# SUBCELLULAR DISTRIBUTION OF CALCIUM IN THE ISOLATED PERFUSED HEART UNDER VARIOUS EXPERIMENTAL CONDITIONS

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CHARLES W. TOMLINSON

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# ABSTRACT

The subcellular distribution of calcium in isolated perfused rat heart under different experimental conditions was studied. The hearts were homogenized and mitochondrial (800 to 8,000 x g), sarcoplasmic reticular (8,000 to 40,000 x g) and supernatant (post 40,000 x g) fractions were separated by differential centrifugation. Increasing the concentration of calcium from 0 to 5 mM in the perfusion medium increased the levels of calcium in heart homogenate as well as in all the fractions. The levels of calcium in the homogenate and all subcellular fractions of hearts perfused for 20 minutes with 5 mM calcium were higher than those in hearts perfused with 1.25 mM calcium whether these hearts were homogenized or perfused in the presence of 5 mM azide. On perfusing the hearts with 5 mM calcium myocardial contractility increased to a maximum within 2 minutes and thereafter declined towards the control level. Homogenate calcium increased progressively and that of mitochondria reached a maximum within 2 minutes whereas reticular calcium reached to a maximal level in 20 minutes. On perfusing the hearts with calcium-deficient medium, there was an immediate reduction in myocardial contractility and the levels of calcium in the homogenate and mitochondria, but no significant decrease in the reticular calcium occurred within 40 minutes. These results reveal that changes in the levels of calcium in the mitochondrial fraction were greater and faster in comparison to the reticular fraction on perfusing the hearts with different concentrations of calcium.

In another series of experiments the effects of changes in heart rate and contractile force due to electrical stimulation, alteration of the temperature of the perfusion medium and varying degrees of stretch tension on the subcellular distribution of calcium in the isolated perfused heart were investigated. Increasing the frequency of stimulation from 80 to 320 pulses/minute decreased myocardial contractility without appreciable changes in the levels of calcium in the tissue homogenate, sarcoplasmic reticular or supernatant fractions. On the other hand, mitochondrial calcium was more in hearts stimulated at 120 pulses/minute in comparison to hearts stimulated at 80 pulses/minute; further increase in the frequency of stimulation resulted in a decrease in the level of mitochondrial calcium. Increasing the temperature of the perfusion medium from 25 to 37°C increased the

heart rate and decreased the contractile force without any changes in the levels of calcium in the homogenate or subcellular fractions. Increasing the stretch tension on the hearts from 0 to 5 g increased the developed contractile force and the level of reticular calcium without changing the heart rate or the levels of calcium in the homogenate, mitochondria and supernatant. These results do not reveal any cause-effect relationship between changes in heart function and subcellular distribution of calcium.

The effects of both substrate and oxygen lacks on the subcellular distribution of calcium in the isolated perfused rat heart were also studied. The levels of calcium in both mitochondrial and reticular fractions decreased whereas supernatant calcium increased in hearts which failed to generate contractility on perfusion with substrate-free medium for 2 hr. The presence of glucose in the perfusion medium maintained contractile force and partially prevented the changes in subcellular distribution of calcium. When the hearts were perfused for 30 minutes with hypoxic medium the contractility declined by about 80% of the control and changes in subcellular distribution of calcium similar to those in substrate-depleted hearts were observed. These changes in calcium distribution due to substrate or oxygen-lack were reversible. The hearts failed to generate contractility within 10 minutes on perfusion with substrate-free hypoxic medium and showed changes in subcellular distribution of calcium comparable to those seen in hearts perfused with hypoxic medium for 30 minutes or substrate-free medium for 2 hours. These results indicate the importance of supply of substrate and oxygen for the maintenance of the mitochondrial and reticular calcium pools in the heart.

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# I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Calcium ions are considered to play an important role in the process of excitation-contraction coupling during the heart cycle (1 - 3). Although it is clear from the experiments of Niedergerke (4) and Winegrad (5, 6) that calcium enters the heart cells during depolarization, adequate information concerning its subcellular distribution is not available in the literature. Numerous studied have shown that both cardiac sarcoplasmic reticulum and mitochondria in vitro are capable of accumulating calcium under a wide variety of conditions (7 - 12); however, on the basis of their rates of calcium transport it is thought that sarcoplasmic reticulum mainly and mitochondria to a certain extent are involved in the regulation of the intracellular concentration of calcium in the heart. On the other hand, mitochondria, which are present in abundance in the myocardial cell, are claimed to play a major role in the regulation of intracellular calcium since these organelles were found to contain the highest specific activity upon exposure of the whole heart to radioactive calcium (13 - 15).

The present investigation was undertaken to examine the subcellular distribution of calcium in rat heart under different conditions. Furthermore, the experiments were designed to determine which cellular components are most actively involved in accumulating calcium in the isolated rat heart perfused with various concentrations of extracellular calcium. An attempt was also made to find a relationship between changes in the subcellular distribution of calcium and contractile force of the heart perfused under these conditions. Recently, a report on subcellular distribution of calcium in rabbit heart perfused with various concentrations of calcium has appeared in literature (16). However, these investigators have neither studied the time-course nor have determined the changes in subcellular distribution of calcium in the presence of inhibitors of mitochondrial calcium transport. Furthermore, their experiments were not designed to gain information concerning the relation between contractile state and subcellular distribution of calcium in the heart. In another series of experiments, we have investigated the relationship between changes in myocardial contractility and subcellular distribution of calcium in the isolated perfused rat heart. The contractile function of the heart in these experiments was altered by varying the frequency of electrical stimulation, temperature of the perfusion medium and the degree of

stretch tension. A preliminary report concerning these results was presented before the Canadian Physiological Society (17) and the Canadian Federation of Biological Sciences (18).

Since some reports have appeared in literature indicating a defect in the abilities of both mitochondria and sarcoplasmic reticulum obtained from a wide variety of failing hearts to accumulate calcium in vitro (8, 11, 19 - 23), we have attempted to gain some information concerning the changes in the levels of calcium in subcellular particles obtained from failing hearts due to the lack of substrate and oxygen. It may be mentioned that both substrate-lack and hypoxia have been shown to depress myocardial contractility in the isolated perfused rat hearts (24 - 29).

