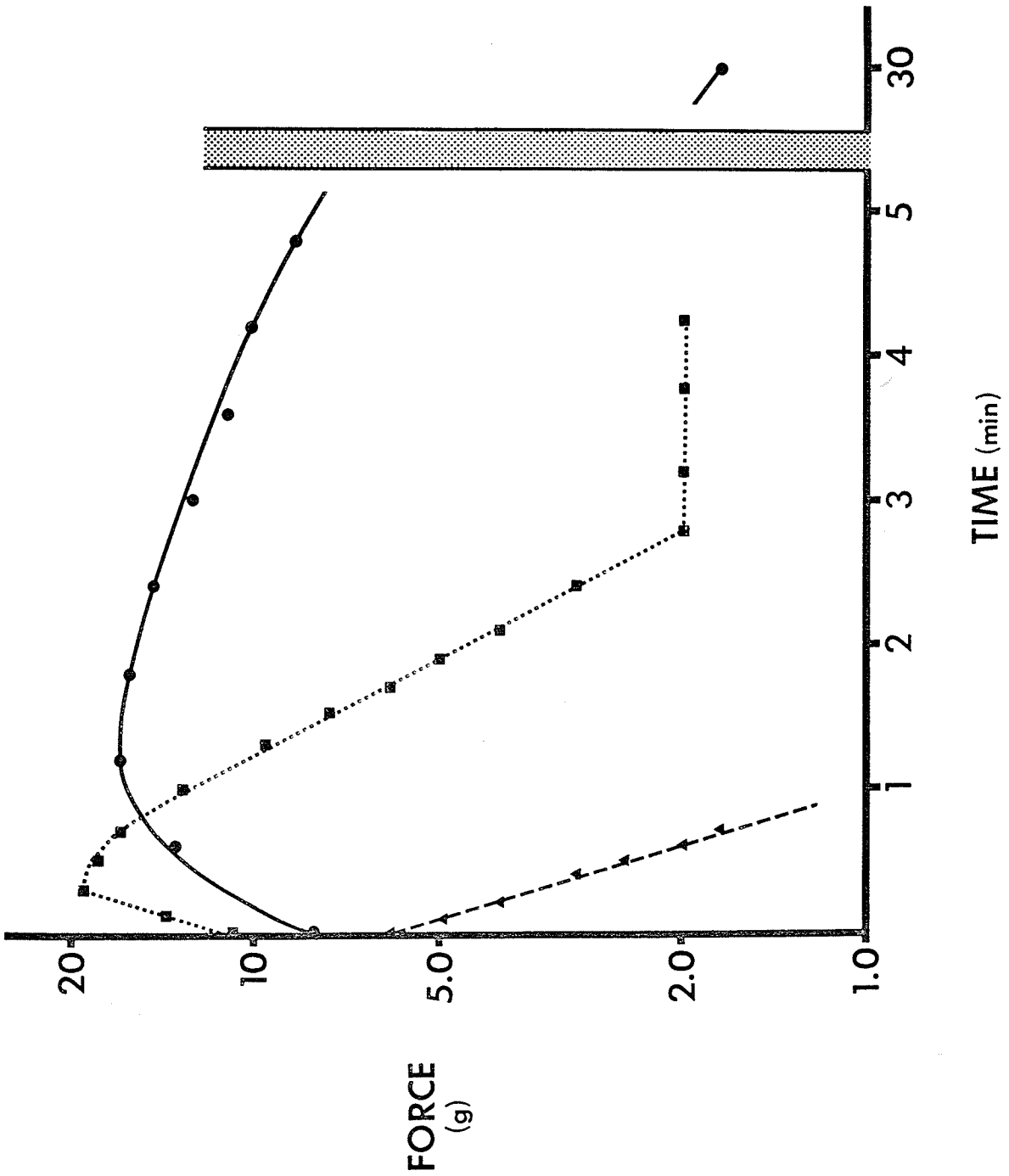
[b] Significant difference ($P < .05$) in Ni concentrations between 5 sec and 30 min gas perfusion.

the highest amount of Ca. The Ca content in each subcellular fraction was not affected by the period of gas perfusion. For example, Ca content of the mitochondrial fraction was not significantly different ($P > .05$) in hearts gas perfused either for 5 sec or 30 min. This suggests that there was no redistribution of Ca during the 30 min gas perfusion after Ni-Krebs perfusion. In contrast, there was a significantly greater ($P < .05$) amount of Ni in the mitochondrial fraction obtained from hearts gas perfused for 5 sec than for 30 min. During 30 min of gas perfusion after Ni-Krebs perfusion, contractile force was gradually restored to approximately 60% of its pre-Ni level and this was associated with a decrease in Ni content in the mitochondrial fraction. There was no significant increase ($P > .05$) in Ni content in any of the other subcellular fractions. The results show that Ni concentration was highest in the mitochondria when contractile force was minimal.

6. Effect of anoxia and 2,4 di-nitrophenol (DNP) on contractile force.

Figure 15 shows a plot of the logarithm of contractile force measured during perfusion with K-H solution bubbled with 95% N_2 - 5% CO_2 (anoxic conditions), K-H solution containing 0.1 mM DNP and Ni-Krebs containing 4 mEq Ni/l. Perfusion of the heart with K-H solution bubbled with 95% N_2 - 5% CO_2 caused an initial increase in force for the first 3 min, then force gradually decayed to about 1.5 g after 30 min. DNP (0.1 mM) caused an initial increase of contractile force

Figure 15: The effect of anoxia, 0.1 mM DNP and 4.0 mEq Ni/l on contractile force. Contractile force was measured under anoxic conditions (●——●), during perfusion with 0.1 mM DNP (■·····■) and during perfusion with 4.0 mEq Ni/l (▲— — —▲). Note that the time scale has been interrupted.


FORCE (g)
20
10
5.0
2.0
1.0
1
2
3
4
5
30
TIME (min)

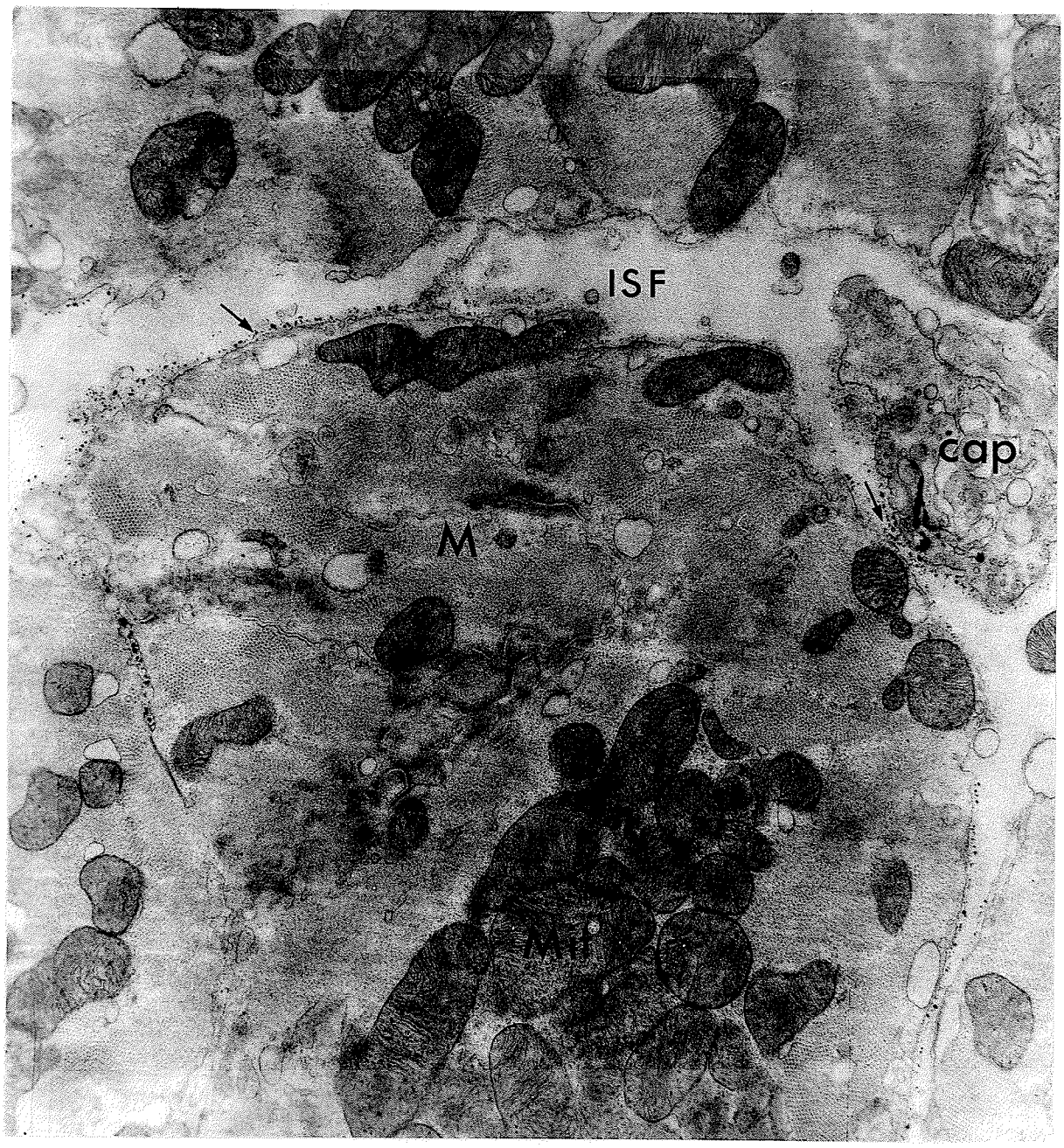
during the first 60 sec of perfusion which was then followed by a gradual decay of contractile force ($T_{1/2}$ < 60 sec) to a level approximately 20% of that measured before DNP treatment (Figure 15). Contractile force was maintained at this reduced level for at least 3 min in the presence of 0.1 mM DNP. Substitution of the perfusion fluid containing 0.1 mM DNP with gas mixture of 95% O_2 - 5% CO_2 did not improve contractile force of the isolated heart. In contrast, Ni-Krebs (4 mEq/l) caused an immediate monoexponential decrease in contractile force ($T_{1/2}$ = 23.4 ± 2.4 sec) without an initial increase. Subsequent perfusion of the heart with 95% O_2 - 5% CO_2 gradually restored contractile force to approximately 60% of that measured before Ni treatment (see above).

C. Uncoupling of Excitation from Contraction by La.

1. <u>The distribution of La ions in liquid and gas perfused hearts</u>.

Electron dense particles of La ions were distributed outside the muscle cell when sections were taken from cat hearts perfused with La after liquid perfusion (Plate II). We observed dense granules lining the capillary wall, in the pinocytotic vesicles of the endothelial cells and also in the intercellular spaces of the capillary endothelial wall. They were very dense on the basement membrane of the endothelial wall and the sarcolemma membrane. The granules appear to be lining the basement membranes. Few granules were found in the interstitial space away from the basement membrane. However, Revel and Karnorvky

Plate II: Electronmicrograph of cardiac muscle (M), transversly sectioned, and a capillary (cap) showing the distribution of La ions in a gas perfused heart. Note that the electron dense granules of La are distributed along the inner surface of the capillary endothelium, along the outer surface of the muscle membrane and the intercellular spaces of the capillary endothelium (indicated by the arrows). Free La granules were not observed in the interstitial fluid (ISF). x 17,940.


ISF
cap
M
MII

(1967) observed dense particles of La in the interstitial spaces. It should be pointed out that La was not added to the fixing solutions in our preparations as is the usual practice (Laszlo et al. 1952; Revel and Karnorvky, 1967) and thus La may have been removed during the fixing procedure in our tissues. Despite this, many dense granules of La were observed along the basement membranes of the muscle cell (Plate II). This suggests that the La remaining is more tightly bound and therefore may not be removed during fixation. That is, loosely bound or free La particles in the vascular and interstitial spaces may have been removed during fixing. When the heart was perfused with La after gas perfusion, a similar distribution of electron dense granules of La were observed as shown in Plate II. Therefore, there was no observable differences between the distribution of La ions in liquid or gas perfused hearts. La granules were not found in the intracellular spaces which was in agreement with reports by other workers (Revel and Karnorvky, 1967; Laszlo et al. 1952).

2. The effect of La on contractile force.

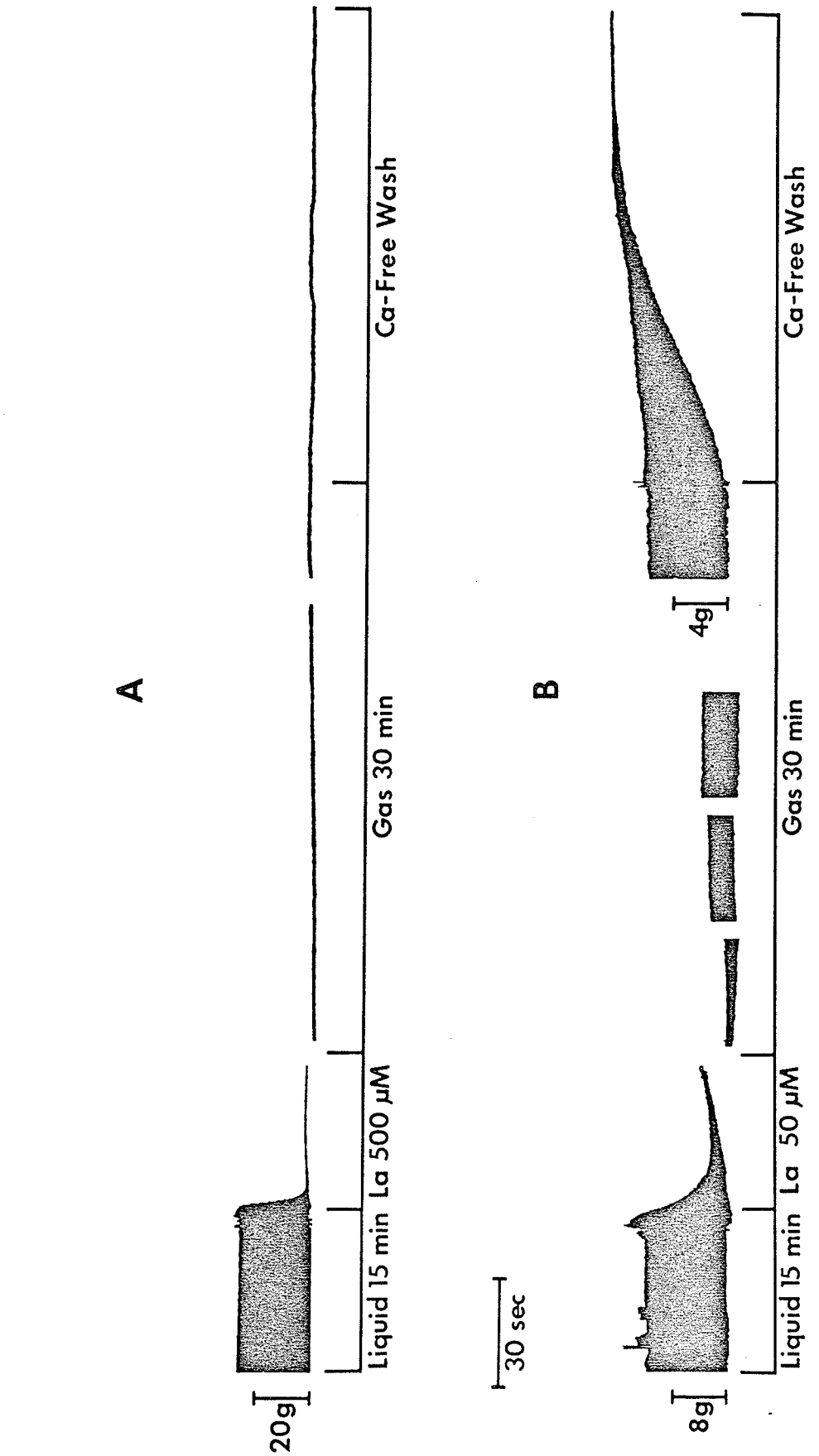
a. Liquid perfused hearts.

The decay of contractile force in a typical heart following a change from Hepes solution to La-Hepes containing 500 uM La (Figure 3, Procedure A) is shown in Figure 16A. Initiation of La-Hepes perfusion caused an immediate monoexponential decrease in contractile

Figure 16: The effect of 500 uM and 50 uM La on contractile force in liquid perfused hearts.

Panel A: The effect of 500 uM La on contractile force in a liquid perfused heart. Contractile force did not return during 30 min of gas perfusion and the Ca-free Wash did not cause a contracture.

Panel B: The effect of 50 uM La on contractile force in a liquid perfused heart. Contractile force gradually returned during 30 min gas perfusion and contracture developed during Ca-free Wash. Note the change in sensitivity before the Ca-free Wash. The tracings are interrupted and the time scale applies only to the tracings.


A
B
20g
Liquid 15 min La 500 µM
Gas 30 min
Ca-Free Wash
30 sec
8g
4g
Liquid 15 min La 50 µM
Gas 30 min
Ca-Free Wash

force to less than 1 g. The mean halftime for the decay of contractile force during perfusion with 500 uM La in five hearts was 2.2 $\pm$ 0.2 sec (Table VIII). This was significantly faster ($P < .05$) than the decay of contractile force following a change from K-H solution to Ca-free K-H solution (Figure 5, Wash I), $T_{1/2}$ = 9.3 $\pm$ 0.6 sec (Table VIII). The decay of contractile force was less rapid with lower concentrations of La. Using 50 uM La, the halftime for the decay of contractile force was 7.8 $\pm$ 0.6 sec (Figure 16B) and when 25 uM La was used the halftime was 10.5 $\pm$ 0.5 sec (Table VIII). The rate of decay of contractile force during perfusion with 25 uM La was not significantly different ($P > .05$) from the decay of contractile force during Ca-free perfusion in liquid perfused hearts. In contrast to higher concentrations of La, 5 uM La only partially inhibited contractile force. As shown in Figure 17A, contractile force decreased to a minimum within the first min of perfusion, but when La-Hepes perfusion was continued, contractile force gradually returned during the next min and attained a steady value significantly lower ($P < .05$) than that measured prior to La-Hepes perfusion. This effect of 5 uM La on contractile force was similar in pattern to the response seen when liquid perfusion was changed to gas perfusion (Figure 17B). Electrical activity monitored by bipolar electrodes attached to the left ventricle was not altered when contractile force was abolished during La-Hepes (25 - 500 uM) perfusion.