## II. METHODS

Male albino rats weighing 300 to 400 g were decapitated, the hearts quickly excised and placed in ice-cold, oxygenated Krebs-Henseleit solution. After removing the connective tissue and fatty material, the hearts were arranged for coronary perfusion by the conventional Langendorff technique as described previously (30). These hearts were equilibrated for a period of 10 minutes with Krebs-Henseleit solution containing 1.25 mM calcium and then switched to a closed circulation system with 50 ml of the perfusion medium containing different concentrations of calcium. The composition of the normal Krebs-Henseleit solution was as follows (mM): NaCl, 120; $NaHCO_3$, 25; KCl, 4.8; $KH_2PO_4$, 1.25; $MgSO_4$, 1.25; $CaCl_2$, 1.25; and glucose, 8.6. The perfusion medium was oxygenated with a gas mixture (95% $O_2$ and 5% $CO_2$) and maintained at a temperature of 37°C. The flow rate was kept constant at 10 ml/min by a Harvard peristaltic pump. The contractile force was monitored on a Grass polygraph recorder with a force displacement transducer (FT03). A resting tension of 1 g was applied to the heart on starting the experiment. The hearts were removed from the perfusion apparatus after the specified time period and soaked in ice-cold homogenizing solution containing 0.32 M sucrose and 5 mM histidine, pH 7.4, blotted extensively with filter paper and soaked again in fresh homogenizing medium. The procedure was repeated three times. It has been considered that most of the perfusate in the heart is removed and contamination by extracellular calcium is minimized by this procedure (13). The sucrose, special enzyme grade, was purchased from Mann Research and treated with Dowex 50 in order to remove contaminant calcium. In several experiments when the hearts were not perfused the excised hearts were placed directly in cold homogenizing medium, trimmed, washed and blotted before homogenization.

The hearts were homogenized in a cold room at 0 to 4°C with 10 volumes of the homogenizing medium in a VirTis homogenizer at a medium speed for 3 20-second intervals. Both mitochondrial (800 to 8,000 x g) and sarcoplasmic reticular fractions (8,000 to 40,000 x g) were isolated according to methods described earlier (8). Each pellet was suspended in 1 ml of homogenizing solution per g tissue weight and the protein concentration was determined by the procedure of Lowry et al (31). The cytochrome oxidase, glucose-6-phosphatase and AMPase activities of the subcellular

fractions were determined according to methods described elsewhere (11, 32, 33). Cytochrome oxidase was used as a mitochondrial marker while glucose-6-phosphatase and AMPase activities were used as markers for sarcoplasmic reticulum. Calcium uptake by the subcellular fractions isolated by the above procedure was measured by incubating these particles (protein concentration, 0.1 to 0.25 mg/ml) in medium containing 150 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM histidine (pH 7.4), 5 mM ATP, and 0.125 mM $Ca^{45}Cl_2$ in a total volume of 4 ml. The reaction was started by the addition of membrane protein and was stopped by Millipore filtration at 25°C. The essential details of this method are described elsewhere (8, 14). The calcium contents of the isolated fractions were measured with a Zeiss atomic absorption spectrophotometer after extraction with 0.5 N HCl according to the method described by Reynafarje and Lehninger (34). $LaCl_3$ (1%) was added to eleminate interference by other ions during the determination of calcium.

In experiments in which the effects of electrical stimulation were studied, the atria were removed and the electrodes were attached to the interventricular septum and the apex of the heart. These hearts were stimulated at the desired frequency with a current just above threshold and of 10 msec duration. In some experiments the spontaneously beating hearts, after equilibration, were perfused with media at 25°, 31° and 37°C, while 1 g of resting tension was maintained. In another series of experiments, the resting tension of the spontaneously beating hearts was increased from 0 to 5 g after these hearts were equilibrated for 10 min at 37°C. At the desired contractile state, these hearts were removed, homogenized and the subcellular fractions were separated by centrifugation.

In the next series of experiments, after an equilibration period of 10 minutes, the hearts were perfused with substrate-free medium, hypoxic medium or substrate-free hypoxic medium for various intervals as indicated in the text. The hearts equilibrated for a 10 minute period were referred as controls. The aerobic medium was gassed with a mixture of 95% $O_2$ and 5% $CO_2$ whereas hypoxic medium was gassed with a mixture of 95% $N_2$ and 5% $CO_2$ for a minimum period of 30 minutes before starting the experiment. In one series of experiments the recovery of the substrate-depleted or hypoxic hearts was studied by preperfusing these hearts with a desired test medium for the specified time followed by perfusion with aerobic

medium containing 8.4 mM glucose. The subcellular fractions of these hearts were separated and the calcium contents were determined as described above.

# III. RESULTS

## A. The Subcellular Distribution of Calcium in the Rat Heart:

Both mitochondrial and reticular fractions of the heart were characterized in terms of their marker enzymes activities and their abilities to accumulate calcium in vitro in the presence of well-known inhibitors of heart mitochondrial ATP-linked calcium transport such as sodium azide and 2,4-dinitrophenol (11, 14, 22, 23). The results shown in Table I reveal that the mitochondrial fraction is rich in cytochrome oxidase whereas the reticular fraction contains high activities of glucose-6-phosphatase and AMPase. Furthermore, ATP-linked calcium uptake in the presence of oxalate by reticular fraction was not significantly decreased ($P > 0.05$) in the presence of sodium azide or 2,4-dinitrophenol (Table II). It may be pointed out that there was some sacrifice to ultimate purification of these subcellular fractions since the method employed in this study was designed for rapid isolation of these particles in order to minimize movements of calcium between these fractions during the isolation procedure.

The subcellular distribution of calcium in rat heart was studied by homogenizing the tissue in media of different pH or in the presence of various agents. The calcium contents of mitochondrial and reticular fractions were higher ($P < 0.05$) while that of supernatant was lower ($P < 0.05$) when the hearts were homogenized in a medium at pH 6.5 in comparison to that at pH 8.3 (Table III). Homogenizing the hearts in the presence of sodium azide or 2,4-dinitrophenol reduced the mitochondrial calcium level and markedly elevated the calcium content of the supernatant fraction (Table IV). The presence of chelating agents such as ethylenediamine tetraacetate acid disodium salt (EDTA) and Ethylenebis (oxyethlenenitrilo) tetracetic acid (EGTA) in the homogenizing medium reduced the calcium contents of mitochondrial and reticular fractions by 40 to 50% of the control values whereas the levels of calcium in supernatant were markedly increased. These experiments suggest that calcium movements take place during the fractionation procedure and both mitochondrial and reticular calcium pools are labile. Ueba et al (16) have shown that these fractions can also pick calcium from the homogenizing medium during the isolation procedure.

TABLE I

SUBCELLULAR DISTRIBUTION OF MARKER ENZYMES IN RAT HEART

| Enzymes | Enzyme Activities* | | |
|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum |
| Cytochrome C Oxidase (μmoles cytochrome C oxidized/mg protein/10 min) | 1.35 ± 0.08 | 9.10 ± 0.42 | 0.6 ± 0.005 |
| Glucose-6-phosphatase (μmoles Pi/mg protein/10 min) | 0.37 ± 0.05 | 0.08 ± 0.02 | 1.5 ± 0.160 |
| AMPase (μmoles Pi/mg protein/10 min) | 0.25 ± 0.01 | 0.11 ± 0.01 | 1.6 ± 0.017 |

*Each value is a mean ± S.E. of 6 experiments.

TABLE II

INFLUENCE OF SODIUM AZIDE AND DINITROPHENOL ON ATP-LINKED CALCIUM UPTAKE BY RAT HEART SUBCELLULAR FRACTIONS

| | Calcium uptake* (μmoles/mg protein/10 min) | |
|---|---|---|
| Conditions | Mitochondria | Reticulum |
| Control | 0.305 ± 0.018 | 0.676 ± 0.026 |
| Sodium azide (5 mM) | 0.020 ± 0.003 | 0.650 ± 0.031 |
| Dinitrophenol (0.25 mM) | 0.018 ± 0.001 | 0.639 ± 0.033 |

*Each value is a mean ± S.E. of 6 experiments.

TABLE III

SUBCELLULAR DISTRIBUTION OF CALCIUM IN RAT HEART HOMOGENIZED IN SOLUTIONS OF DIFFERENT pH

| pH of homogenizing solution | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 6.5 | 4.9 ± 0.3 | 23.0 ± 1.3 | 18.1 ± 0.8 | 0.4 ± 0.2 |
| 7.4 | 4.8 ± 0.5 | 17.2 ± 0.8 | 15.5 ± 1.1 | 0.6 ± 0.3 |
| 8.3 | 5.0 ± 0.6 | 15.1 ± 0.9 | 14.3 ± 0.6 | 1.5 ± 0.3 |

*Each value is a mean ± S. E. of 6 to 8 experiments.

TABLE IV

SUBCELLULAR DISTRIBUTION OF CALCIUM IN RAT HEART HOMOGENIZED IN THE PRESENCE OF VARIOUS AGENTS

| | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| Additions | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 4.4 ± 0.6 | 16.6 ± 0.9 | 14.5 ± 0.8 | 0.3 ± 0.1 |
| Sodium azide (5 mM) | 4.7 ± 0.3 | 9.3 ± 0.8 | 17.3 ± 0.9 | 7.4 ± 0.8 |
| Dinitrophenol (0.2 mM) | 4.4 ± 0.3 | 6.9 ± 1.0 | 18.2 ± 1.2 | 5.2 ± 0.7 |
| EDTA (1 mM) | 4.6 ± 0.2 | 8.8 ± 0.7 | 8.2 ± 0.6 | 13.0 ± 0.3 |
| EGTA (1 mM) | 4.5 ± 0.4 | 8.5 ± 1.2 | 8.1 ± 1.0 | 17.3 ± 1.3 |

*Each value is a mean ± S.E. of 5 experiments.

The changes in the distribution of calcium in the perfused heart were studied during the equilibration period and the results are shown in Table V. The calcium contents of heart homogenate, mitochondria, reticulum and supernatant increased on perfusion with normal Krebs-Henseleit solution. Since the values for calcium contents in various fractions of hearts equilibrated for 10 minutes were not significantly different ($P > 0.05$) from those in hearts equilibrated for 20 minutes, the hearts equilibrated for 10 minutes with normal Krebs-Henseleit solution were employed in subsequent experiments.

B. Effect of Extracellular Calcium on the Subcellular Distribution of Calcium and Myocardial Contractility:

The isolated hearts were perfused for 40 minutes with media containing different concentrations of calcium and the subcellular distribution of calcium was determined. Increasing the amount of calcium in the perfusion medium was observed to increase the calcium contents in heart homogenate and all other fractions (Table VI). It was interesting to observe that the mitochondrial calcium, but not the reticular calcium, in hearts perfused with 1.25 mM calcium was significantly higher ($P < 0.01$) than that in hearts perfused with calcium-deficient medium. Furthermore, the increase in calcium content in the mitochondrial fraction on perfusing the heart with 5 mM calcium was greater than the corresponding rise in the level of calcium in the reticular fraction. These changes were also evident when the hearts perfused with 1.25 and 5 mM calcium for 20 minutes were homogenized in the presence of azide (Table VII).

The effects of sodium azide and 2,4-dinitrophenol on changes in subcellular distribution of calcium in hearts perfused for 20 minutes with 1.25 and 5 mM calcium were also studied. It was observed that in the presence of azide or dinitrophenol the calcium contents in homogenate, mitochondria, reticulum and supernatant were significantly higher ($P < 0.05$) in hearts perfused with 5 mM calcium than in hearts perfused with 1.25 mM calcium (Table VIII). In another experiment, the hearts were pretreated with sodium azide for 10 minutes and further perfused for 20 minutes in medium containing 1.25 or 5 mM calcium in the presence of azide. The levels of calcium in different fractions obtained from the hearts perfused with 5 mM calcium were markedly higher than in hearts perfused with 1.25 mM calcium (Table IX).

TABLE V

INFLUENCE OF PERFUSION ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART

| Time of perfusion (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 5.2 ± 0.3 | 16.5 ± 0.8 | 14.3 ± 0.7 | 0.4 ± 0.2 |
| 1 | 5.4 ± 0.4 | 17.0 ± 0.7 | 14.9 ± 0.6 | 1.0 ± 0.4 |
| 5 | 6.3 ± 0.4 | 18.9 ± 0.8 | 16.4 ± 0.3 | 1.2 ± 0.3 |
| 10 | 7.4 ± 0.2 | 20.0 ± 0.8 | 17.1 ± 0.7 | 1.3 ± 0.2 |
| 20 | 7.4 ± 0.5 | 21.8 ± 0.6 | 17.4 ± 0.9 | 1.8 ± 0.2 |

*Each value is a mean ± S. E. of 5 experiments.

TABLE VI

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED HEART PERFUSED FOR 40 MINUTES WITH MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

| Concentration of calcium (mM) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 3.8 ± 0.6 | 12.6 ± 1.1 | 14.8 ± 0.7 | 0.2 ± 0.2 |
| 1.25 | 8.5 ± 0.9 | 21.2 ± 1.3 | 16.0 ± 1.1 | 1.2 ± 0.8 |
| 2.50 | 13.2 ± 0.5 | 24.7 ± 1.1 | 20.1 ± 0.9 | 3.6 ± 1.0 |
| 5.00 | 20.5 ± 1.1 | 33.2 ± 0.9 | 21.9 ± 1.3 | 6.2 ± 1.1 |

*Each value is a mean ± S. E. of 7 to 8 experiments.