TABLE VIII

The Effect of La-Hepes Perfusion (5-500 uM) on Decay of Contractile Force in Liquid and Gas Perfused Hearts

	Liquid Perfusion $T_{1/2}$ (sec)	Gas Perfusion $T_{1/2}$ (sec)
Ca-free Perfusion	9.3 ± 0.6[a] (10)	21.6 ± 3.9[b] (7)
500 uM La	2.2 ± 0.2[c] (5)	0 (3)
250 uM La	2.8 ± 0.2[c] (2)	-
50 uM La	7.8 ± 0.6 (3)	-
25 uM La	10.5 ± 0.5 (2)	-
5 uM La	17.1 ± 0.8 (2)	-

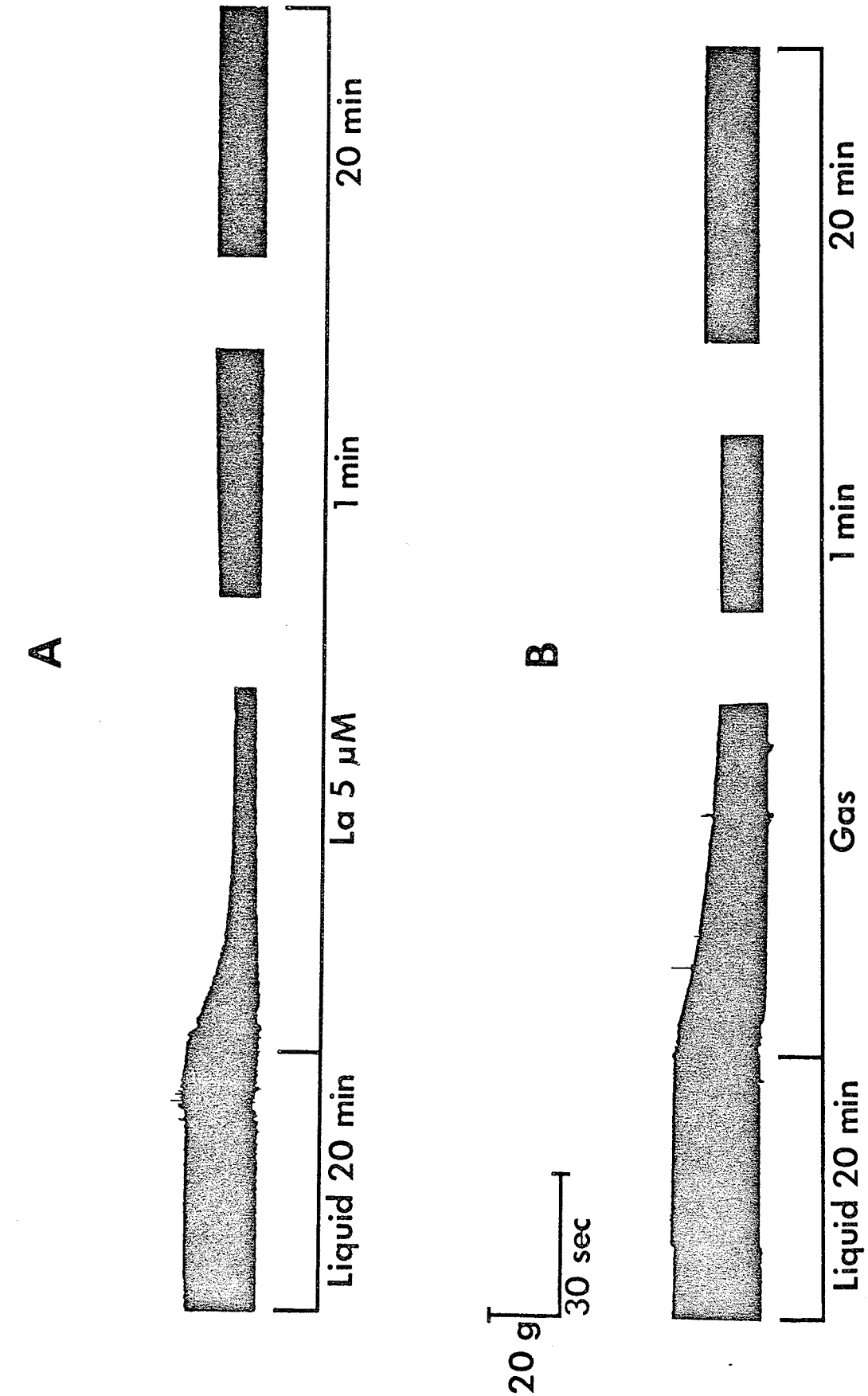
Number in parenthesis indicates the number of hearts.

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

[b] Significant difference ($P < .05$) between gas and liquid perfusion.

[c] Significantly less ($P < .05$) than Ca-free perfusion.

Figure 17: Comparison of the effect of 5 uM La and gas perfusion on contractile force.
Panel A: The effect of 5 uM La on contractile force.
Panel B: The effect of gas perfusion on contractile force. Note that the tracings are interrupted and the time scale applies only to the tracings.


A
Liquid 20 min
La 5 µM
1 min
20 min
B
Liquid 20 min
Gas
1 min
20 min
20 g
30 sec

After a brief period of perfusion with La-Hepes (25 - 500 uM), perfusion with Hepes solution (Figure 3, Procedure A (i)) restored contractile force to a value not different ($P > .05$) from the force prior to La-Hepes perfusion. The rate of restoration of contractile force was dependent on the concentration of La used and also on the time of exposure of the heart to the La-Hepes. That is, after brief exposure to La (25 - 500 uM) contractile force was completely restored, to a value which was not different ($P > .05$) from the value before La-Hepes perfusion. Prolonged exposure (> 5 min) with 25 uM or more of La apparently had secondary effects and contractile force could never be completely restored. Repeated exposure to La had successively less effect on the decay of contractile force. The rate of decay was slower and the effect was less than the preceeding exposure. This is in agreement with the results of Sanborn and Langer (1970).

b. Gas perfused hearts.

Figure 18 shows a typical record of contractile force when the hearts were exposed to 500 uM La after a period of preperfusion with water saturated O_2 (Figure 3, Procedure B). In contrast to liquid perfused hearts, 500 uM La had no effect on contractile force. Contractile force developed by the heart during 500 uM La-Hepes perfusion was the same as that during gas perfusion. Prolonged La-Hepes (500 uM) perfusion (> 10 min) caused contractile force to decrease gradually.

Figure 18: The effect of 500 uM La on contractile force after 30 min gas perfusion. Note that the tracings are interrupted and the time scale applies only to the tracings.

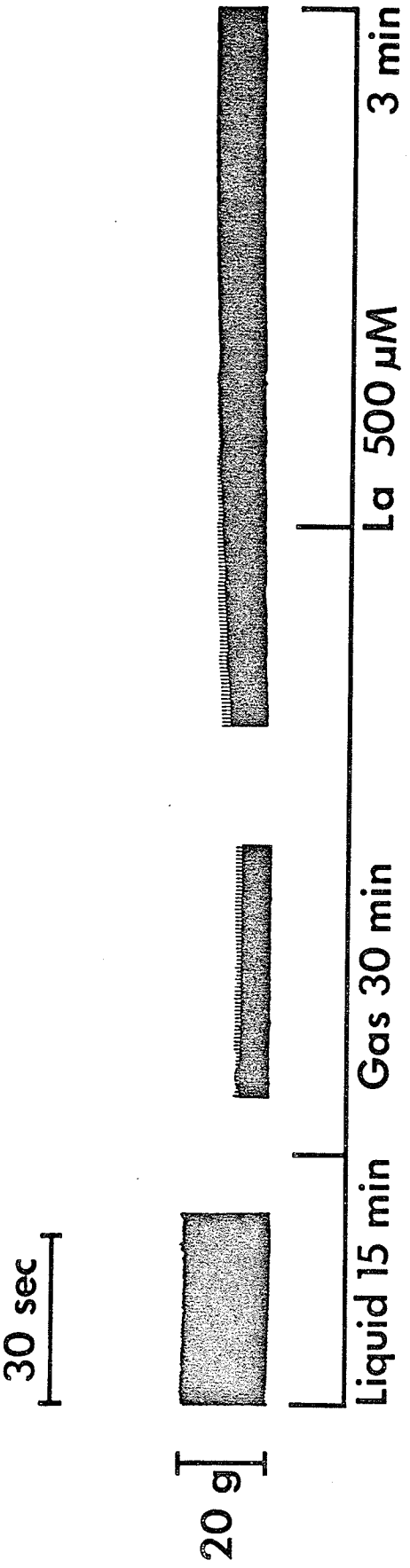

30 sec
20 g
Liquid 15 min
Gas 30 min
La 500 µM
3 min

Figure 16B shows a typical record of contractile force in an experiment where the heart was gas perfused after contractile force was abolished by 50 uM La (Figure 3, Procedure A(ii)). After 30 min of gas perfusion, contractile force was restored to about 40% of that measured immediately before La perfusion (Figure 19). Recovery after higher concentrations of La was less. After 250 uM La, 20% recovery was observed, while recovery after 25 uM La was 80% (Figure 19). However, when contractile force was abolished by 500 uM La, contractile force did not return during subsequent 30 min of gas perfusion (Figure 16A and 19). Ca-free perfusion subsequent to gas perfusion caused a contracture (Figure 16B). The decrease in contractile force during the Ca-free perfusion followed a monoexponential function. In three hearts perfused with 50 uM La, the halftime for the decay of contractile force during Ca-free perfusion after gas perfusion was 24.8 $\pm$ 2.5 sec (Table IX). No contracture was observed during Ca-free washout after gas perfusion when contractile force was abolished by 500 uM La (Figure 16A).

3. Ca washout kinetics after La perfusion.

Figure 20A shows a plot of the logarithm of Ca concentration measured in the Ca-free perfusion (Procedure A(ii) of Figure 3) as a function of time. Graphical analysis yielded a two compartment system. The washout of the first compartment, Ca_I, had a halftime of 6.9 $\pm$ 1.0

Figure 19: The effect of several concentrations of La (25 - 500 uM) on contractile force after 30 min gas perfusion after exposure to La-Hepes. Contractile force is expressed as a percent of the force measured on liquid immediately before La-Hepes perfusion. Number in parenthesis indicates the number of hearts and vertical bars represent $\pm$ S. E. of the mean.

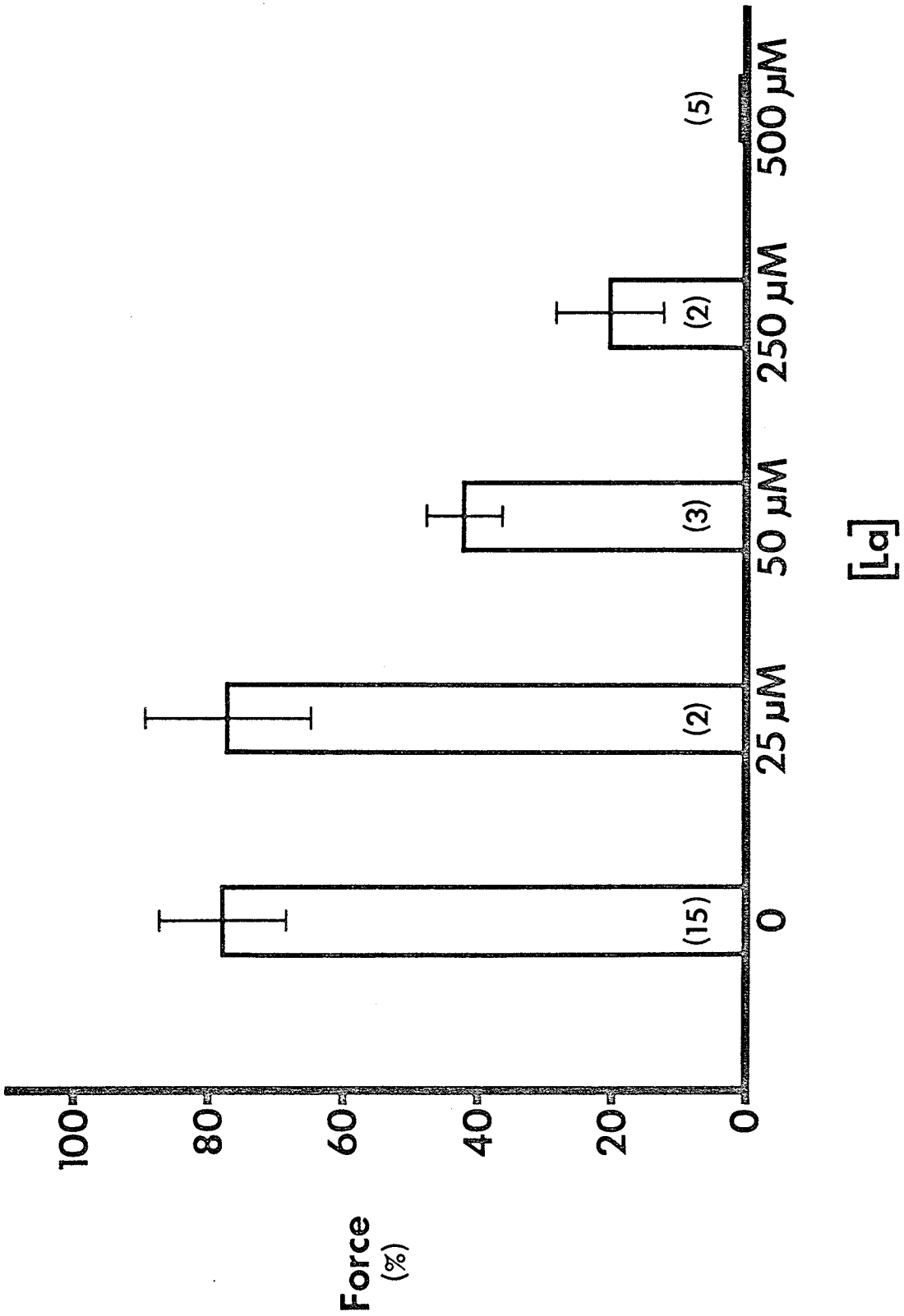

Force
(%)
100
80
60
40
20
0
(15)
0
(2)
25 µM
(3)
50 µM
(2)
250 µM
(5)
500 µM
[La]

TABLE IX

The Effect of La-Hepes Perfusion on Washout of Ca in Ca-free Wash (Figure 3, Procedure A (ii))

	Control (7)		La-treated Hearts (3)	
	$T_{1/2}$ (sec)	Ca Content mEq/kg	$T_{1/2}$ (sec)	Ca Content mEq/kg
Contractile force	21.6 ± 3.9[a]	-	24.8 ± 2.5	-
Ca_I	5.6 ± 0.3	-	6.9 ± 1.0	0.87 ± 0.18
Ca_{II} or Ca_{IIB}	22.7 ± 5.3	1.31 ± 0.18	42.4 ± 1.2[b]	2.59 ± 0.04[b]
Ca_{III}	> 300	-	0	0

Number in parenthesis indicates number of hearts.

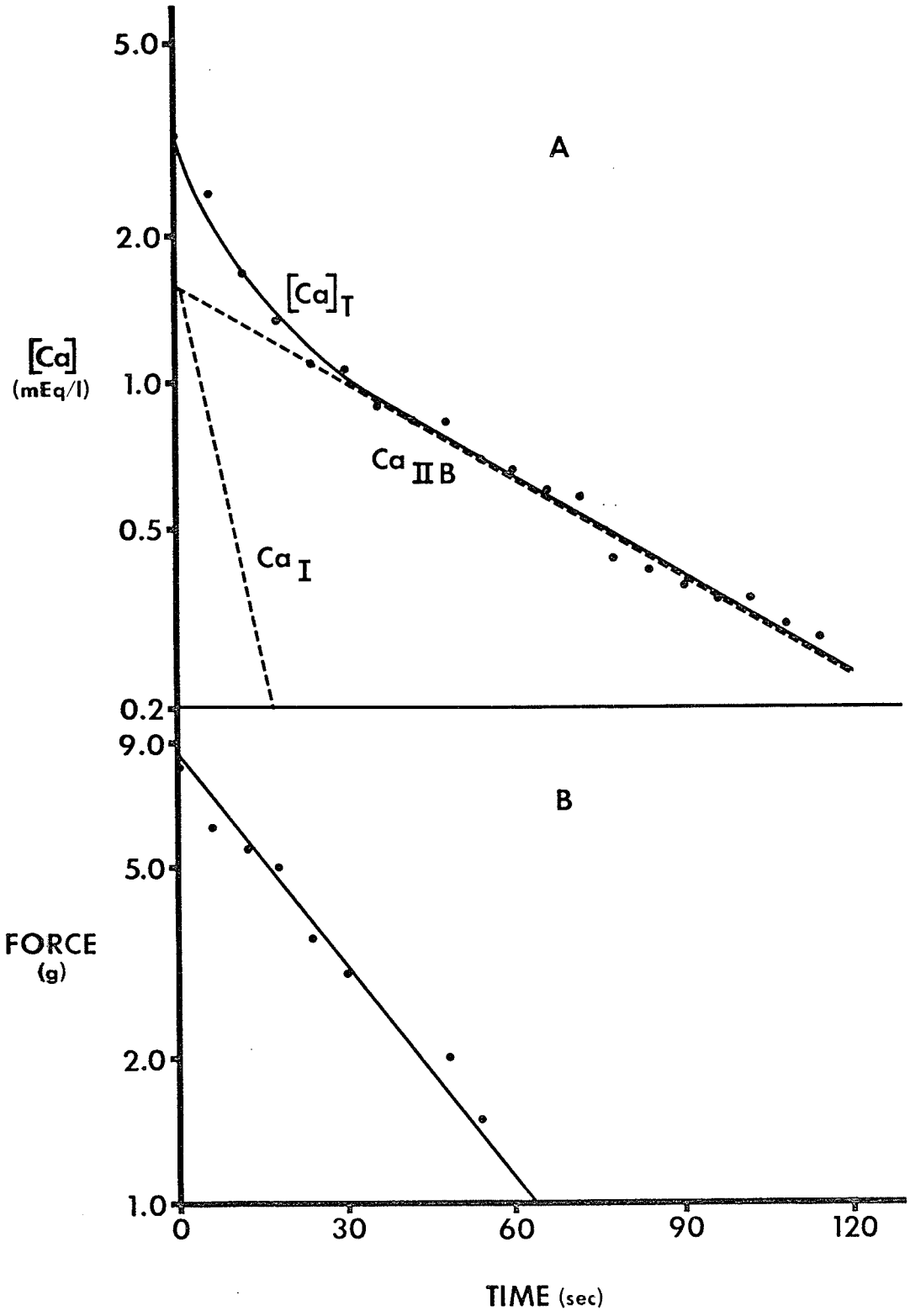
[a] Mean ± S. E.