TABLE VII

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PERFUSED WITH MEDIA CONTAINING 1.25 AND 5 mM CALCIUM FOR 20 MINUTES AND HOMOGENIZED IN THE PRESENCE OF 5 mM SODIUM AZIDE

| Concentration of calcium (mM) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 1.25 | 8.1 ± 0.5 | 8.8 ± 1.0 | 18.7 ± 1.3 | 10.3 ± 1.1 |
| 5.00 | 18.7 ± 1.2 | 17.9 ± 1.3 | 26.1 ± 0.9 | 32.4 ± 1.7 |

*Each value is a mean ± S. E. of 6 experiments.

TABLE VIII

EFFECT OF PERFUSION FOR 20 MINUTES WITH MEDIUM CONTAINING 1.25 OR 5 mM CALCIUM IN THE PRESENCE OF 5 mM SODIUM AZIDE OR 0.2 mM DINITROPHENOL ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART

| Concentration of calcium (mM) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| A. Sodium azide | | | | |
| 1.25 | 5.9 ± 0.5 | 18.3 ± 0.5 | 17.2 ± 1.2 | 5.2 ± 1.1 |
| 5.00 | 15.1 ± 1.1 | 27.1 ± 1.3 | 23.5 ± 1.3 | 16.3 ± 0.9 |
| B. Dinitrophenol | | | | |
| 1.25 | 7.1 ± 0.8 | 14.5 ± 1.1 | 19.6 ± 0.8 | 5.4 ± 0.9 |
| 5.00 | 14.9 ± 1.2 | 22.2 ± 0.5 | 27.5 ± 1.4 | 20.8 ± 1.1 |

*Each value is a mean ± S.E. of 5 to 6 experiments.

TABLE IX

EFFECT OF PERFUSION FOR 20 MINUTES WITH MEDIUM CONTAINING 1.25 OR 5 mM CALCIUM IN THE PRESENCE OF 5 mM SODIUM AZIDE ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PREPERFUSED FOR 10 MINUTES WITH MEDIUM CONTAINING 5 mM SODIUM AZIDE

| Concentration of calcium (mM) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 1.25 | 7.7 ± 0.2 | 21.1 ± 0.7 | 21.3 ± 0.6 | 10.9 ± 1.0 |
| 5.00 | 28.6 ± 1.9 | 36.1 ± 1.1 | 31.7 ± 2.1 | 33.8 ± 3.6 |

*Each value is a mean ± S.E. of 6 experiments.

An attempt was also made to gain information concerning the relationship between changes in subcellular distribution of calcium and myocardial contractility. The hearts were perfused for different intervals with medium in the absence or presence of 5 mM calcium and contractile force and levels of calcium in homogenates, mitochondria and reticulum were determined. On perfusing the hearts with 5 mM calcium the contractile force increased by 80% of the control within 2 minutes and thereafter declined gradually towards the control level during 40 minutes of perfusion (Fig. 1), whereas calcium levels in the heart homogenate increased progressively (Fig. 2). The calcium content in mitochondria in these hearts increased to a maximal level within 2 minutes (Fig. 3), while reticular calcium showed a peak at 20 minutes of perfusion with 5 mM calcium (Fig. 4). The hearts failed to generate contractile force within 30 seconds after initiation of perfusion with calcium-deficient medium (Fig. 1). The levels of calcium in homogenate and mitochondrial fraction decreased significantly ($P < 0.05$) within 30 seconds (Fig. 2 and 3), but reticular calcium did not show any significant change ($P > 0.05$) during 40 minutes of perfusion with calcium-deficient medium (Fig. 4).

C. Effect of Electrical Stimulation on the Subcellular Distribution of Calcium and Myocardial Contractility:

The perfused ventricles were stimulated with various frequencies for 10 minutes and contractile force and subcellular distribution of calcium were determined. On increasing the frequency of stimulation from 80 to 320 pulses per minute the contractile force of the myocardium decreased (Fig. 5). The negative inotropic action of high frequency of stimulation has also been observed in the isolated perfused heart preparations by other investigators (35,36). No changes in the levels of calcium in homogenate, reticular or supernatant fractions were apparent in these hearts (Table X). On the other hand, mitochondrial calcium in hearts stimulated at 120 pulses per minute was higher ($P < 0.05$) than that in hearts stimulated at 80 pulses per minute, but thereafter increasing the frequency of stimulation was found to lower the mitochondrial calcium significantly ($P < 0.05$). This biphasic change in mitochondrial calcium due to electrical stimulation was also observed when the hearts were homogenized in the presence of 5 mM sodium azide (Table XI). When the hearts were preperfused with medium containing sodium azide, the level of mitochondrial