[b] Significant difference ($P < .05$) between control and La-treated hearts.

Figure 20: The effect of 50 uM La on Ca washout and decay of contractile force.

Panel A: A typical Ca washout curve $[Ca]_T$ after exposure to 50 uM La and 30 min gas perfusion. The filled circles (●) are the concentrations of Ca measured in the serial samples during the washout and the dashed lines, the two compartments resolved by graphical analysis. Ca_I and Ca_{IIB} are defined in the text.

Panel B: The decay of contractile force in the same heart.

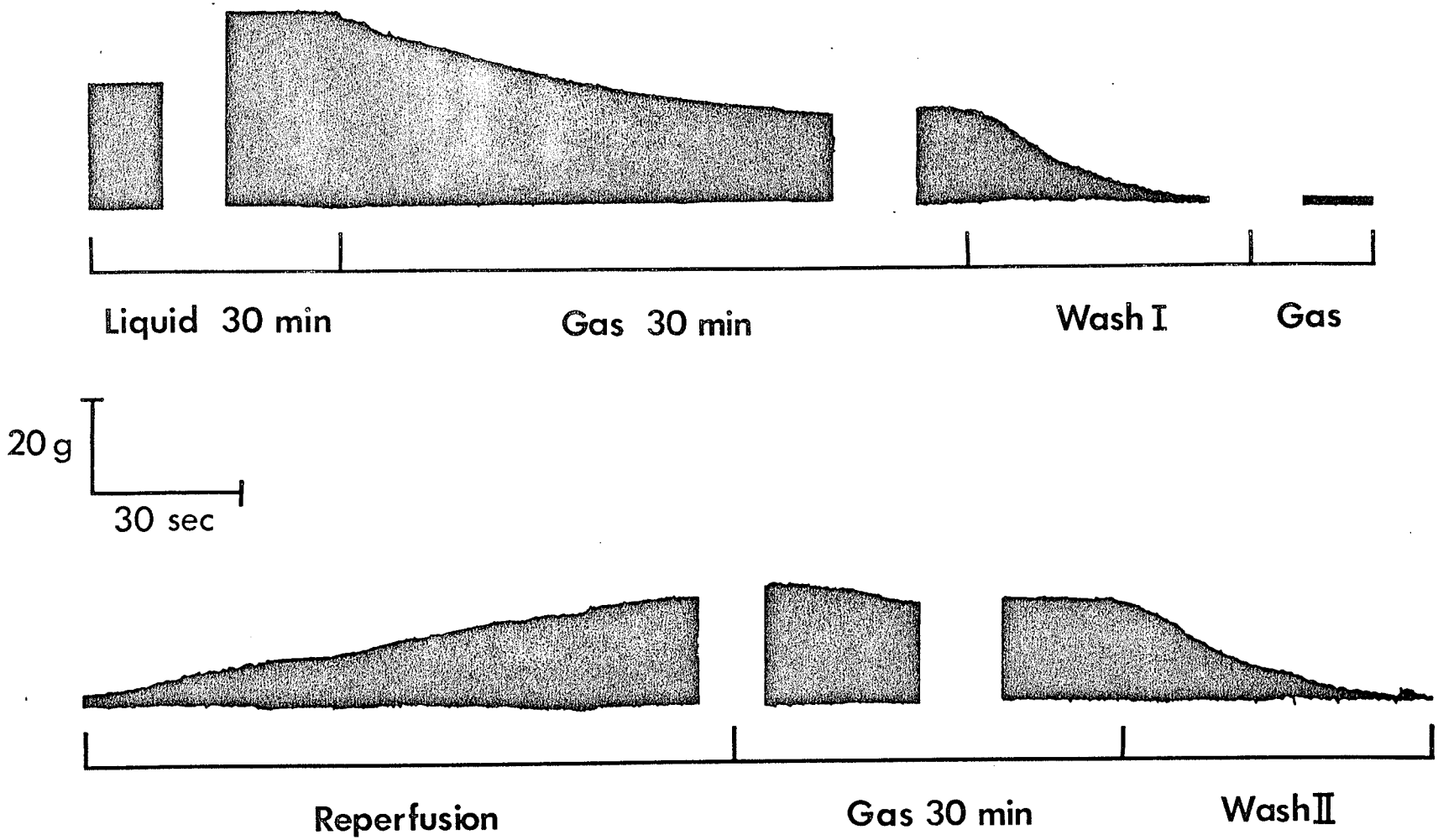

5.0
2.0
1.0
0.5
0.2
[Ca]
(mEq/l)
A
[Ca]T
Ca II B
Ca I
9.0
5.0
2.0
1.0
FORCE
(g)
B
0
30
60
90
120
TIME (sec)

sec which was not significantly different ($P > .05$) from the washout of Ca_I in control Ca-free perfusions after gas perfusion (Table IX). The halftime for the washout out of Ca from the second compartment, Ca_{IIB}, was 42.4 ± 1.2 sec, and this was not correlated with the decay of contractile force, $T_{1/2}$ = 24.8 ± 2.5 sec (Table IX, Figure 20A-B). The rate of Ca efflux from Ca_{IIB}, was significantly slower ($P < .05$) than Ca_{II} of control hearts (Table IX) and there was no evidence of the slow third compartment, Ca_{III}. The quantity of Ca washed out of Ca_{IIB}, 2.59 ± 0.04 mEq Ca/kg, was significantly greater ($P < .05$) than the Ca washed out from Ca_{II} in control hearts.

4. The effect of La on the restoration of contractile force in Ca-depleted hearts.

Figure 21 shows a tracing of contractile force in a typical experiment described in Figure 4, Procedure A. During preperfusion with Hepes solution contractile force gradually increased until it attained a steady level after about 10 min of perfusion. When the liquid perfusion medium was replaced by water saturated O_2, contractile force decayed to a new level, perhaps by redistribution of Ca within the several pools of the heart (Bailey and Dresel, 1968; Krip et al. 1971). However, contractile force during gas perfusion was still significantly less ($P < .05$) than that measured during the liquid preperfusion. When the hearts were washed out with Ca-free perfusion (Wash I) contractile force decayed monoexponentially to less than 1 g. Reperfusion of the hearts with ^{45}Ca

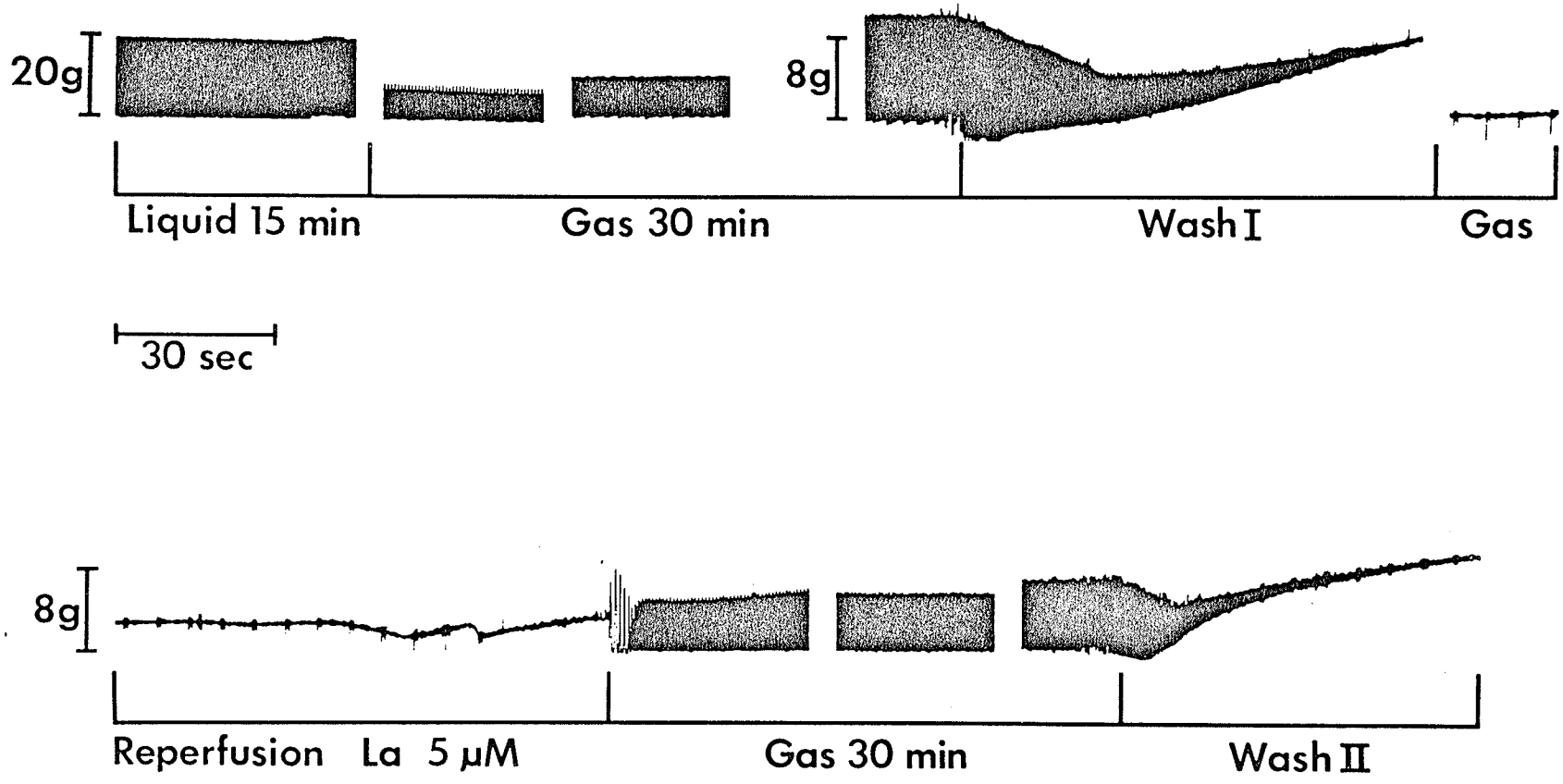
Figure 21: The effect of various perfusion media on contractile force in a control uptake experiment. Note the return of contractile force when the hearts were reperfused with Ca containing solution after Wash I. The tracings are interrupted and the time scale refers to the contractile force tracings only. See text for details.


Liquid 30 min
Gas 30 min
Wash I
Gas
20 g
30 sec
Reperfusion
Gas 30 min
Wash II

labelled perfusate containing normal concentrations of unlabelled Ca (5 mEq/l) restored contractile force to a steady level which was usually lower than that seen during liquid preperfusion. The restoration of contractile force was rapid and reached a steady level after approximately 90 sec (Bailey and Sures, 1971). Contractility was assumed to be completely restored when contractile force attained this steady level. Subsequent gas perfusion produced changes in contractile force similar to the previous period of gas perfusion. That is, contractile force decayed during the first 2 min of gas perfusion and then gradually returned to a level somewhat below that recorded during liquid perfusion before gas perfusion (Figure 21). The second Ca-free washout (Wash II, Figure 4) again caused a monoexponential decay in contractile force without a significant increase in resting tension (Figure 21).

Figure 22 shows a record of contractile force in a heart described in Figure 4, Procedure B. Hearts were reperfused with La-Hepes (5 uM La) after Ca-free Wash I. Contractile force was not restored. However, when gas perfusion was initiated contractile force immediately returned to 3-4 g and during the 30 min of gas perfusion gradually returned to 6.6 $\pm$ 1.5 g. When the heart was washed out with Ca-free perfusate (Wash II), a contracture developed (Figure 22). The halftime for the decay of contractile force was 17.2 $\pm$ 2.8 sec. This was not significantly different ($P > .05$) from the decay of contractile force during

Figure 22: The effect of 5 uM La on restoration of contractile force in Ca-depleted hearts. Note that there was no return of contractile force when the heart was reperfused with La-Hepes (5 uM) after Wash I. See text for details. The tracings are interrupted and the time scale refers to the contractile force tracings only. Note the change in sensitivity before Wash I.


20g
8g
Liquid 15 min
Gas 30 min
Wash I
Gas
30 sec
8g
Reperfusion La 5 µM
Gas 30 min
Wash II

Ca-free perfusion after gas perfusion ($T_{1/2}$ = 21.6 ± 3.9 sec).

5. Ca uptake by Ca-depleted hearts.

a. Uptake under normal conditions.

The logarithm of Ca measured in the effluent during reperfusion with K-H solution is shown in Figure 23 (Figure 4, Procedure A). Subtraction of the effluent Ca concentration from the Ca concentration in the perfusate yielded the curve R shown in Figure 23. The mean half-time for the extraction of Ca from the reperfusion medium in Ca_1 and Ca_2 are given in Table X. The rate of approach of Ca uptake to the steady state by Ca_1 was very rapid, $T_{1/2}$ = 5.6 ± 1.7 sec (Table X). This was not significantly different ($P > .05$) from the uptake of ^{3}H-Inulin, $T_{1/2}$ = 6.4 ± 1.0 sec in six experiments. It is to be noted that the uptake of ^{3}H-Inulin into the heart follows only a monoexponential function. This implies that Ca_1 may represent Ca uptake into the extracellular space. The total amount of Ca in Ca_1 was 0.92 ± 0.13 mEq/kg tissue wet weight. If this Ca was distributed homogeneously in tissue water, the concentration would be 1.15 mEq Ca/l total tissue water. However, if this Ca was distributed homogeneously in interstitial water estimated by the volume of distribution of ^{3}H-Inulin, the concentration of Ca in Ca_1 would be 4.18 mEq Ca/l which approximated the concentration of Ca in the perfusate (5.0 mEq Ca/l). This suggests that if Ca_1 represent Ca uptake in the extracellular space, it is probably not bound.

Figure 23: 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 squares 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, Ca_1 (– – – –) and Ca_2 (·····) resolved by graphical analysis.