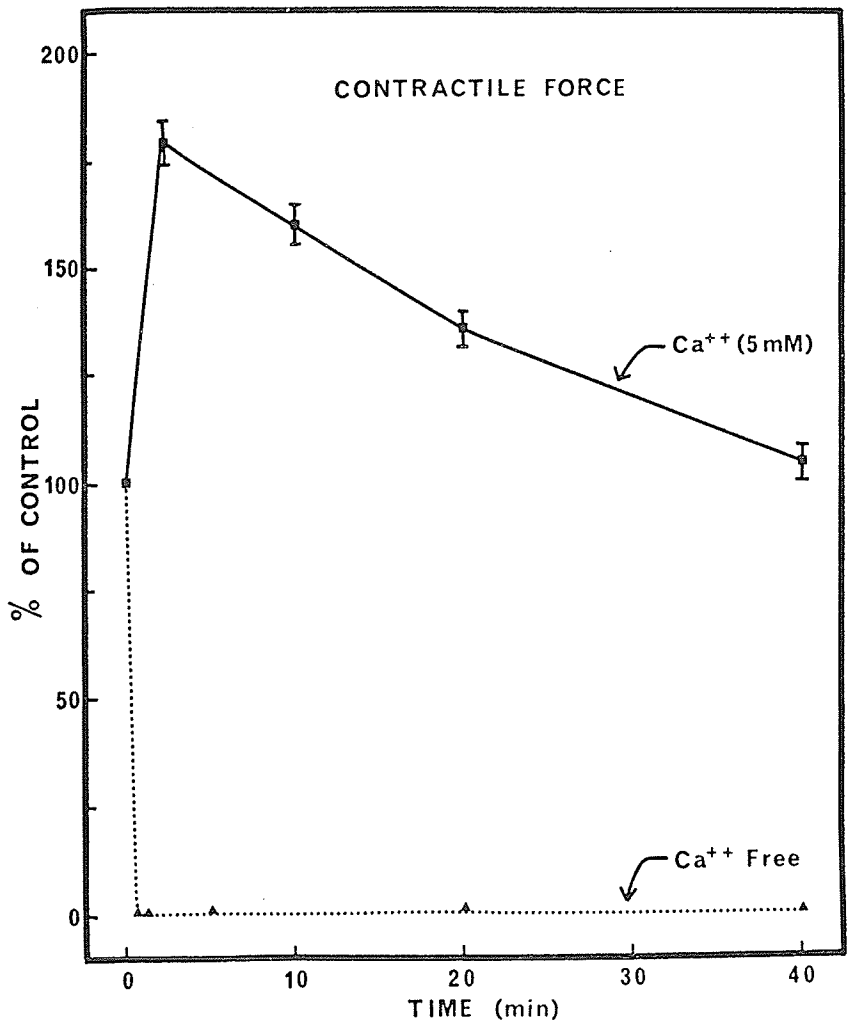


FIGURE 1: Contractile force of the isolated rat heart perfused with medium in the absence or presence of 5 mM calcium. ▲.....▲ calcium-deficient medium; ▩——▩ medium containing 5 mM calcium. Each value is a mean ± S. E. of 6 experiments.

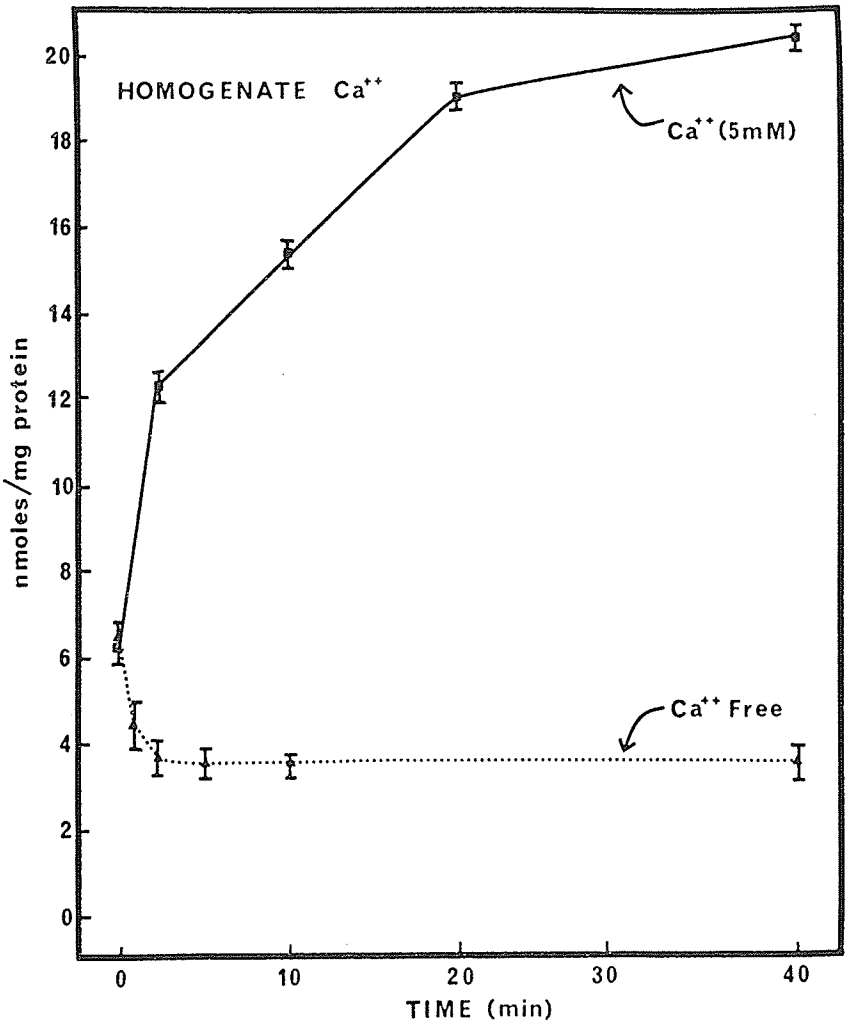


FIGURE 2: Levels of calcium in the homogenates of the isolated rat heart perfused with medium in the absence or presence of 5 mM calcium. ▲.....▲ calcium-deficient medium; ■——■ medium containing 5 mM calcium. Each value is a mean ± S. E. of 6 experiments.

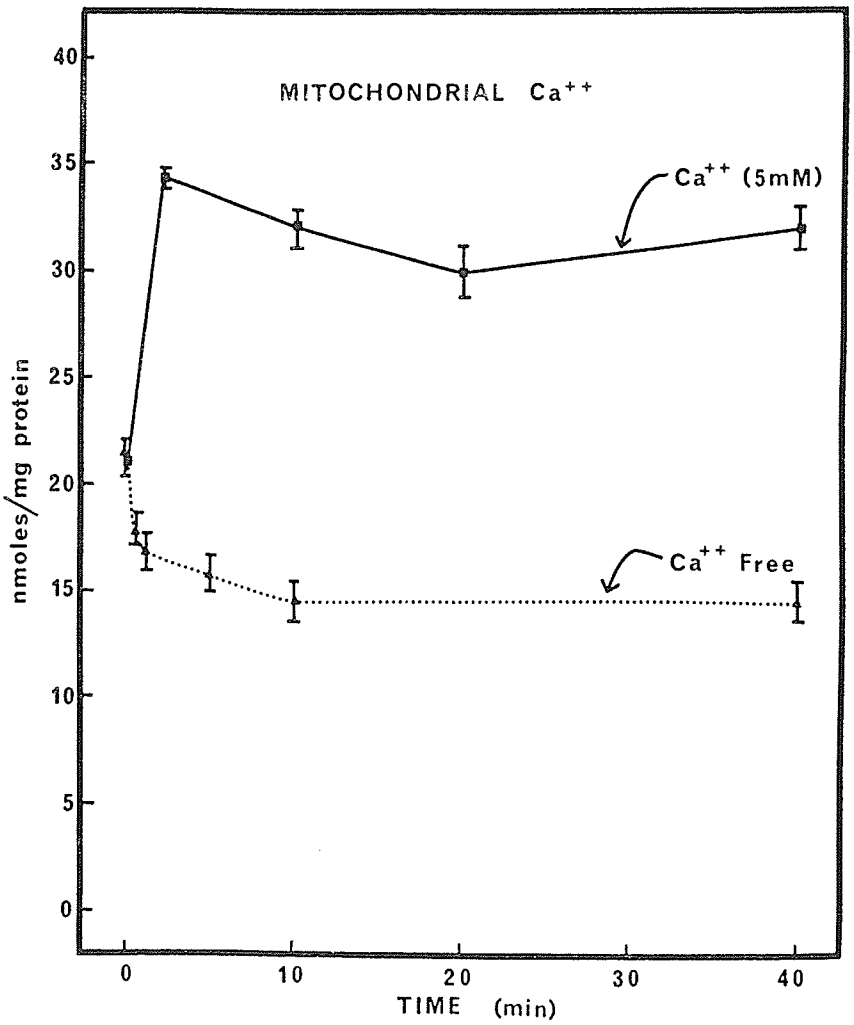


FIGURE 3: Levels of calcium in the mitochondrial fraction of the isolated rat heart perfused with medium in the absence or presence of 5 mM calcium. ▲·····▲ calcium-deficient medium; ■——■ medium containing 5 mM calcium. Each value is a mean ± S.E. of 6 experiments.

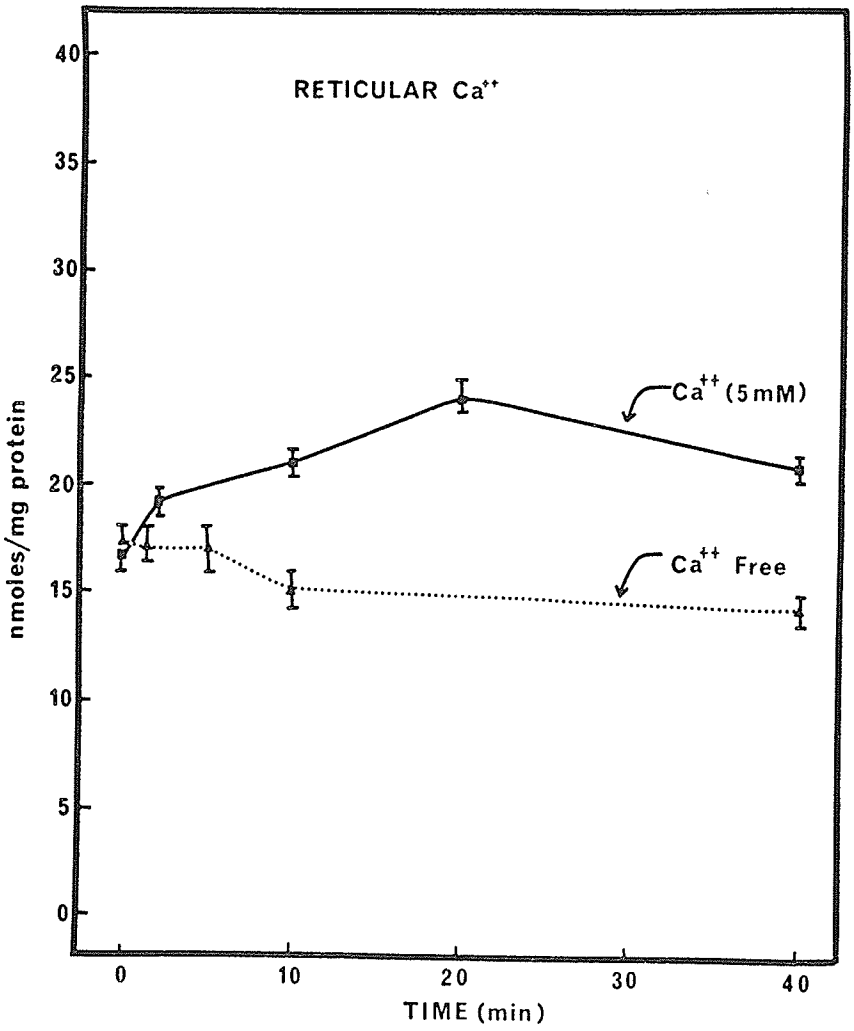


FIGURE 4: Levels of calcium in the sarcoplasmic reticular fraction of the isolated rat heart perfused with medium in the absence or presence of 5 mM calcium. ▲.....▲ calcium-deficient medium; ■——■ medium containing 5 mM calcium. Each value is a mean ± S. E. of 6 experiments.

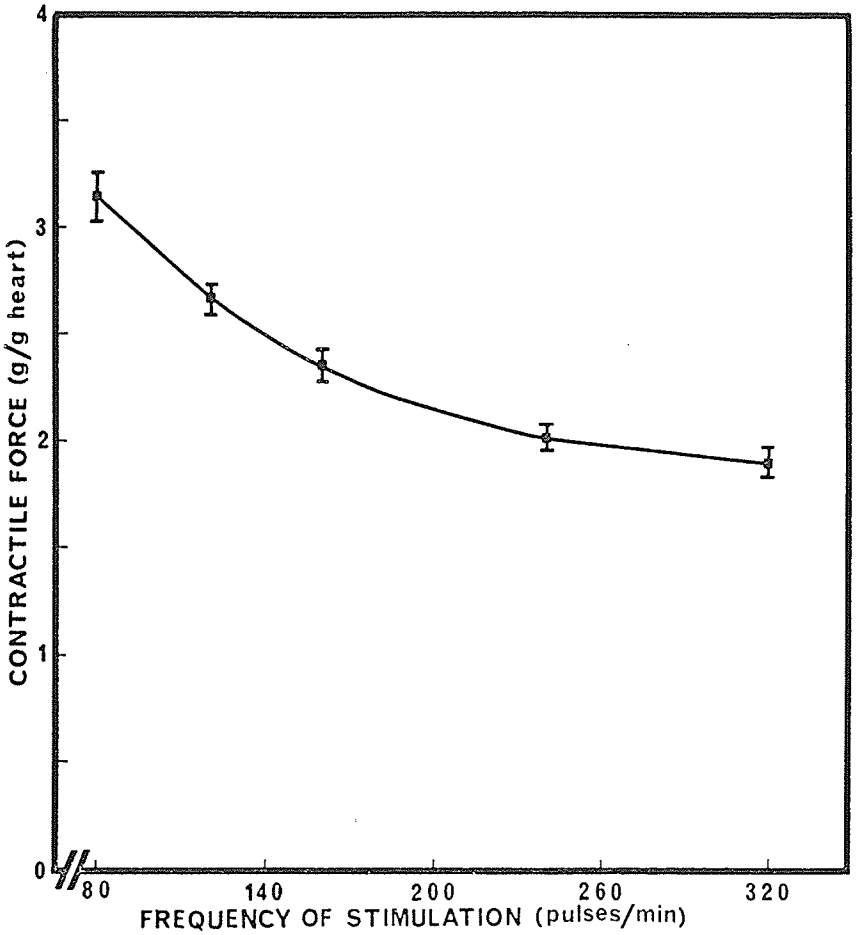


FIGURE 5: Contractile force of the isolated rat heart perfused with 1.25 mM calcium and stimulated at various frequencies. Each value is a mean ± S. E. of 6 experiments.