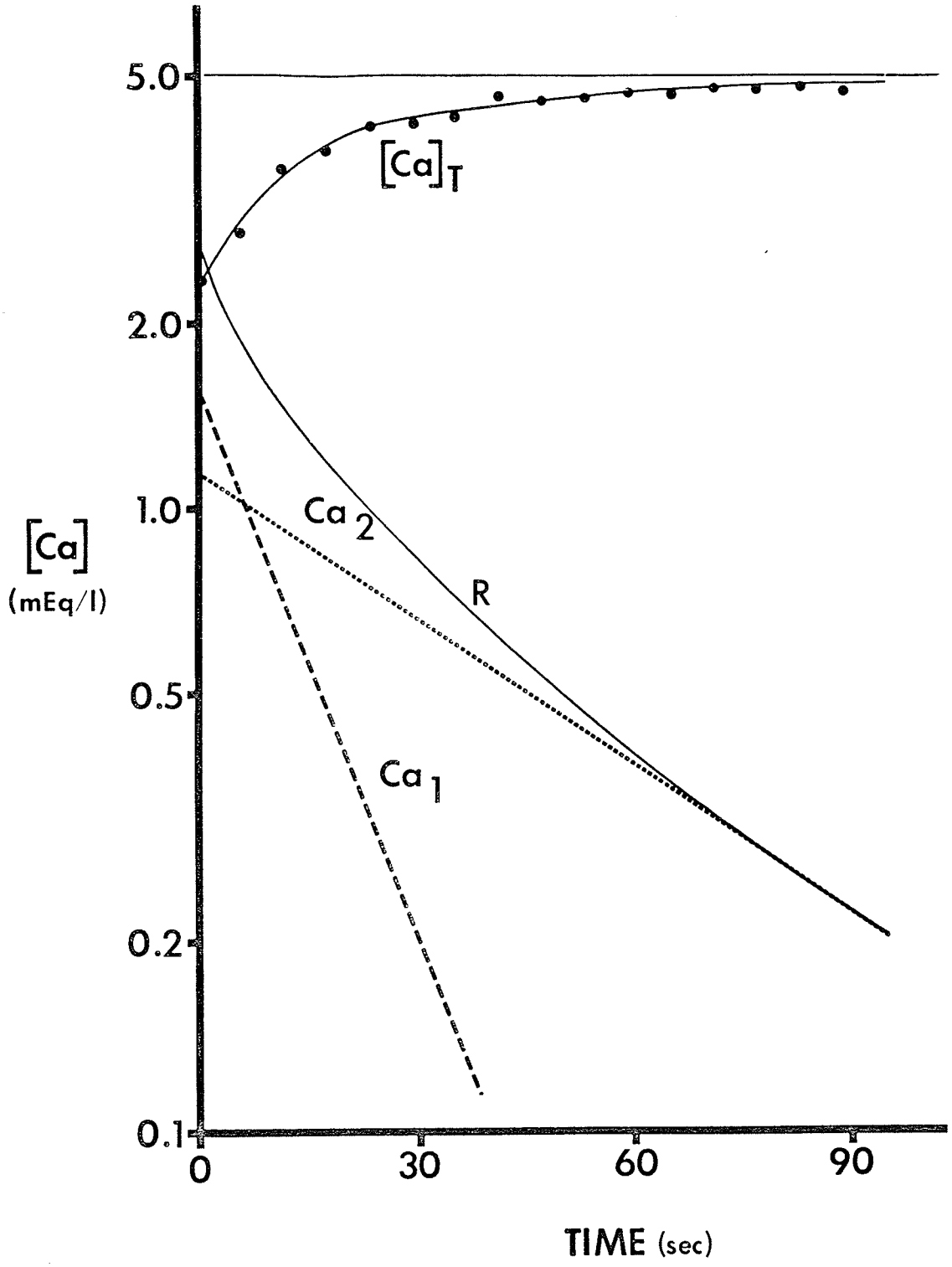

5.0
2.0
1.0
0.5
0.2
0.1
[Ca]
(mEq/l)
[Ca]T
Ca 2
R
Ca 1
0
30
60
90
TIME (sec)

TABLE X

The Effect of 5 uM La on Ca Uptake in Ca-depleted Hearts

	Control (5)		La (5)
	Ca_1	Ca_2	Ca_2
Ca			
$T_{1/2}$ (sec)	5.6 ± 1.7[a]	46.2 ± 6.2[b]	33.4 ± 7.9
t = 0 (mEq Ca/l)	1.54 ± 0.20	1.02 ± 0.11[b]	1.90 ± 0.29
Content (mEq Ca/kg tissue wet weight)	0.92 ± 0.13	2.87 ± 0.33[b]	3.96 ± 0.38[c]
^{45}Ca			
$T_{1/2}$ (sec)	5.6 ± 1.0	41.4 ± 5.7[b]	31.98 ± 1.63
t = 0 (cpm x 10^6/l)	32.88 ± 3.86	24.80 ± 3.27[b]	32.43 ± 4.26
Content (cpm x 10^6/kg tissue wet weight)	19.15 ± 4.33	58.45 ± 5.96[b]	79.98 ± 9.15[c]

Number in parenthesis indicates number of hearts.

[a] Mean ± S. E.

[b] Significant difference ($P < .05$) between Ca_1 and Ca_2 in control hearts.

[c] Significant difference ($P < .05$) between control and La-reperfused hearts.

The rate of approach to the steady state for Ca_2 was significantly slower ($P < .05$) than Ca_1, $T_{1/2} = 46.2 \pm 6.2$ sec, and because of its slower rate of approach to the steady state may represent Ca uptake into intracellular stores.

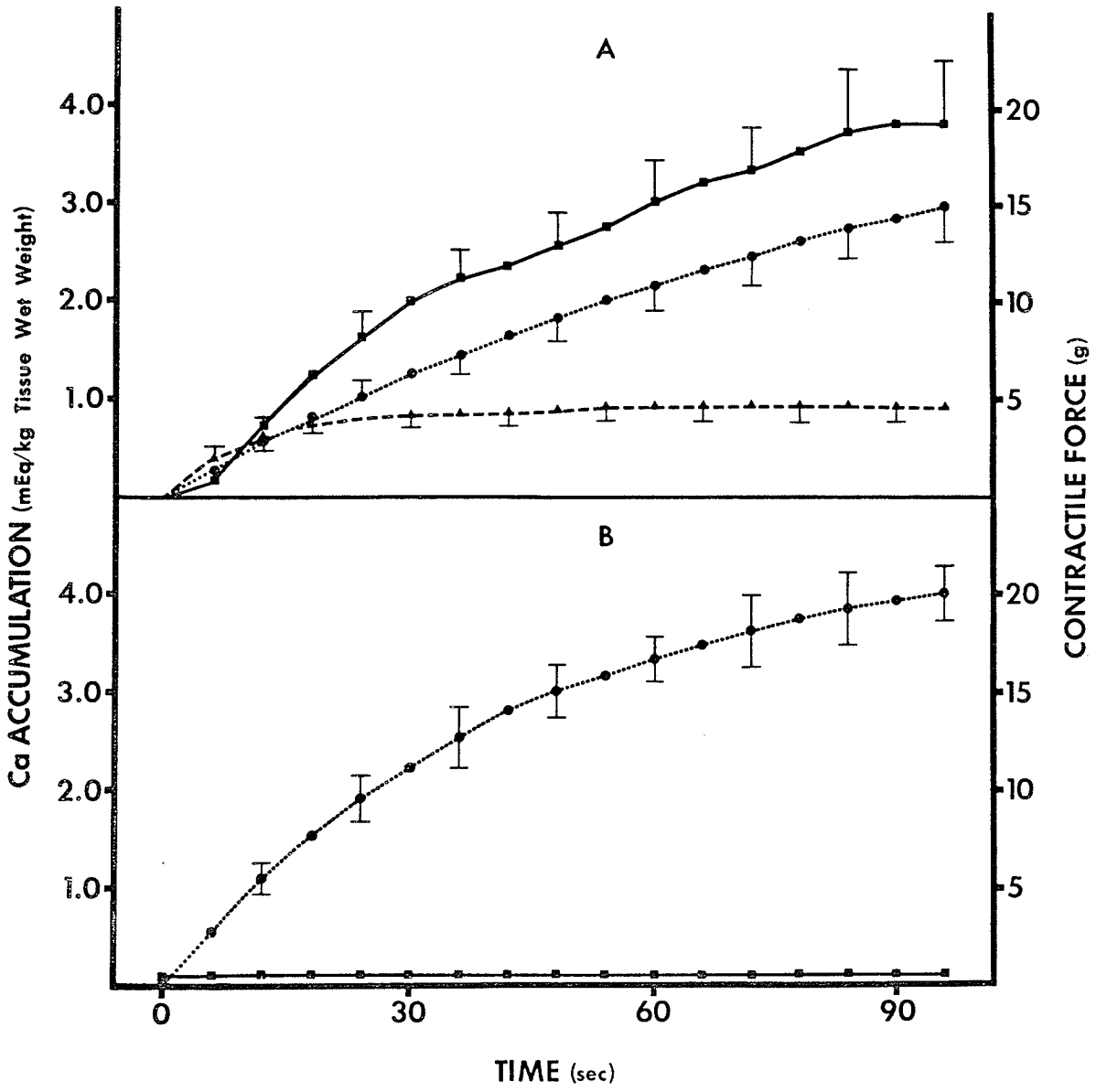
The reduction in the Ca concentration of the perfusate as a result of Ca extraction by Ca_1 during the first 6 sec of reperfusion, that is the intercept of the line representing $[Ca]_{1t=0}$, was significantly greater ($P < .05$) than the intercept for the second compartment, $[Ca]_{2t=0}$. Therefore, the quantity of Ca taken up by Ca_1 was significantly greater ($P < .05$) than the quantity of Ca taken up by Ca_2 during the first 6 sec of reperfusion. However, since Ca_1 reached the steady state more rapidly than Ca_2, the total quantity of Ca accumulated in Ca_1 was significantly less ($P < .05$) than the quantity taken up by Ca_2.

The accumulation of Ca in Ca_1 and Ca_2 is expressed as the quantity of Ca accumulated as a function of time in Figure 24A. The restoration of contractile force during the reperfusion, plotted as a function of time is also shown in Figure 24A and is similar in pattern to the accumulation of Ca by Ca_2. In contrast, the pattern of Ca accumulation by Ca_1 was very different from the pattern of restoration of contractile force. The accumulation of Ca in Ca_1 reached an asymptote of 0.92 ± 0.13 mEq Ca/kg tissue wet weight after 20 sec of reperfusion while contractile force attained the steady level after 90 sec.

Figure 24: The effect of 5 uM La on Ca accumulation and restoration of contractile force in Ca-depleted hearts.

Panel A: The accumulation of Ca_1 (▲— — —▲), Ca_2 (●·····●) and the restoration of contractile force (■——■) in 5 hearts during reperfusion with 5.0 mEq Ca/l are plotted as a function of time.

Panel B: The effect of La (5 uM) on the accumulation of Ca and restoration of contractile force. The symbols are the same as in Panel A. Vertical lines indicates $\pm$ S.E. of the mean.


A
B
4.0
3.0
2.0
1.0
20
15
10
5
0
30
60
90
Ca ACCUMULATION (mEq/kg Tissue Wet Weight)
CONTRACTILE FORCE (g)
TIME (sec)

The return of contractile force was a linear function of the quantity of Ca accumulated by Ca_2 at that time, but was not linearly related to the accumulation of Ca by Ca_1 (Figure 25). When the restoration of contractile force was expressed as a function of the quantity of Ca accumulated by Ca_2 during the reperfusion in five hearts, the mean coefficient of correlation, r, between the two variable was 0.93 ($P < .01$) (Figure 25).

b. Uptake in the presence of La.

Figure 26 shows a plot of the Ca concentration measured in the effluent during reperfusion with La-Hepes (5 uM) (Figure 4, Procedure B). In contrast to control hearts, contractile force was not restored during the reperfusion and subtraction of the Ca concentration in the perfusate yielded a line which was described by a monoexponential function instead of the double exponential function of time measured in the control hearts (Figure 23 and 26). The slope of the line, represented by the halftime was not different ($P > .05$) from the halftime of the uptake of Ca in Ca_2 in the control hearts (Table X). Ca uptake by Ca_1 was not evident in hearts reperfused in the presence of 5 uM La-Hepes.

The quantity of Ca extracted by the heart in the first 6 sec of reperfusion, that is, the intercept of the line $[Ca]_{2t=0}$ was significantly greater ($P < .05$) than the Ca extracted by Ca_2 in control hearts. Since the rate of approach to the steady state was not different, the quantity

Figure 25: The relationship between the restoration of contractile force and the accumulation of Ca in Ca_1 and Ca_2 during reperfusion of 5 hearts with 5.0 mEq Ca/l.

▲— — — —▲ indicates the restoration of contractile force as a function of the accumulation of Ca in Ca_1.

●——— indicates the restoration of contractile force as a function of the accumulation of Ca in Ca_2.

The mean coefficient of correlation, for the restoration of contractile force and Ca accumulation in Ca_2 is 0.93. The relationship between the restoration of contractile force and Ca accumulation in Ca_1 was not linear.

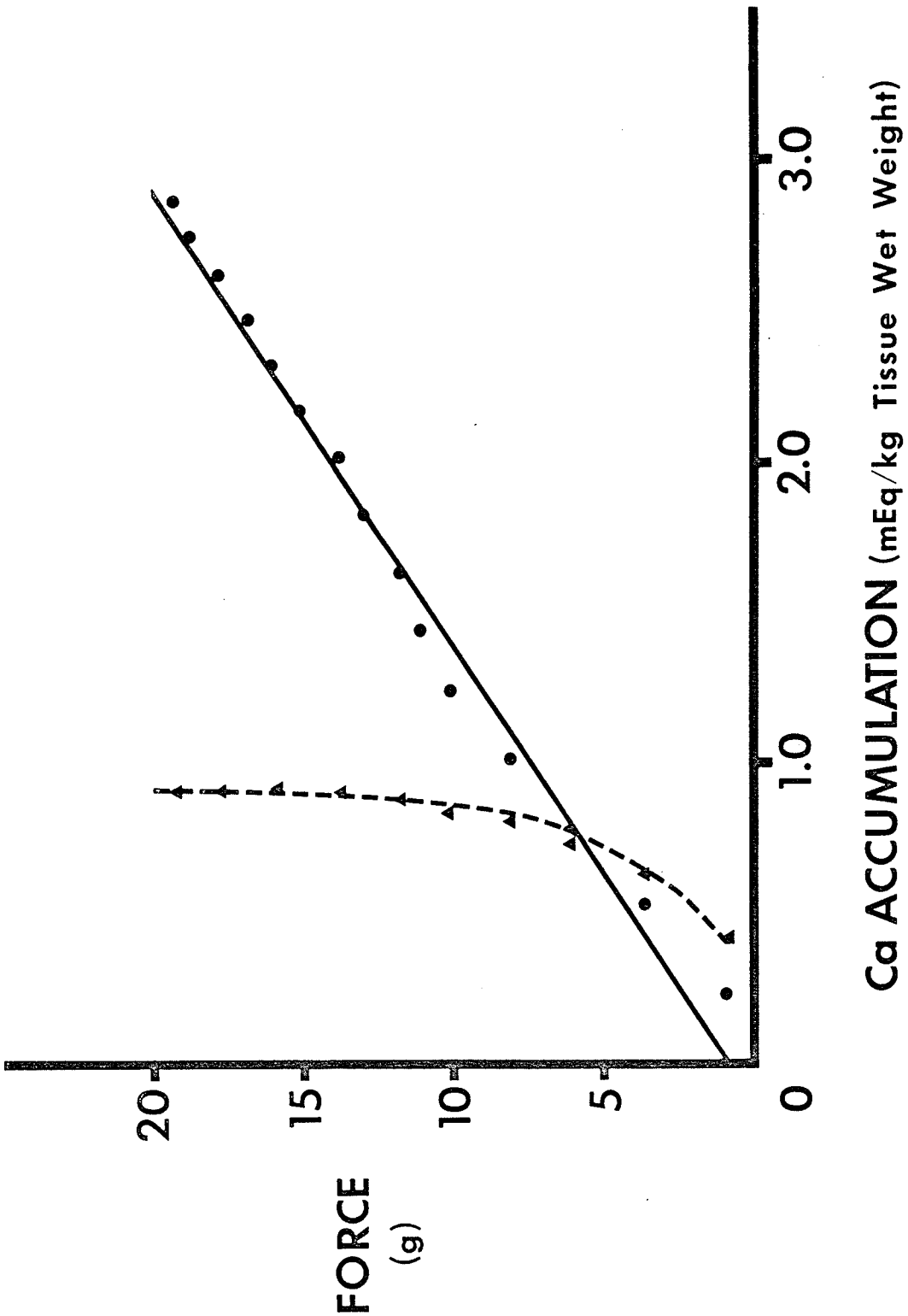
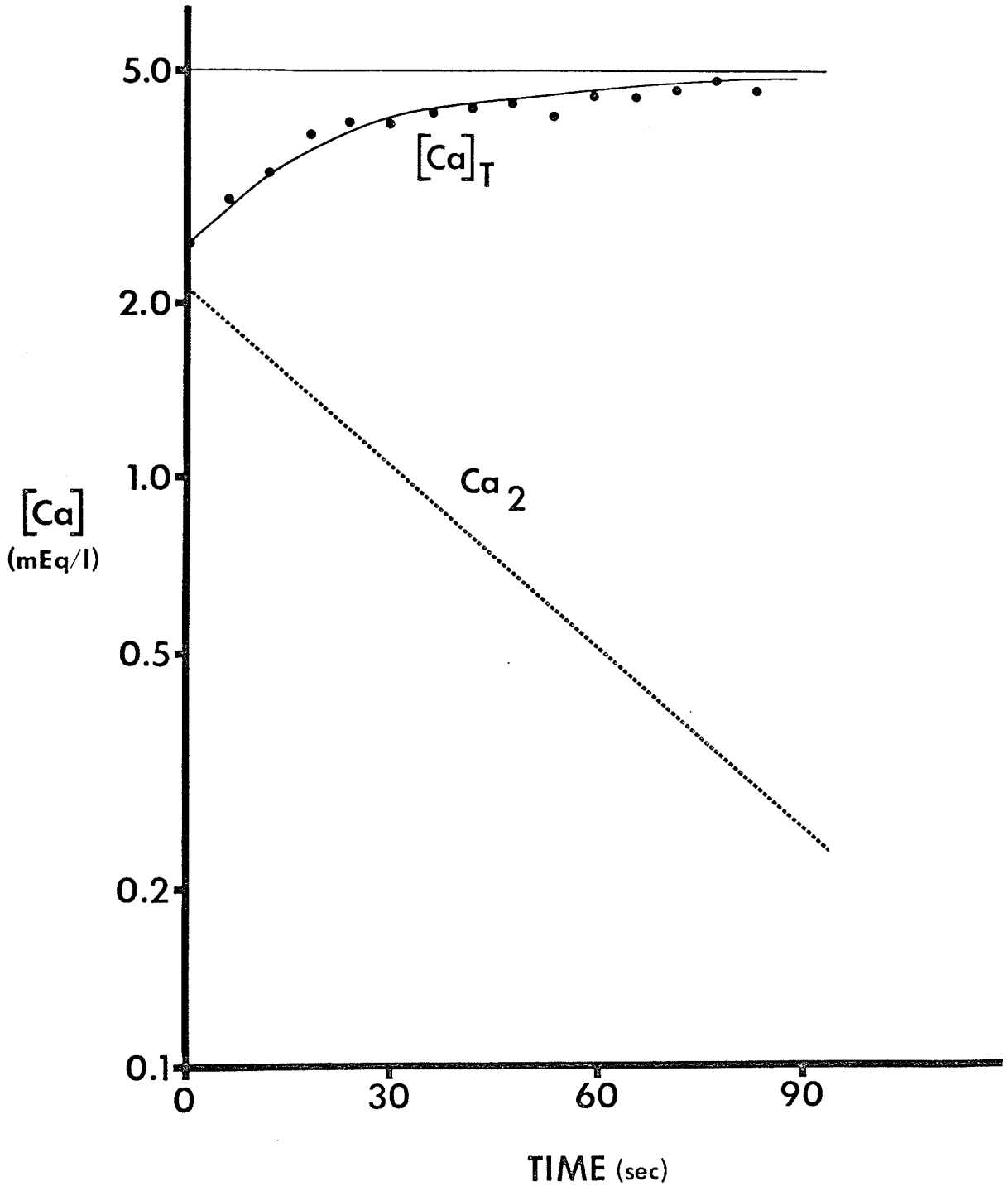

FORCE
(g)
20
15
10
5
0
1.0
2.0
3.0
Ca ACCUMULATION (mEq/kg Tissue Wet Weight)

Figure 26: The effect of 5 uM La on Ca uptake in Ca-depleted hearts. The uptake of Ca in a typical experiment during reperfusion with 5.0 mEq Ca/l plus 5 uM La. The heart was first depleted of Ca by Wash I. The logarithm of Ca concentration measured in the effluent was plotted as a function of time. The curve, $[Ca]_T$, is the least squares best fit line for the data points indicated by the filled circles (●). The horizontal line indicates the concentration of Ca in the reperfusion medium, 5.0 mEq Ca/l. The diagonal line, Ca_2 (·····) is the least squares best fit line for the difference between the Ca concentration measured in the effluent and the reperfusion Ca concentration.


5.0
2.0
1.0
0.5
0.2
0.1
[Ca]
(mEq/l)
[Ca]T
Ca2
0
30
60
90
TIME (sec)

of Ca taken up during reperfusion with La-Hepes was significantly greater ($P < .05$) than the quantity of Ca taken up by Ca_2 in control hearts (Table X). However, the total quantity of Ca taken up by Ca_2 during reperfusion with La-Hepes was not significantly different ($P > .05$) from the total quantity ($Ca_1 + Ca_2$) of Ca taken up in control hearts (Table XI).

The quantity of Ca accumulated in these hearts is plotted as a function of time in Figure 24B. The pattern of Ca accumulation was similar to the Ca accumulation in Ca_2, although contractile force was not restored in hearts reperfused with La-Hepes (Figure 22 and 24B).

In both the control hearts and hearts reperfused with La-Hepes (5 uM La), the uptake of ^{45}Ca from the perfusate followed the same pattern as Ca. A summary of the uptake of ^{45}Ca in both the control hearts and hearts reperfused with La-Hepes are given in Table X and XI.

6. <u>The effect of La on the distribution and fate of Ca taken up by Ca-depleted hearts</u>.

The concentration of Ca measured in Ca-free Wash II (Figure 4, Procedure A) of a heart reperfused until contractile force has been completely restored is shown in Figure 27A. The washout of Ca was characteristic of the usual three compartment system (Bailey and Dresel, 1968). The washout of the second compartment was parallel to the decay of contractile force. In sharp contrast, when the hearts were reperfused with La-Hepes (5 uM), the pattern of the washout of Ca was characteristic of a two compartment system (Figure 28A). There was no

TABLE XI

The Effect of 5 uM La on the Total Ca Uptake in Ca-depleted Hearts

	Control (5)	La (5 uM) (5)
Total Ca uptake (mEq/kg tissue wet wt.)	3.78 ± 0.34[a]	3.96 ± 0.38
Total ^{45}Ca uptake (cpm x 10^6/kg tissue wet wt.)	77.60 ± 5.60	79.98 ± 9.15

Number in parenthesis indicates number of hearts.

[a] Mean ± S. E.

Figure 27: The washout of Ca and decay of contractile force in Wash II.

Panel A: A typical washout curve, (Wash II), from a heart first depleted of Ca by Wash I and then reperfused with perfusate containing 5.0 mEq Ca/l. The concentrations of Ca measured in the serial samples indicated by filled circles (●) are connected by the least squares best fit line, $[Ca]_T$. The dashed lines indicate the compartments, Ca_{II} and Ca_{III} resolved by graphical analysis. Ca_I was omitted from the figure for clarity.

Panel B: The decay of contractile force during Wash II in the same heart.

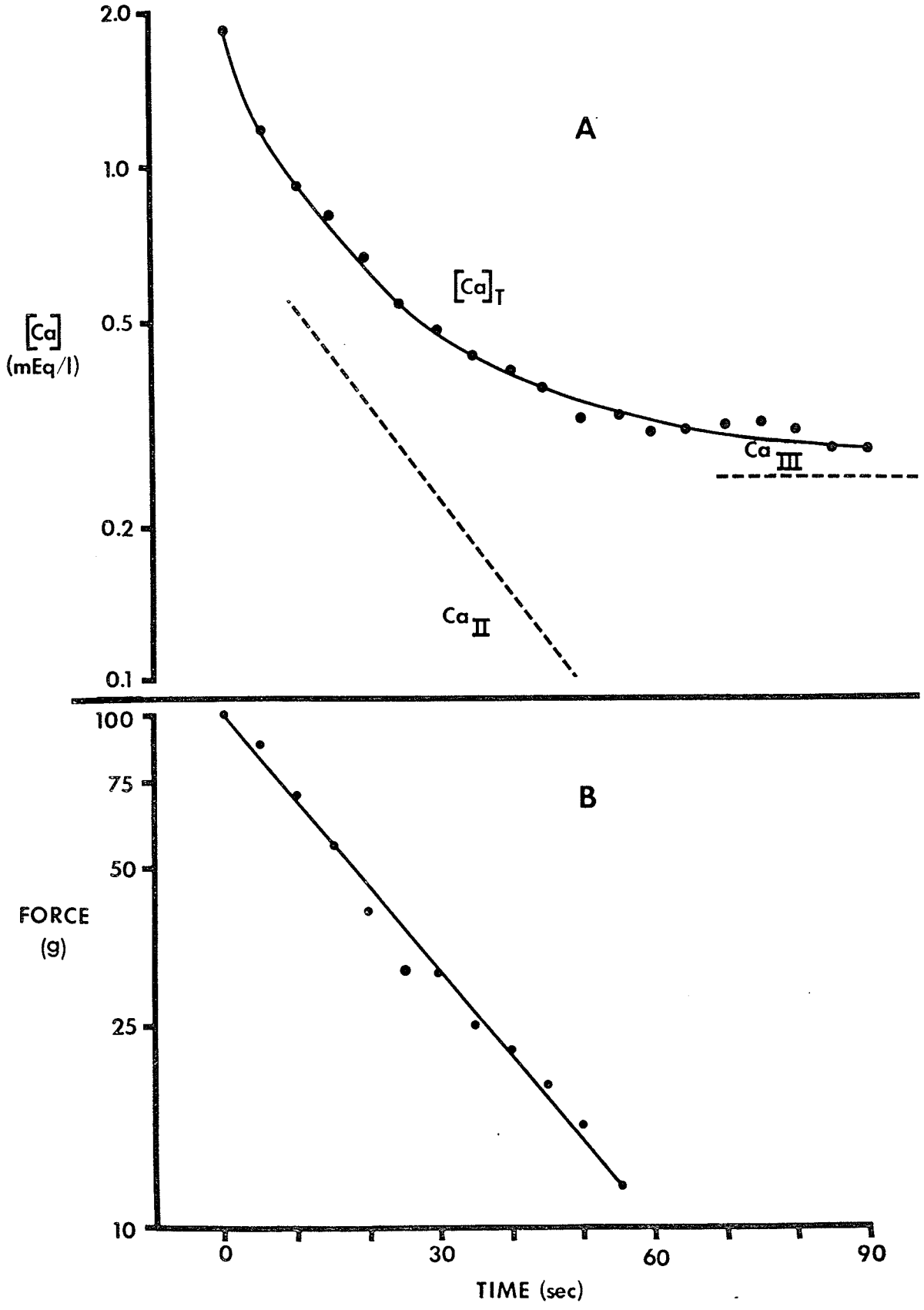
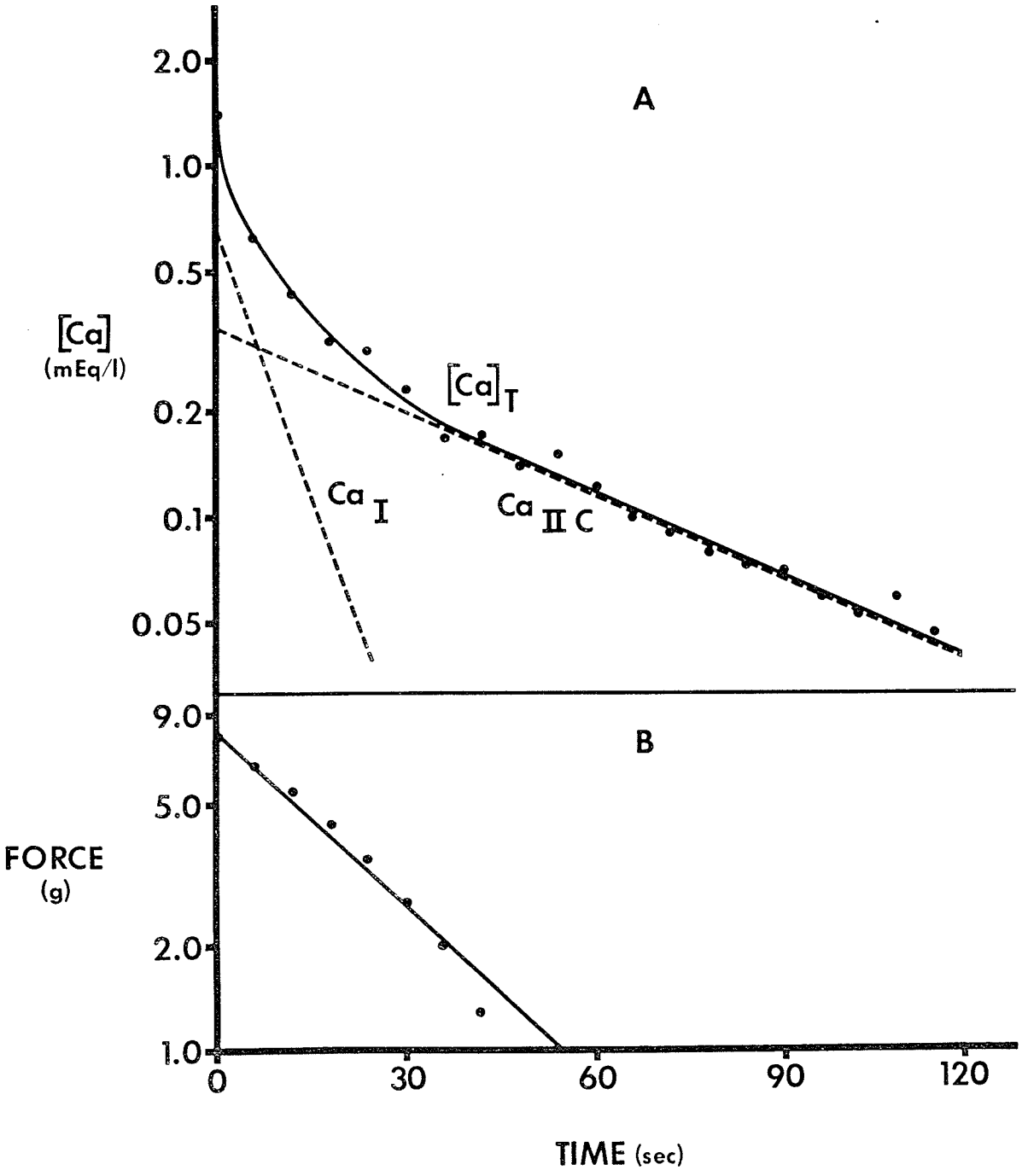

2.0
1.0
0.5
0.2
0.1
[Ca]
(mEq/l)
A
[Ca]T
CaIII
CaII
100
75
50
25
10
FORCE
(g)
B
0
30
60
90
TIME (sec)

Figure 28: The effect of 5 uM on Ca washout and decay of contractile force in Wash II.

Panel A: The washout (Wash II) of Ca from a heart first depleted of Ca by Wash I and then reperfused with perfusate containing 5.0 mEq Ca/l plus 5 uM Ca. The concentrations of Ca measured in the serial samples, indicated by the filled circles (●) are connected by the least squares best fit line, $[Ca]_T$. The dashed lines indicate the two compartments, Ca_I and Ca_{IIC}, resolved by graphical analysis.

Panel B: The decay of contractile force during Wash II in the same heart.


2.0
1.0
0.5
0.2
0.1
0.05
[Ca]
(mEq/l)
A
[Ca]T
Ca I
Ca II C
9.0
5.0
2.0
1.0
FORCE
(g)
B
0
30
60
90
120
TIME (sec)

evidence of a third compartment, Ca_{III}, in the 2-3 min washout. The linear portion of the washout curve, Ca_{IIC}, was not parallel to the decay of contractile force. The mean halftimes for the washout of Ca_{II}, Ca_{IIC} and contractile force from the control hearts and hearts reperfused with La-Hepes (5 uM) are tabulated in Table XII. There was no evidence of a Ca compartment which was washed out at the rate corresponding to the rate of decay of contractile force in the hearts reperfused with La-Hepes. The linear portion of the washout curve, Ca_{IIC}, in the La reperfused hearts had a halftime approximately twice that of the halftime for the washout of Ca_{II} and the decay of contractile force (Table XII). The halftime for the decay of contractile force in the control hearts was not significantly different ($P > .05$) from the decay of contractile force in hearts reperfused with La-Hepes even though there was a contracture during Ca-free Wash II in the La reperfused heart.

The intercepts, $[Ca]_{IIt=0}$ and $[Ca]_{IICt=0}$ measured in Ca-free Wash II for hearts reperfused with Hepes solution and La-Hepes are given in Table XII. The initial concentrations of Ca arising from the second compartment in the washout in hearts restored in the absence or in the presence of La were not significantly different ($P > .05$) (Table XII). However, a significantly greater ($P < .05$) amount of Ca was washed out of the second compartment in hearts reperfused with La-Hepes (Table XII). The Ca remaining in the tissues after the Ca-free Wash II was

TABLE XII

The Effect of La-Hepes (5 uM) Reperfusion on Wash II (Figure 4)

	Control (5)	La (5)
$T_{1/2}$ of Ca_{II} and Ca_{IIC} washout (sec)	22.7 ± 5.3[a]	45.5 ± 3.9[b]
$T_{1/2}$ of decay of contractile force (sec)	21.6 ± 3.9	17.0 ± 2.8
$[Ca]_{IIt=0}$ (mEq/l)	0.85 ± 0.13	0.71 ± 0.15
Ca_{II}, Ca_{IIC} content (mEq Ca/kg tissue wet wt.)	1.31 ± 0.18	2.70 ± 0.16[b]
Residual tissue Ca (mEq Ca/kg tissue wet wt.)	0.75 ± 0.10	0.43 ± 0.05[b]

Number in parenthesis indicates number of hearts.

[a] Mean ± S. E.

[b] Significant difference ($P < .05$) between control and La-Hepes reperfused hearts.

significantly less ($P < .05$) in hearts reperfused with La-Hepes than the control hearts (Table XII).

The pattern of ^{45}Ca washout in Ca-free Wash II was the same as the Ca washout described above. The washout of ^{45}Ca in the La-Hepes reperfused hearts was a two compartment system in contrast to the three compartment system obtained from the control hearts. Again, a significantly greater ($P < .05$) amount of Ca was washed out of the second compartment in hearts reperfused with La-Hepes. Of the total amount of ^{45}Ca taken up by the hearts $16.0 \pm 5.2\%$ was recovered from Ca_{II} in the control hearts. In contrast, more than 50% of the ^{45}Ca taken up was recovered in Ca_{IIC} from hearts reperfused with La-Hepes.

SECTION IV

DISCUSSION

A. Experimental Preparation.

1. Ca-free perfusion.

Electron microscopic studies showed that the morphological structures of the cardiac muscle were normal after perfusion with a Ca-free solution. In other words, there were no gross differences between sections taken from hearts perfused with K-H solution, freshly removed from the animals or after 3 min of Ca-free perfusion. These data are in contrast to those reported by Zimmerman and Hulsmann (1966) and Zimmerman et al. (1967). They introduced the term 'Ca paradox' for the loss of electrical and mechanical activity of the heart as well as for the extensive morphological changes and loss of myocardial cell contents which occurred after perfusion of isolated rat hearts with Ca-free perfusion fluid for 3 min or more, followed by perfusion with the normal Ca containing solution. This phenomenon was postulated to be the result of an excessive influx of Ca into the cells during reperfusion of the Ca containing solution, which resulted in the destruction of large parts of the hearts. Kitten hearts appeared to be more resistant to the 'Ca paradox'. There was no evidence of myocardial damage after Ca-free perfusion. 'Ca paradox' did not occur in the kitten hearts after perfusion with Ca-free solution in that normal contractile force could be restored by reperfusion of hearts with Ca containing perfusion fluid after Ca-free washout (Bailey and Sures, 1971).