TABLE X

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED RAT HEART STIMULATED AT VARIOUS FREQUENCIES FOR 10 MINUTES

| Frequency of stimulation (pulses/min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 80 | 8.2 ± 0.5 | 20.3 ± 0.8 | 13.3 ± 0.6 | 1.6 ± 1.2 |
| 120 | 8.6 ± 0.3 | 26.9 ± 0.9 | 12.3 ± 0.6 | 1.8 ± 0.8 |
| 160 | 8.0 ± 0.4 | 19.9 ± 0.7 | 12.7 ± 0.5 | 1.3 ± 0.4 |
| 240 | 8.0 ± 0.6 | 17.0 ± 0.9 | 11.2 ± 1.2 | 1.4 ± 1.0 |
| 320 | 8.0 ± 0.4 | 16.4 ± 0.6 | 11.6 ± 0.6 | 1.5 ± 0.7 |

*Each value is a mean ± S. E. of 5 to 7 experiments.

TABLE XI

EFFECT OF ELECTRICAL STIMULATION FOR 10 MINUTES ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED RAT HEART HOMOGENIZED IN THE PRESENCE OF 5 mM SODIUM AZIDE

| Frequency of stimulation (pulses/min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 80 | 7.3 ± 0.5 | 12.2 ± 0.3 | 16.5 ± 0.6 | 12.9 ± 0.9 |
| 120 | 7.6 ± 0.4 | 16.5 ± 0.8 | 17.0 ± 0.6 | 14.3 ± 1.1 |
| 320 | 7.0 ± 0.3 | 9.0 ± 0.5 | 16.4 ± 0.7 | 14.8 ± 1.2 |

*Each value is a mean ± S. E. of 4 to 6 experiments.

calcium in hearts stimulated at 120 pulses per minute was not higher ($P > 0.05$) than that in hearts stimulated at 80 pulses per minute (Table XII). However, a marked decrease ($P < 0.005$) in mitochondrial calcium was observed in hearts stimulated at 320 pulses per minute under these conditions. A time course of increments in mitochondrial calcium due to stimulation of the hearts at 120 pulses per minute is shown in Table XIII. In a preliminary study a time-dependent reduction in mitochondrial calcium was noted on stimulating the hearts at 320 pulses per minute. In all experiments on electrical stimulation, the levels of calcium in homogenate, reticulum and supernatant did not change significantly.

D. Effect of Perfusion Temperature on the Subcellular Distribution of Calcium and Myocardial Contractility:

In another series of experiments the spontaneously beating hearts were perfused for 10 minutes with media at 25°, 31° and 37°C. The hearts perfused at 25°C developed greater contractile force and exhibited decreased heart rate in comparison to the hearts perfused at 37°C (Fig. 6). This is consistent with the well-known findings of Hajdu (37) that during hypothermia, the amplitude and duration of cardiac muscle contraction are increased. In these experiments, no differences in the levels of calcium were apparent in the tissue homogenates or any subcellular fractions of the hearts (Table XIV). Perfusing the hearts at 25°C for different intervals also did not show any change in the subcellular distribution of calcium (Table XV).

E. Effect of Stretch Tension on the Subcellular Distribution of Calcium and Myocardial Contractility:

The effects of varying degrees of stretch tension for 10 minutes on the isolated perfused hearts were also studied. Increasing the amount of stretch from 1 to 5 g was found to increase contractile force without any changes in heart rate (Fig. 7). These effects of increasing the stretch tension on contractile function of the perfused heart are similar to those reported earlier from this laboratory (30). No significant changes ($P > 0.05$) in levels of calcium in homogenate, mitochondria or supernatant were noted on applying 0 to 5 g tension of perfused hearts (Table XVI), but the reticular calcium pool was significantly higher ($P < 0.05$) in hearts with 1 to 5 g of tension in comparison to hearts without any tension. These results were

TABLE XII

EFFECT OF ELECTRICAL STIMULATION FOR 10 MINUTES ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PREPERFUSED FOR 10 MINUTES WITH MEDIUM CONTAINING 5 mM SODIUM AZIDE

| Frequency of stimulation (pulses/min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 80 | 7.4 ± 0.2 | 19.3 ± 1.1 | 21.8 ± 1.0 | 11.1 ± 1.4 |
| 120 | 7.8 ± 0.4 | 20.7 ± 0.8 | 19.6 ± 1.2 | 8.7 ± 1.1 |
| 320 | 7.6 ± 0.4 | 12.6 ± 0.5 | 19.3 ± 1.3 | 10.2 ± 1.2 |

*Each value is a mean ± S.E. of 6 experiments.

TABLE XIII

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED RAT HEART STIMULATED AT A FREQUENCY OF 120 PULSES PER MINUTE FOR DIFFERENT INTERVALS

| Period of stimulation (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 8.1 ± 0.4 | 20.0 ± 0.9 | 13.4 ± 1.3 | 1.3 ± 0.3 |
| 2 | 8.3 ± 0.3 | 23.4 ± 1.2 | 13.6 ± 1.4 | 1.6 ± 0.5 |
| 5 | 8.2 ± 0.5 | 25.4 ± 0.8 | 14.9 ± 0.9 | 1.6 ± 0.9 |
| 10 | 8.5 ± 0.4 | 28.0 ± 0.9 | 12.0 ± 0.8 | 1.8 ± 0.7 |

*Each value is a mean ± S.E. of 4 to 5 experiments.