There appears to be some species difference in the response of myocardial cells to Ca-free perfusions or perfusion with solutions in which the Ca ion concentration was reduced or removed by the addition of Ca-binding agents. Extensive necrosis of the human myocardium after perfusion with potassium citrate had been reported by Meesen and Poche (1963 - in Zimmerman et al. 1967). Potassium citrate was used to induce cardiac standstill for surgical intervention and necrosis was found in hearts after normal circulation had been resumed. However, in experiments on dogs, no significant structural changes were observed in biopsies taken from the hearts after citrate induced arrest (Lohr et al. 1960 - in Zimmerman et al. 1967). Lee and Visscher (1970) were able to restore rhythmic contractions in rabbit hearts after perfusion with Ca-free perfusate containing normal concentrations of potassium (5 mM) for 12 min. Shine et al. (1971) perfused rabbit papillary muscle preparations with Ca-free perfusate for 7 min without any outward sign of myocardial damage. 'Ca paradox' seems to be related to rat hearts only. Bielecki (1969) showed that the occurence of 'Ca paradox' in rat hearts was influenced by changes in the pH of the perfusion fluid. He showed that perfusion of the heart with Ca-free solution at pH 7.4 for 3 min or more, followed by perfusion with normal Ca containing solution resulted in 'Ca paradox'. In this study we have therefore, limited the duration of Ca-free perfusion to 3 min or less, to prevent any possible

damage to the myocardial cells even though rat and kitten hearts probably behave differently under such circumstances.

Ca-free perfusion was used as a means to relate changes of contractile force with Ca fluxes in the myocardium. One disadvantage to the measurement of tracer Ca flux under steady state conditions of contractility is that it has been virtually impossible to correlate a specific Ca pool in the heart with the maintenance of contractile force. One cannot be certain that a Ca pool is related to the maintenance of contractility in the heart unless changes in Ca flux in a single Ca compartment induced by cardioactive drugs or other interventions are accompanied by simultaneous and parallel changes in contractile force. However, when the Ca steady state in the heart was disturbed by perfusion with a Ca-free K-H solution the washout of a single Ca compartment, which Bailey and Dresel (1968) have called Ca_{II}, was found to be directly related to the decay of contractile force. Ca fluxes measured under the non-steady state condition induced by the Ca-free perfusion are certainly not physiological and little biological significance can be attached other than changes in the parameters for Ca flux. This technique has eliminated or at least minimized back diffusion of Ca into the heart, a factor which may interfere with ^{45}Ca flux measurements made under steady state conditions. Back diffusion or back flux of Ca into the tissue was negligible during washout with Ca-free media since the diffusion

gradient for Ca out of the tissue into the vascular space was infinite and was maintained by constant replenishment of washout fluid.

In the interpretation of the mechanical effects of Ca-free perfusions we have assumed that no important electrical alteration occured to interfere with the kinetics of Ca movement. Electrical activity of the myocardium monitored by bipolar electrodes attached to the left ventricle showed no gross changes during Ca-free perfusions. In 1913, Mines observed that electrical activity of the frog heart persisted in Ca-free solution even when contractile force was abolished. Hoffman and Suckling (1956) observed a slight prolongation of the action potential in dog papillary muscle when the Ca concentration of the medium was reduced to 0.027 mM. In rabbit papillary muscle, Gibbs and Vaughan (1968) showed that the action potential was unaffected after 90 min in Ca-free solution at 32°C despite a decline of tension to less than 1% of control value. Abe and Goto (1964) showed that complete removal of Ca from the medium brought about a slight decrease of resting potential, a slight lowering of plateau and a prolongation of action potential. The decline of contractile force in isolated kitten hearts used in these experiments was continuous and smooth during Ca-free perfusions, which implies that no important conduction disturbances developed.

2. <u>The gas perfused heart</u>.

The gas perfused heart was the main preparation used in this

investigation. Various workers have reported that the isolated heart could be maintained with O_2 or a mixture of O_2 and CO_2 for at least 8 hours (e.g. Magnus, 1902; Burns et al. 1958; Sabiston et al. 1959; Talbert, 1960). Gabel and coworkers (1966) demonstrated that gas perfused hearts (95% O_2 - 5% CO_2 mixture) beat more strongly, perform more work and fail much more slowly than hearts perfused with substrate-free K-H solution. Electron-micrographs of sections of kitten cardiac tissue taken after gas perfusion showed intact sympathetic nerve endings, normal muscle cells and mitochondria. This suggests that the cardiac tissue was not damaged even though the heart was not perfused with a physiological liquid medium. Therefore, the gas perfused heart served as a useful tool because perfusing the heart with gas prior to the washout removed Ca and other substances in the vascular space as a contributing factor in the maintenance of contractile force (Krip et al. 1971). Contractile force during gas perfusion was thus determined entirely by the store of Ca in the tissue and the Ca present in the interstitial spaces. Krip et al. (1971) reported that Ca in the vascular space contributed to a certain percentage of the force developed by the heart, however, the major proportion of the contractile force was maintained by Ca_{II} and the Ca in the interstitial spaces. The removal of vascular Ca during gas perfusion eliminated a source of error and interference in the analysis of the Ca washout curves with respect to the decay of contrac-

tile force during Ca-free washouts. Gas perfusion, by eliminating the liquid dead space, provided convenient and exact starting and ending points for relating changes in contractile force induced by liquid perfusion with changes in Ca fluxes. However, every useful technique is not without disadvantages. The major drawback of gas perfusion is that hearts perfused in this manner are not functioning under physiologic conditions and conclusions drawn from them may be rather misleading. In any event, we believed that the advantages offered by the technique of gas perfusion for studies of ion flux far outweighed the disadvantages.

Our results show that the kinetics of Ca movement during excitation-contraction coupling and the utilization of Ca to activate contraction was different depending upon whether the hearts were perfused with liquid or gas. The rate of decay of contractile force during Ca-free Wash I (Figure 5) in liquid perfused hearts (i.e. washout without a prior period of gas perfusion) was more rapid than the decay of contractile force observed during Ca-free wash after gas perfusion (Bailey and Dresel, 1968; Bailey et al. 1972). However, the rate of decay of contractile force whether in liquid perfused hearts or after gas perfusion was correlated with the washout of a single Ca compartment. Contractile force was partially restored during gas perfusion after being abolished by a brief Ca-free washout following liquid perfusion. Since gas perfusion essentially removed the influence of substances contributing to

the maintenance of contractile force in the vascular space and also prevents further removal from or addition of Ca to the heart, the results suggest that contractile force during gas perfusion in this case was maintained in the absence of interstitial Ca and only by the Ca remaining in the heart after Ca-free Wash I. That is, the return of contractile force could be attributed only to the redistribution of Ca from the various intracellular pools to the Ca pool(s) essential for contraction, Ca_{II}.

B. Ca Kinetics in Heart Muscle.

1. Washout studies.

The gas perfusion technique enabled Bailey and Dresel (1968) to identify a pool of Ca (Ca_{II}) involved in the maintenance of contractile force in the isolated kitten heart. Langer (1965) showed a similar parallelism between the washout of a single Ca compartment he called phase 2 and the decay of contractile force in arterially perfused dog papillary muscle preparation, and suggested that phase 2 was the Ca pool responsible for contraction in the heart. However, there are significant discrepancies between the halftimes for the washout of Ca from phase 2 and the washout of Ca_{II}, and between the Ca capacity of phase 2 and Ca_{II} content. The mean halftime for Ca efflux from Ca_{II} was 21.6 sec. Langer (1965) reported that the halftime for the washout of Ca during Ca-free perfusions from phase 2 was 360 sec and showed that the decay of contractile force had a similar halftime. It should be

noted that in these experiments, the halftime for the washout of Ca from Ca_{II} was comparable to the halftime for the washout of Ca from phase 1, 35 sec. However, later reports from the same laboratory (Shine et al. 1971) show that the washout of Ca from phase 1 was correlated with the decay of contractile force in isolated blood perfused rabbit interventricular septum. But in these more recent experiments the halftime for the washout of Ca from phase 1, $T_{1/2}$ = 72 sec, differed significantly from the washout of Ca_{II}, $T_{1/2}$ = 21.6 sec. No explanation was given for this discrepancy between the halftimes for the washout of phase 1 in his previous study (1965) and those reported in the recent paper (1971). Bailey and coworkers (1968, 1971) suggested that the discrepancy between their results and that of Langer and coworkers was due to the difference in flow rates employed in the experiment. There was about a 5-fold difference between the rate of perfusate flow in our experiments and those of Langer (1965), 4.5 ml/g/min and 1 ml/g/min, respectively. On the other hand, although differences in flow rates may be the explanation for this discrepancy in washout rates, Shine and coworkers (1971) found no difference in rate constants of phases 2 and 3 when flow rate was doubled. The rate constant for phase 1 washout increased only when the perfusate flow was doubled at high Ca concentrations (5-10 mM). At the lower Ca concentrations (5 mEq/l) employed in our experiments the rate constant was independent of the rate of perfusate flow (Shine et al.

1971). It should be noted that even though these rate constants were measured under similar experimental conditions, that is, during Ca-free perfusion, the washouts by Bailey and coworkers (1968, 1971, 1972) were performed after a period of gas perfusion while those obtained by Langer and coworkers were obtained by changing directly from the liquid perfusate containing Ca to the Ca-free perfusate.

In order to compare Langer's data with ours, hearts were washed out with Ca-free K-H solution after 30 min of preperfusion with K-H solution (Wash I, Figure 5). In order to minimize mixing of the perfusates, great care was taken to ensure that the cannula was cleared of liquid before the Ca-free perfusate was introduced. The halftime of decay of contractile force was 9.3 sec and was correlated with the washout of Ca from a single compartment ($T_{1/2}$ = 9.6 sec). The halftime compares favourably with the washout of Ca from Langer's phase 0 ($T_{1/2}$ = 12 sec) which Langer assumed to be vascular Ca. In his experiments, however, the washout of phase 0 was not correlated with the decay of contractile force. Therefore, not only are there discrepancies between our results for Ca efflux and those reported by Langer and coworkers, but the rates of decay of contractile force reported from that laboratory are also different. The halftime for the decay of contractile force in our experiments, 9.3 sec and 21.6 sec after gas perfusion, differed by about an order of magnitude from that by Shine et al. (1971), 72 sec and Langer (1965), 360 sec. This

discrepancy between the rate of washout of Ca and the decay of contractile force during Ca-free perfusions is perplexing. The slower rate of decay of contractile force during Ca-free perfusions reported by Langer (1965) and Shine et al. (1971) may be accounted for by the mixture of Ca-free perfusate with the Ca containing perfusate, since they did not mention if precautions were taken to avoid mixing of the perfusion fluids. We observed approximately the same halftime for the decay of contractile force as Langer (1965) in our experiments if the cannula to which the aorta was attached was not cleared of the Ca containing perfusate before Ca-free solution was introduced.

In any event, Saari and Johnson (1971) reported similar Ca washout kinetics in rabbit hearts under very similar conditions. However, they observed a double exponential decay of contractile force with a halftime for the faster component similar to ours, 14 sec. Two phases of decay of contractile force have also been reported by other workers (Lüllman and Holland, 1962; de Caro, 1967). Teiger and Farah (1967) reported a mean halftime of 114 sec for the washout of a Ca compartment they postulated to be associated with contractility in isolated rabbit atria. However, they have measured ^{45}Ca efflux into Ca containing solution, that is under steady state conditions. Sabatini-Smith and Holland (1967) reported a single exponential decay of contractile force for rabbit atria when transferred from Ringers solution containing 2.4 mM Ca to

0.24 mM Ca solution, with a halftime of 51 sec. Thus, there are wide differences in the rates of Ca washout and decay of contractile force reported in the literature. We are unable to offer an explanation for these differences except to suggest that they may be due to different experimental conditions employed.

2. Uptake studies.

The uptake of Ca was determined in hearts initially depleted of Ca by Ca-free perfusion. Reperfusion of hearts with K-H solution after Ca-free perfusion during which contractile force was abolished restored contractile force. The initial restoration of contractile force approximated a linear function of time and after 90 sec reached a steady level. The extraction of Ca from the perfusate followed a double exponential function. The restoration of contractile force was correlated with the accumulation of Ca in a pool in the heart corresponding to the slower phase of Ca uptake, Ca_2. Bailey and Sures (1971) reported similar results for the restoration of contractile force, but they detected only a monoexponential extraction of Ca from the perfusate. The accumulation of Ca by the heart during the reperfusion was correlated with the restoration of contractile force. They concluded that the monoexponential rate of removal of Ca from the heart probably represents the sum of Ca uptake into Ca_I and Ca_{II}. This discrepancy could be explained on the basis of a better method of Ca determination and a non-biased method of compart-

mental analysis by the computer program as compared to the manual method of analysis. In any event, our present results are qualitatively in agreement with those reported by several workers. Langer and Brady (1963) reported that there were at least two kinetically distinct systems in their ^{45}Ca loading experiments. The halftimes were 6 min for the fast phase which they suggested to be involved in the contractile process and 70 min for the slow phase. These values were considerably slower than the rates we obtained, the mean halftimes for Ca_1 and Ca_2 were 5.6 sec and 46.2 sec respectively. These differences may be accounted for by the fact that our experiments were conducted under non steady state conditions of Ca flux and contractility. Langer and Brady (1963) in contrast, determined ^{45}Ca uptake into dog papillary muscle which was not initially depleted of Ca with a rate of perfusate flow of only 1 ml/g/min while in our experiments the flow rate was 4.5 ml/g/min. Similarly, Grossman and Furchgott (1964b), Teiger and Farah (1967) and Wasserman and Holland (1971) reported at least two phases of Ca influx in cardiac muscle. Like Langer and Brady, they related their fast phase of Ca exchange to the initiation and maintenance of contractile activity in the heart, but they neither upset the Ca steady state of the heart by initially depleting the tissue of Ca, nor was there a change in contractile force during ^{45}Ca loading. Niedergerke et al. (1969a) also reported two phases of Ca uptake into heart cells under steady state

conditions and suggested that Ca movement occurs in two compartments of the myocardium; one compartment provides space for the rapid uptake and release of Ca, while the other contain sites for the more slowly exchanging fraction of this ion. However, they did not correlate their findings with contractile activity.

The restoration of contractile force was linearly correlated with the accumulation of Ca in Ca_2 which suggests that Ca_2 was directly involved in the contractile process. The relationship between the accumulation of Ca in Ca_1 and the restoration of contractile force was not linear.

However, because of the limitations of kinetic analysis in localizing the various pools of Ca to specific anatomical sites in the myocardium, it was not possible to conclude with any certainty the location of Ca_1 and Ca_2. Ca_1 may represent Ca taken up into the extracellular space. If Ca_1 represents the Ca taken up into the extracellular space, then Ca_2 is most likely intracellular Ca because of the longer halftime for the approach to the steady state. The halftime for the approach of Ca_2 uptake to the steady state was similar to that obtained with ^3H-Inulin. It should be emphasized that comparisons between ^3H- Inulin and Ca uptake and washout can be misleading since Ca is known to be bound to the cell membrane and cellular proteins in or on the cells (Nanninga, 1961), while binding of inulin to cellular structures is unknown (D. Ilse,

Personal Communications). Moreover, the rates of diffusion across the capillary endothelium of inulin and Ca may differ considerably (Pappenheimer, 1953). Thus, it may be fortuitous that the inulin spaces obtained in these experiments are comparable to those measured by other investigators (e.g. Bleechen and Fisher, 1954; Cotlove, 1954; Page, 1962). That is, because of the large size of the inulin molecule it is difficult to believe that it had reached equilibrium after only 2 to 3 min of perfusion while other workers measured inulin spaces in cardiac muscle after 30 min equilibration. Therefore, although inulin and Ca uptake kinetics into Ca_1 were similar, these results cannot be considered as conclusive that Ca_1 represents extracellular Ca. The concentration of Ca in Ca_1 if distributed in the interstitial water was calculated to be 4.18 mEq/l tissue water which approximated the concentration of Ca in the perfusate (5.0 mEq/l). Although this strongly indicates that Ca_1 probably represents free Ca in the interstitial space and not Ca bound to superficial binding sites, later evidence suggests that Ca_1 does not represent free interstitial Ca alone (see below).

Another possibility is that Ca_1 represents carrier-mediated uptake of Ca and Ca_2 uptake is a slower, passive diffusion of Ca into the myocardial cell down the electrochemical gradient. This possibility is discussed below.

C. The Uncoupling of Excitation from Contraction.

1. Ni.

The results show that Ni abolished contractile force in cardiac muscle without affecting the electrical activity of the muscle cell. The parallel shift in the dose-response curves caused by Ni indicates that this effect of Ni probably occured at some step in the process coupling excitation to contraction, perhaps by direct competition with Ca for sites to activate the contractile apparatus. These results support the hypothesis proposed by Kaufmann and Fleckenstein (1965) who observed that the inhibition of contractility of isolated guinea pig papillary muscles by Ni can be completely overcome by an excess of Ca. Our experiments with isolated perfused cat hearts yield further evidence for a competition between Ca and Ni for sites to activate the contractile mechanism. First, the effect of Ni on contractile force was similar in hearts perfused with Ni-Krebs after gas perfusion and in hearts not gas perfused. Second, the exchange of Ca between the vascular space and the myocardium was not affected by the presence of Ni in the perfusion fluid. Third, the return of contractile force during gas perfusion following Ni-Krebs perfusion was related directly to a simultaneous decrease of Ni content in a pool we have called Ni_{II}. We have assumed that Ni_{II} and Ca_{II} represent Ca and Ni bound to the same morphological location because of the similar kinetic behaviour of these ions during washout. Therefore, since

gas perfusion effectively removed the vascular compartment as a source (or a sink) for substances which affect the contractile process, we have concluded that contractile force was restored because the Ni in Ni_{II} was redistributed to other tissue compartments during gas perfusion.

Bailey and Sures (1971) have reported that an increase in the quantity of extracellular Ca utilized for contraction is associated with the positive inotropic response to ouabain. If Ni inhibits contractile force by competing with Ca, then one might expect that in the presence of an effective concentration of ouabain, Ni would have less inhibitory effect on contractile force than in the absence of the drug. Our results show that ouabain partially antagonized the effect of Ni on contractile force in normal extracellular Ca (5 mEq/l). Ouabain increased Ca_{II} content, which supports the proposed mechanism for the effect of Ni on contractile force. However, Bailey and Sures (1971) did not detect an increase in Ca_{II} content in their ouabain treated hearts as compared to their control hearts. They reported a Ca_{II} content in the ouabain treated hearts similar to that measured in these experiments, but their control values were higher. No explanation can be given for this discrepancy in the effect of ouabain on Ca_{II} content.

Nayler (1964) suggested that Ni and several other divalent cations release Ca from a small surface located component of the Ca exchange system, and unlike its effect on skeletal muscle Ni does not release

bound cellular Ca (Frank, 1962). If Ni released superficial Ca to inhibit contraction of the heart, a decrease in Ca_{II} content should have been detected since Ca_{II} is apparently a superficial Ca pool (Bailey and Dresel, 1968; Bailey and Sures, 1971; Bailey et al. 1972). In any event, Ca_{II} content measured when contractile force was completely abolished by Ni was not different from Ca_{II} content measured when contractile force was restored after 30 min of gas perfusion. In contrast to the mechanism suggested by Nayler (1964), we observed that only tightly bound tissue Ca was decreased after Ni-Krebs perfusion. One explanation for the discrepancy between our results and those of Nayler simply may be the difference in experimental techniques and in the identification of the Ca pools. That is, the pool which Nayler identified as tightly bound cellular Ca may in fact not be related to what we identify as the residual tissue Ca, and the surface located pool may not be analogous to Ca_{II}.

Subcellular fractionation studies showed that a greater concentration of Ni was present in the mitochondrial fraction in hearts which were removed when contractile force was completely inhibited after Ni perfusion. This suggests that the mitochondria may be the morphological location of Ca_{II}. Several investigators have suggested, on the one hand, that mitochondrial Ca may be involved in the process of excitation-contraction coupling based on the fact that mitochondria can accumulate

and release Ca (Fanburg, 1964; Haugaard et al. 1969; Dhalla, 1969, 1970). Patriarca and Carafoli (1968) reported that a large percentage of the ^{45}Ca found in the heart after administration of the isotope in vivo was present in the mitochondria. Horn et al. (1971) showed that the mitochondria contain most of the Ca in the intact heart and that the exchange of the mitochondrial Ca with the extracellular Ca was extremely rapid. Ueba and coworkers (1971) showed that there was a striking increase in mitochondrial Ca as extracellular Ca was increased from 0.59 to 9.0 mM while microsomal Ca was little affected. However, active tension was not carefully determined or correlated with mitochondrial Ca content. In any event, these observations support the view that the mitochondrial Ca play a significant role in the movements of Ca occurring during the cycle of contraction and relaxation of the myocardium. This would be in agreement with our suggestion that Ca_{II} may be mitochondrial Ca. On the other hand, we find evidence which contradicts this possibility. The in vitro studies with mitochondria can be criticized in that clear separation of the subcellular fractions are difficult and mitochondrial preparations are contaminated with the fragments of sarcoplasmic reticulum, which could invalidate the conclusions (Dhalla et al. 1970). Secondly, Ca is known to redistribute during homogenization of the tissue, and Ca is known to be taken up by the mitochondria during the isolation procedure (Horn et al. 1971; Kubler and Shinebourne, 1971).

This may account for the additional mitochondrial Ca measured in the hearts perfused with high Ca reported by Patriarca and Carafoli (1968) and Ueba et al. (1971). Tomlinson et al. (1971) found that changes in mitochondrial Ca were not related to changes in cardiac contractility. Kubler and Shinebourne (1971) concluded that the uptake and release of Ca by the mitochondria are not related to the contraction-relaxation process in the heart.

It is possible that the mitochondrial fractions obtained in this investigation were contaminated with other subcellular structures. However, our isolation procedure was identical for hearts treated with Ni and gas perfused for either 5 sec or 30 min, thus the changes in Ni concentrations measured under these conditions may be valid since contamination with other subcellular structures would be similar for all the hearts. If mitochondria do take part in the regulation of intracellular Ca concentration at the site of the contractile proteins, extrusion of Ca from mitochondria must occur and be regulated by changes in the appropriate cellular components. Little is known about the mechanism of Ca efflux from mitochondria. Therefore, further investigation and better controlled subcellular fractionation studies of these processes are essential to even postulate that the Ca which regulates contractile force in the kitten heart originates from the mitochondria.

It is conceivable that Ni inhibited contractile force by acting as

a metabolic poison and not by competing with Ca for sites to activate the contractile elements. If this were the case, we would expect the decay of contractile force to be very much slower than we have shown. Clark et al. (1932) reported that frog ventricle continued to contract for approximately an hour under anaerobic conditions. Clark et al. (1937) also showed that normal contractility was maintained in frog ventricles in the presence of iodoacetate for as long as 2 hours in the presence of oxygen. Webb (1950) reported that cyanide (0.01 - 2.0 mM) produced a temporary stimulation before depression of contractile amplitude in rabbit atria. We have also shown that DNP in concentrations known to block ATP formation had a much slower rate of depression of contractile force than the decay seen with Ni. When the hearts were deprived of oxygen, the heart continued to contract with less force for more than 30 min. These results suggest that Ni probably does not affect contractile force by acting as a metabolic poison uncoupling oxidative phosphorylation in the mitochondria.

It is well known that the force of muscular contraction in cardiac muscle is related directly to the amount of ionized intracellular Ca available to activate the contractile elements (e.g. Niedergerke, 1955, 1956). Recent evidence indicated that Ca forms a complex with troponin, which removes the inhibition of myosin ATPase to initiate contraction (Ebashi and Endo, 1968). Thus, on the one hand, Ni may have inhibited

contractile force by blocking the formation of the Ca-troponin complex or may have displaced Ca from the Ca-troponin binding sites, thereby inhibiting contraction. However, Fuchs et al. (1970) have shown that Ni and other divalent cations do not affect Ca binding on skeletal muscle troponin. Although troponin obtained from skeletal and cardiac muscle has similar physical properties (Katz, 1970), it is not certain if these similarities extend to the binding of Ca by cardiac muscle troponin. On the other hand, there have been reports that the Ca bound to troponin is not the regulator of the contraction-relaxation cycle but is the Ca bound to myosin which initiates the contractile process (Dancker, 1970; Drabikowski, 1970). Thus, Ni could inhibit the binding of Ca to myosin or release the Ca bound to myosin and inhibit contraction.

Voegtlin (1915) studied the mechanism of the toxic action of the heavy metals on isolated hearts, and suggested that the heavy metals, including Ni, produce their toxic effect by affecting the permeability of the cells of the heart. However, in these early experiments, no measurement of permeability was made. If Ni had affected permeability of the cell membrane, this was not reflected as a change in the kinetics of Ca exchange between the vascular space and the myocardium. Ni could have affected the movement of Ca from Ca_{II} to the contractile elements, but we were unable to detect direct effects on Ca movement with our present method. If a change had occured, one would suspect that the

rate of decay of contractile force and rate of washout of Ca_{II} would have differed in the Ni perfused hearts since the Ca movement from Ca_{II} to the contractile mechanism and out of the cell has been shown to be a unidirectional pathway (Bailey et al. 1971).

We have concluded therefore that Ni inhibits contractile force only when it is present in a pool similar kinetically to a Ca pool necessary for contraction, Ca_{II}. Ni apparently competes with Ca at some step in the excitation-contraction process subsequent to its release from Ca_{II}. Perhaps the competition occurs during the interaction of divalent cations in the troponin-tropomyosin complex, and actin-myosin interaction. This effect of Ni is reversed either by removing Ni from Ca_{II} by increasing extracellular Ca which indirectly increases the Ca content of Ca_{II}, or by increasing the Ca content in this pool by pretreatment with ouabain.

2. La.

La may have interrupted the process coupling excitation from contraction by first, interfering with Ca for the activation of the contractile process as observed with Ni. However, La (25-500 uM) abolished contractile force in liquid perfused kitten hearts without affecting the electrical activity of the muscle (Takenaka and Yumoto, 1968; Sanborn and Langer, 1970), and had no effect on contractile force in hearts preperfused with gas. These facts coupled with the observation that La does not enter the intracellular space as shown by the electron-micro-

graphs and also as reported by Laszlo et al. (1952) and Revel and Karnorvky (1967) suggests that La did not interfere with Ca for activation of the contractile proteins as observed with Ni.

Secondly, La may have displaced Ca from Ca_{II}, known to be involved directly in the maintenance of contractile force. Sanborn and Langer (1970) concluded that La (5-40 uM) displaced Ca from a pool, phase 2, which they suggest represents 'contractile dependent Ca'. However, their results were not conclusive since 30-40 uM La reduced tension by 81% - 96%, but less than two-thirds of phase 2 Ca was displaced. They reconciled this conflict by assuming that phase 2 was composed of two or more morphologically distinct but kinetically unresolvable fractions. Later, Shine et al. (1971) suggested that another pool of Ca, phase 1, was responsible for the maintenance of contractile force while phase 2 represents a Ca storage pool. However, Sanborn and Langer (1970) reported that La did not displace Ca in phase 1. Our results show that La did not displace Ca from Ca_{II}, and subsequent washout of La treated hearts showed that Ca_{II} content was, in fact, significantly greater than that obtained in control hearts. Again, we were not able to detect a significant increase in Ca efflux when Ca containing perfusate was changed to La-Hepes solution. This suggests that either La did not displace Ca or that our experimental technique was too gross to observe small changes in Ca efflux. The effect of La (25-500 uM)

on contractile force was very rapid ($T_{1/2}$ < 10 sec) and since we were collecting the effluent at 6 sec intervals, more transient effects on Ca flux may not have been detected.

Thirdly, La may have blocked the uptake of Ca into the cell, and thereby abolished contractile force as has been reported by other investigators for smooth and skeletal muscle (e.g. van Breemen, 1969; van Breemen and McNaughton, 1970; Weiss, 1970). When the hearts were reperfused with La-Hepes (5 uM) after Ca-free perfusion, contractile force was not restored. The extraction of Ca from the perfusate in the presence of La was a monoexponential function in sharp contrast to the double exponential function for extraction of Ca in the absence of La (control hearts). The rate constant represented by the halftime for the approach of Ca flux to the steady state was not significantly different from the halftime of Ca_2 uptake in control hearts. Thus, we can conclude that La blocked the uptake of Ca in Ca_1. It is clear then that when Ca uptake into Ca_1 was blocked, contractile force was not restored, even though there was significantly more Ca taken up by Ca_2. The results strongly suggest that perhaps the Ca contained in Ca_1 plays an essential role in the activation of contraction even though the accumulation of Ca in this pool was not linearly correlated with the restoration of contractile force. This implies that the Ca which entered Ca_1 may 'trigger' or in some way cause the release of Ca in Ca_2 to activate the contractile

process. At present, we have no evidence for or against the possibility that Ca_1 directly interacts with the contractile mechanism.

Since one component of Ca uptake could be selectively blocked by La, one is tempted to speculate on the possibility that there are at least two processes regulating the movement of Ca in the heart cells. The first mode of Ca transport through the membrane may be by a carrier mechanism, or via the fixed negative charges on the membrane. Van Breemen and van Breemen (1969) have shown that the fixed negative charges of the phosphate and carboxyl groups on artificial phospholipid membrane facilitate Ca transport and that this transport is more rapid than occurs by simple diffusion. La blocks this facilitated transport of Ca in artificial membranes. According to van Breemen and van Breemen (1969), La is probably bound more tightly to the fixed negative charges on the membrane and thus prevents the binding of Ca to these sites. It is thus conceivable that the carriers may be the fixed negative charges and La blocked this carrier-mediated transport of Ca represented by Ca_1 uptake. Reuter and Beeler (1969) reported that there is a significant Ca current during the plateau phase of the action potential in ventricular muscle fibres. Sanborn and Langer (1970) reported that the action potential of ventricular muscle was not affected by La in quantities sufficient to block contractile force. Thus, on the one hand, the pathway for Ca movement not affected by La, i.e. into Ca_2, may be

the major carrier of Ca current. On the other hand, the pathway for Ca transport blocked by La is probably mediated by a carrier which neutralized the positive charges of the Ca ion, thus under normal conditions does not contribute significantly to the Ca current during the action potential. In addition, the electron-micrographs showed that La was bound on the outer surface of the membrane and by binding to these sites may have inhibited Ca_1 uptake, and consequently the restoration of contractile force. However, the second pathway of Ca transport through the membrane, that is, the Ca which enters during Ca_2 may simply be movement of Ca down its electrochemical gradient as occurs in skeletal muscle (Gilbert and Fenn, 1957) and nerve (Hodgkin and Keynes, 1957). This pathway for Ca movement was also suggested by Niedergerke (1963a) to exist in the frog cardiac muscle. The possibility that the second pathway of Ca transport may be carrier mediated as well, but by carriers which are not sensitive to La blockade cannot be disregarded.

Our results showing that Ca entered the muscle cells in the presence of La are at variance with the reports on the action of La by all other investigators. Van Breemen and coworkers (1969, 1970) have shown that La blocked the Ca transport through an artificial phospholipid cholesterol membrane and through smooth muscle cell membranes and has concluded that La completely blocked the influx of Ca into cells. Weiss and coworker (1969, 1970) concluded that La prevented the uptake of

Ca to various cellular sites apart from its effect on the superficial Ca binding sites in smooth and skeletal muscle. Later Miledi (1971) indicated that La prevented inward movement of Ca in nerve terminals. However, larger concentrations of La (0.5 - 5mM) were used in these studies than were used in our experiments. We found a specific blockade of Ca uptake into Ca_I with 5 uM La. Higher doses of La (50 - 500 uM) or prolonged perfusion with 5 uM of La may have multiple effects on ion flux and the secondary effects of La may have masked the interpretation of the results obtained by these investigators. However, our results agree in some respects with the studies by these investigators in that La probably blocked one of the pathways of Ca movement across the cell membrane, and that is probably the carrier-mediated transport via the binding sites on the membrane. Palmer and van Breemen (1970) have also suggested that La blocked the inward movement of Ca in cardiac muscle cells, thereby inhibiting contractile force.

Further evidence suggesting that Ca was taken into the cells in the presence of La was observed in Wash II (Figure 4). If Ca was taken up only into the extracellular space, the washout would contain small quantities of Ca, and would be very rapid. However, we observed a slower efflux of Ca from hearts reperfused in the presence of La as compared to the efflux of Ca_{II} in the control hearts. This slow efflux of Ca from the heart in the washout indicated that Ca must have entered

the cell during the reperfusion. Since one of the carrier-mediated forms of Ca transport was probably blocked by La, the efflux of Ca out of the cell would also be inhibited if it is by the same carrier mechanism. A carrier-mediated Ca exchange system and a carrier-mediated efflux of Ca has been shown to exist in cardiac muscle by Reuter and Seitz (1968) and Reuter (1970). Whether these systems are related to the carrier mechanism blocked by La is open to question. Thus, the slower efflux of Ca measured in the presence of La may simply reflect a passive movement of Ca down the infinite concentration gradient during Ca-free perfusion. Ca does not diffuse passively out of the cell under physiological conditions because the electrochemical gradient favours the movement of Ca into the cell. Because of this, Niedergerke (1963a) postulated an active efflux of Ca from the cell. However, in our experiments, a passive Ca efflux from the cell is conceivable since we were measuring Ca efflux into Ca-free perfusate which may have resulted in Ca diffusion down the infinite concentration gradient. This gradient was maintained by continuous renewal of Ca-free perfusate through the heart. Alternatively, La could have altered the steric configuration of the membrane and changed the permeability of the membrane to Ca ions (D. Ilse, Personal Communication). These changes may be reflected in the different washout kinetics for Ca in the hearts treated with La.

In summary, our results show that La abolished contractile force

by preventing the uptake of Ca into a superficial site which is involved in the process coupling excitation to contraction. La apparently blocked 'trigger Ca' thus blocking the release of Ca in Ca_{II} to activate the contractile elements. By binding to the negative sites on the cell membranes, La probably altered the steric configuration of the membrane which would account for the different washout kinetics for Ca in hearts treated with La.

D. A Hypothetical Model for Ca Involvement in Excitation - Contraction Coupling.

The present observations suggest that the process coupling excitation to contraction in cardiac muscle is very similar to that of skeletal muscle. In his review, Sandow (1965) concluded that although excitation of skeletal muscle is accompanied by an enhanced uptake of Ca, contraction in skeletal muscle is not activated by the particular Ca which enters the muscle in association or as a direct result of the excitation. He concluded that an internal release and translocation of ionized Ca almost certainly provides the basis for activation of contraction in skeletal muscle. The Ca causing contraction was released from the Ca-loaded membraneous sacs, the terminal cisternae of the triads (a specialized part of sarcoplasmic reticulum) possibly in response to the increase influx of Ca associated with the excitatory stimulus. Bianchi and Bolton (1967) introduced the term 'trigger calcium' for that Ca which enabled the terminal cisternae to release stored Ca to act as activator Ca for

contraction in skeletal muscle. According to this hypothesis, 'trigger Ca' is located in the T-tubules and is released during excitation or during membrane depolarization.

Though mammalian ventricles contain more sarcoplasmic reticulum than amphibian heart, Fawcett and McNutt (1969) have pointed out that the sarcolemmal cisternae are far less 'capacious' and are apposed to the sarcolemmal membrane to a much smaller extent in mammalian myocardium than in skeletal muscle. The T-tubules of cardiac muscle are fewer but larger than those of skeletal muscle. The effect of various divalent ions and drugs on excitation-contraction coupling in skeletal and cardiac muscle are different. On the basis of these observations, various investigators (Winegrad, 1961; Niedergerke, 1963a, 1963b; Nayler, 1964; Langer, 1968) have postulated a different process for the participation of Ca in coupling excitation to contraction in cardiac muscle. The studies of Winegrad (1961) and Niedergerke (1963a) indicated that activation of contraction in cardiac muscle directly involves and is dependent upon those Ca ions which enter the muscle during or as an immediate result of excitation. Niedergerke (1963a) suggested that Ca from the extracellular space traversed the cell membrane in combination with a carrier, CaR, and was then released to activate the contractile process. He defined the final pool as 'activator Ca' and has postulated that an equilibrium exists between this pool and CaR, the Ca bound to carriers

and to the inner surface of the cell membrane. After interaction with the contractile proteins, 'activator Ca' is then taken up by a second Ca pool to initiate the relaxation phase of the cycle, and is then expelled from the cell, probably by an active process since the electrochemical gradient favours the accumulation of Ca in the cell. Since then, many investigators (e.g. Nayler, 1967; Langer and Brady, 1963; Reuter and Beeler, 1969; Langer and Serena, 1970; Manring and Hollander, 1971; Bailey et al. 1972) have substantiated this hypothesis.

On the basis of Niedergerke's hypothesis (1963a), the Ca which activates the contractile process comes directly from the extracellular space. He postulated that Ca is transported across the cell membrane on excitation as CaR. Once inside the cell, Ca is freed from its carrier and goes on to activate the contractile process. More recently, however, Chapman and Niedergerke (1970a, b) suggested that a second pool which accumulates Ca after interaction with the contractile proteins plays a significant role in activation of the contractile mechanism. The second Ca pool was assumed to be a storage pool in the previous model (Niedergerke, 1963a). According to their hypothesis, the increase in tension development in response to an increase in external Ca concentration is brought about by the co-operative action of two pools of Ca inside the heart cells. The first pool initiates contraction and the second pool maintains the tension developed. However, we observed that at least

two kinetically distinct pools of Ca are essential to couple excitation to contraction in cardiac muscle. Ca_1 may be identical to the pool Niedergerke (1963a) originally defined as CaR, and may 'trigger' the release of Ca from an intracellular pool of Ca probably Ca_{II} to activate contraction. Ca_1 may or may not initiate contraction while Ca_{II} is essential to activate the contractile process. Thus, there is a discrepancy between our results and the model proposed by Niedergerke (1963a) and also by Chapman and Niedergerke (1970a, b) for Ca movement during contraction in the heart. Perhaps, the difference resides in the fact that in the earlier studies Ca influx was investigated in tissues not previously depleted of Ca and therefore changes in contractile force could not be correlated with changes in Ca influx. In addition, Ca influx was measured over 20 to 30 min while we have found that 2 to 3 min is sufficient time for all of the Ca involved in contraction to turnover. The restoration of contractile force is rapid and if transient changes in Ca flux are not detected then the interpretation of the results may be misleading.

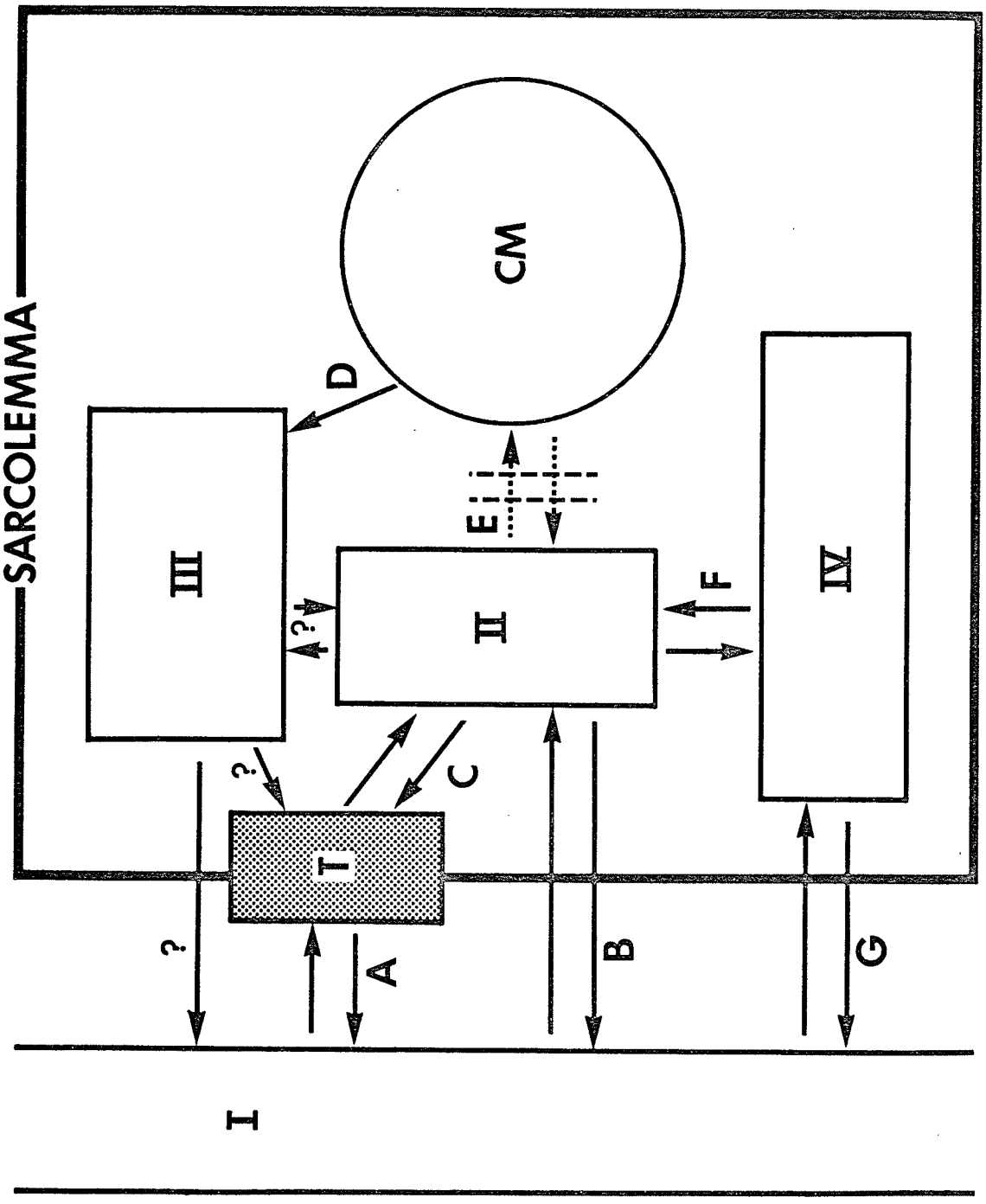
A schematic diagram of a model for the movement and distribution of Ca in coupling excitation to contraction is shown in Figure 29. According to this model, depolarization of the sarcolemma causes an inward movement and release of Ca from site T (Ca_1) which 'triggers' the release of Ca from an internal Ca pool (Ca_{II}). The released Ca,

Figure 29: A schematic diagram of the hypothetical model of 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 washouts described in text.

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.

 probable site of action of Ni.

 probable site of action of La.


SARCOLEMMA
CM
D
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I

'activator Ca', then activates the contractile elements to give rise to contraction. 'Activator Ca' is then probably actively accumulated by Ca_{III} to initiate relaxation. Ca_{III} may represent the sarcoplasmic reticulum which is known to accumulate Ca actively and to lower the intracellular Ca concentration after contraction (Martonisi and Feretos, 1964; Weber et al. 1963). The Ca accumulated in Ca_{III} could then either be actively pumped out of the cells or returned to Ca_{II}.

The inward movement of Ca from the extracellular space measured during excitation may be visualized as replenishing the superficial site T and also Ca_{II} either via T and arrow C, or by the alternative pathway of transport indicated by arrow B (Winegrad and Shanes, 1962; Niedergerke, 1963b; Reuter and Beeler, 1969). The filling of Ca_{III}, assuming it is sarcoplasmic reticulum, may have occurred by active accumulation of Ca after it had participated in contraction, that is, via arrow D, and is only after Ca_{II} content has been fully restored (Bailey et al. 1972). The active efflux of Ca out of the cell from Ca_{III} could be through site T, probably visualized as the Ca-Ca exchange and Na-Ca exchange postulated by Reuter and Seitz (1968) and Reuter (1970), or it may be accumulated by Ca_{II} and then pumped out of the cells by the same mechanism. There is little or no evidence for or against the direct exchange of Ca between Ca_{II} and Ca_{III}.

The residual Ca pool, Ca_{IV}, represents tightly bound tissue Ca,

and probably exchanges directly with the vascular space via arrows G or from Ca_{II} via arrow F (Bailey and Dresel, 1968; Bailey et al. 1972). Which of the two sets of exchange, F or G, is operating at any given time or whether both processes occur is questionable. Bailey et al. (1972) have shown that there is no exchange between the residual tissue pool and Ca_{III}. They have shown that Ca_{II} and the residual pool, Ca_{IV}, could be labelled simultaneously with ^{45}Ca, but without labelling Ca_{III}. Therefore, Ca_{III} and Ca_{IV} represent entirely different Ca pools in the heart which do not exchange with each other. Thus, these observations show that the previous assumption that Ca_{III} represents tightly bound tissue Ca (Bailey and Dresel, 1968) is in error.

The movement and distribution of Ca so far discussed is only operational when the hearts are perfused with a physiological solution and functioning under relatively normal physiological conditions. However, when the hearts were gas perfused, the process coupling excitation to contraction is different from the conditions operating under liquid perfusion. Ca probably exchanges between Ca_{II} and the contractile elements during gas perfusion as indicated by the broken arrows E. Ni blocked the interaction of Ca with the contractile elements, therefore the effect of Ni on contractile force is the same in either liquid perfused or gas perfused hearts.

Bailey et al. (1972) showed that in hearts in which the Ca in

Ca_{II} was specifically restored without restoring Ca_{III}, the Ca from Ca_{II} did not move to Ca_{III} during 30 min of gas perfusion. That is, once Ca had entered Ca_{II} in hearts in which only Ca_{II} was restored, it remained there during the subsequent gas perfusion and contractile force was maintained. They concluded that Ca was released from Ca_{II} to participate in contraction and was again accumulated selectively by Ca_{II} during the relaxation phase of the contractile cycle. Thus, the Ca released from Ca_{II} is not redistributed to the other Ca pools of the heart. These observations are consistent with earlier reports that during gas perfusion Ca slowly redistributed between Ca_{II} and the other tissue pools including Ca_{III} only when Ca_{II} content was 0.911 ± 0.17 mEq/kg tissue wet weight or greater (Bailey and Dresel, 1968; Bailey and Krip, 1971). Ca_{III} probably acts as a storage region or a buffer compartment which accumulates the excess Ca in Ca_{II} and in the vicinity of the contractile elements, and may in fact represent the sarcoplasmic reticulum.

It is of interest to note that the process governing the release of Ca from Ca_{II} to activate the contractile elements is also different in gas perfused hearts. This was evident in the case when the hearts were perfused with Ca-free perfusate immediately after perfusion with Ca containing solution. The rate of decay of contractile force during the Ca-free perfusion was very rapid ($T_{1/2} < 10$ sec) and was correlated with the rate of washout of a Ca pool and with the rate of washout of ^{3}H-Inulin.

The rate of decay of contractile force during Ca-free perfusion after gas perfusion was much slower and correlated with the rate of washout of Ca_{II} (Bailey and Dresel, 1968; Krip and Bailey, 1971; Bailey et al. 1972). These results suggest that the maintenance of contractile force during liquid perfusion is dependent on a more superficial pool of Ca, perhaps located on the external surface membrane sites, while maintenance of contractile force during gas perfusion is dependent on Ca_{II} which is assumed to be intracellular or situated near the intracellular surface based on the longer halftime of washout ($T_{1/2}$ > 20 sec). Another difference between gas and liquid perfusion is shown by the fact that contractile force gradually returned to 50% of control levels during gas perfusion subsequent to Ca-free washout only in hearts not exposed to gas perfusion prior to the washout. However, if the hearts were washed out subsequent to gas perfusion, contractile force failed to return (Bailey and Krip, 1972). During the brief Ca-free perfusion in hearts not exposed to gas perfusion prior to the washout, contractile force decreased to less than 1 g and we have assumed that the washout removed interstitial Ca and probably the Ca located on the surface of the sarcolemma represented by T in the model (Figure 29). These results suggest that the maintenance of contractile force in the kitten heart during gas perfusion may not rely on the superficial Ca pool which we have postulated to be 'trigger Ca' as in liquid perfused hearts. Moreover, since La only

blocked the superficial Ca pool, 'trigger Ca', we therefore observed that La in relatively high concentrations (500 uM) had no effect on contractile force in hearts preperfused with gas.

On the other hand, since contractile force is less during gas perfusion after a brief Ca-free washout or after treatment with La, 'trigger Ca' or the Ca in extracellular space may contribute to part of the force maintained by the heart during gas perfusion. This is conceivable since contractile force was partially restored during gas perfusion, after hearts previously depleted of Ca were perfused with perfusate containing 5 uM La. Contractile force was also only about 50% of the force developed in the absence of vascular Ca. Since 5 uM La blocks the 'trigger Ca' pool, these results suggest that although 'trigger Ca' is not essential to activate the release of Ca_{II} for the maintenance of contractile force during gas perfusion, it could probably contribute to part of the force developed during gas perfusion. However, when hearts which were not depleted of Ca were perfused with varying concentrations of La (25 - 250 uM) and then gas perfused, restoration of contractile force during the subsequent gas perfusion depended directly on the concentration of La used and the duration of La-Hepes perfusion. When force was abolished by 25 uM La, restoration of force during gas perfusion was about 80% of force developed during liquid perfusion (Figure 19). Although this concentration of La was more than the La concentration used in Ca-depleted hearts, contrac-

tile force was restored to a higher level than in hearts previously depleted of Ca. Perhaps not all of the 'trigger Ca' was displaced by the La even though a higher concentration of La was used since the La had to compete at the same site with the Ca already present.

Excitation-contraction coupling in cardiac muscle is not only controlled by Ca but other ions normally present in body fluids such as Na, K, and Mg also play a role in this process. Therefore, logical extensions of the present study would be firstly to investigate the interrelationships of Ca, Na, K, and Mg in the regulation of excitation-contraction coupling, secondly to study the exact role of 'trigger Ca' and Ca_{II} in this process, and finally to elucidate the exact pathways of intracellular Ca exchange.

The Value of Hypothesis

"Now I do not regret these hypotheses, or even the titles of the papers; because they have set people (including myself) thinking and devising new experiments. That indeed is the chief purpose of hypotheses. I have long believed, and am still inclined to believe, that all theories of muscular contraction are wrong. But they have been very useful in stimulating new research. In fact many of the theories are self-destructive, by provoking fresh inquiry and leading to new facts which they cannot explain. The only useless theories are those that cannot be tested and can 'explain' everything."-

Archibald Vivian Hill

E. Summary

The objective of this investigation primarily was to elucidate the probable pathway of Ca movement during excitation-contraction coupling in cardiac muscle, and to identify, if possible, the location of the Ca responsible for activation of contraction to a specific anatomical site within the myocardial cell.

Gas perfusion provided a valuable tool, but the validity of its use is questionable in the study of Ca fluxes in excitation-contraction coupling under physiological conditions. The limitations of this technique are that Ca movement and Ca utilization in the heart during gas perfusion is different from that during liquid perfusion. Its use has no physiological basis. Although this technique has enabled us to evaluate the routes travelled by Ca during the process coupling excitation to contraction in cardiac muscle we therefore, must be cautious with the interpretation of results obtained with this preparation.

Ni abolished contractile force only when it was present in a pool similar kinetically to a Ca pool necessary for contraction, Ca_{II}. Ni apparently competed with Ca at some step in the excitation-contraction process subsequent to its release from Ca_{II}. Perhaps the competition occurs at the point of interaction between Ca and the troponin-tropomyosin complex. The inhibition of contractile force to Ni is reversed by removing Ni from Ca_{II}, by increasing extracellular Ca which indirectly

increases Ca in Ca_{II}, or by increasing Ca in Ca_{II} by treatment with ouabain.

In contrast to Ni, La does not compete with Ca for sites to activate the contractile proteins. There was also no evidence that La displaces Ca from Ca_{II}, the Ca known to be directly involved in the maintenance of contractile force. In fact, more Ca was washed out of Ca_{II} though at a slower rate after La treatment. In any event, our results show that La abolished contractile force by preventing the uptake of Ca into a superficial site, Ca_I which may be involved in the process 'triggering' the release of Ca in Ca_{II} to activate the contractile elements.

We have postulated a probable pathway of movement of Ca in kitten heart during the process coupling excitation to contraction when hearts were perfused with a liquid perfusate. During excitation 'trigger Ca' is released from the surface membrane sites to activate the release of Ca from an intracellular pool, Ca_{II}. The released Ca, then interacts with the contractile elements giving rise to contraction. The Ca is then accumulated by Ca_{III} which may represent sarcoplasmic reticulum or some other storage sites, and subsequently actively pumped out of the cell. The resultant lowering of intracellular Ca concentration terminated the contraction and initiated relaxation.

In the current study, various Ca compartments were defined by kinetic analysis of Ca efflux curves and uptakes were assumed to repre-

sent a pool or pools of exchangeable Ca in the tissue. However, the limitations of kinetic analysis do not allow a precise identification of any Ca pool with a specific anatomical origin. Any Ca with similar kinetics of uptake or efflux characteristics, even though arising from sites of different anatomical origin, cannot be differentiated by this technique.

SECTION V

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