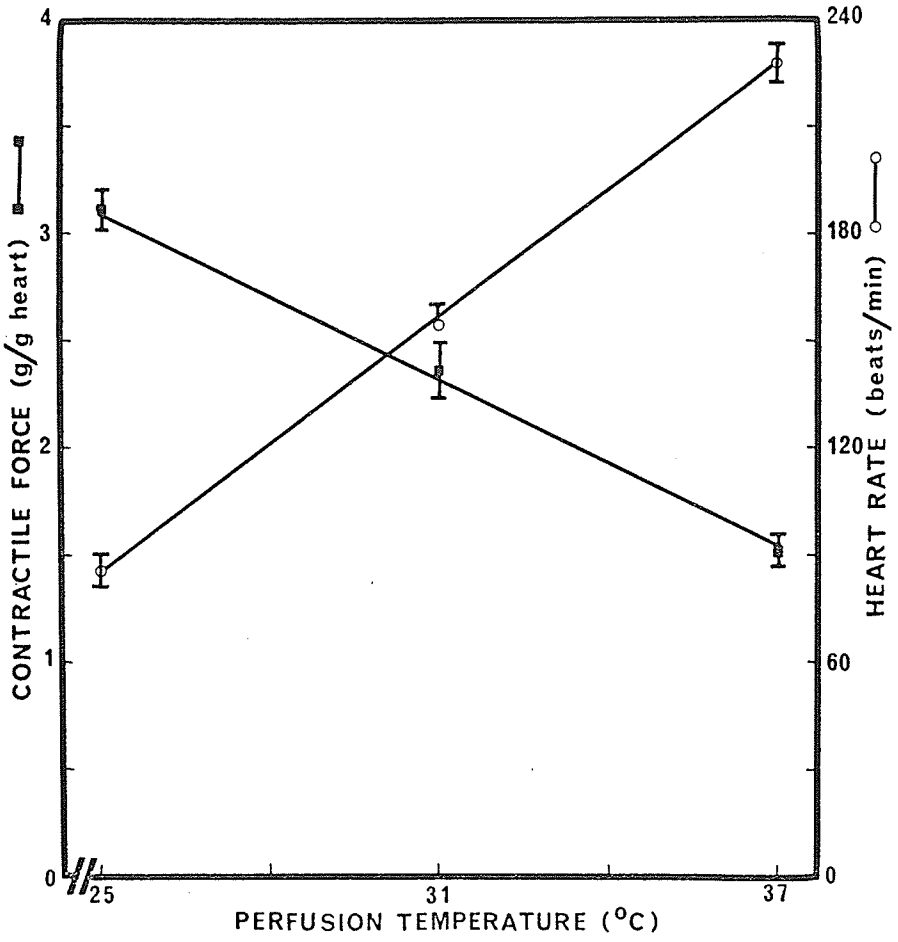


FIGURE 6: Contractile force and heart rate of the isolated rat heart perfused with medium at different temperatures. ■—■ contractile force; ○—○ heart rate. Each value is a mean ± S. E. of 6 experiments.

TABLE XIV

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PERFUSED FOR 10 MINUTES WITH MEDIA MAINTAINED AT DIFFERENT TEMPERATURES

| Perfusion temperature (°C) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 25 | 7.4 ± 0.3 | 21.6 ± 0.6 | 18.0 ± 1.4 | 1.3 ± 0.6 |
| 31 | 6.9 ± 0.4 | 21.4 ± 1.5 | 16.6 ± 1.5 | 1.8 ± 0.6 |
| 37 | 6.9 ± 0.2 | 20.5 ± 0.9 | 16.5 ± 0.6 | 1.6 ± 0.6 |

*Each value is a mean ± S. E. of 6 to 8 experiments.

TABLE XV

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PERFUSED WITH MEDIUM MAINTAINED AT 25°C FOR DIFFERENT INTERVALS

| Period of perfusion (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 7.4 ± 0.7 | 20.9 ± 0.9 | 18.3 ± 1.2 | 1.2 ± 0.7 |
| 2 | 7.5 ± 0.5 | 22.0 ± 0.8 | 17.4 ± 1.4 | 0.8 ± 0.4 |
| 5 | 7.0 ± 0.4 | 21.3 ± 0.9 | 16.8 ± 1.4 | 0.6 ± 0.3 |
| 10 | 7.3 ± 0.4 | 21.0 ± 0.6 | 18.0 ± 1.5 | 0.8 ± 0.6 |

*Each value is a mean ± S. E. of 5 to 6 experiments.

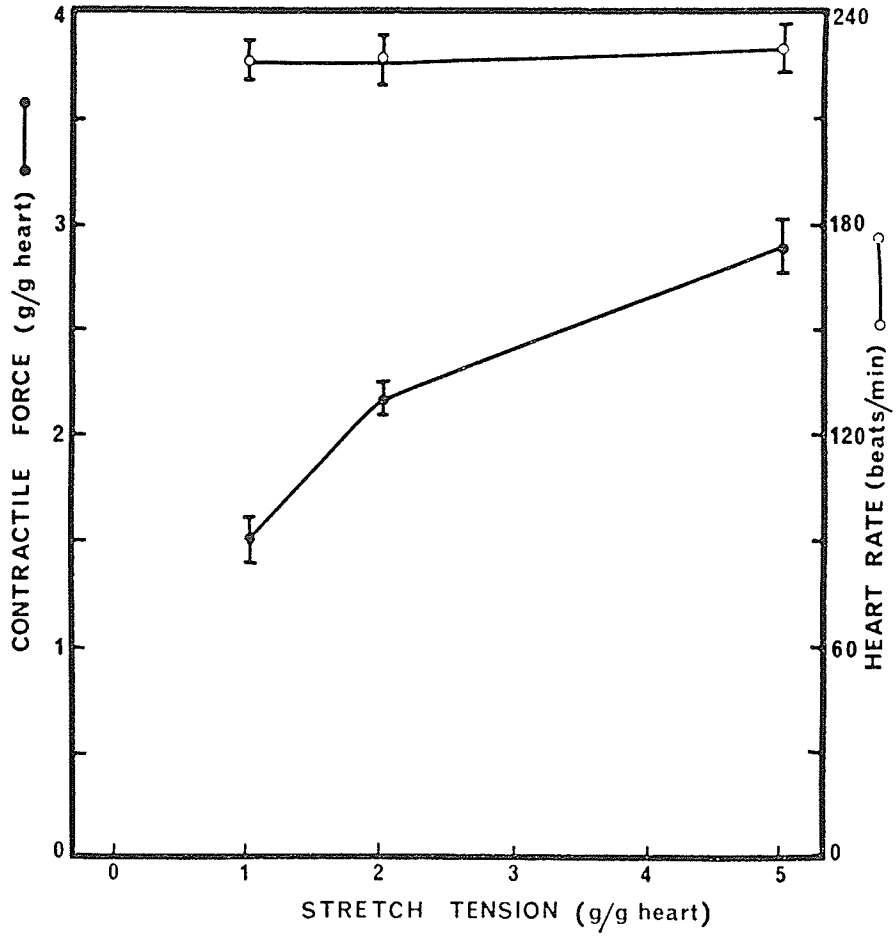


FIGURE 7: Contractile force and heart rate of the isolated rat heart perfused with 1.25 mM calcium and subjected to different degrees of stretch tension. ■—■ contractile force; O—O heart rate. Each value is a mean ± S.E. of 6 experiments.

TABLE XVI

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED HEARTS SUBJECTED TO VARIOUS DEGREES OF STRETCH TENSION FOR 10 MINUTES

| Tension (g) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 7.1 ± 0.3 | 20.7 ± 0.9 | 15.1 ± 0.5 | 1.3 ± 0.5 |
| 1 | 7.1 ± 0.3 | 21.3 ± 1.1 | 18.9 ± 1.2 | 2.1 ± 0.6 |
| 2 | 6.7 ± 0.5 | 21.5 ± 0.8 | 19.5 ± 1.3 | 2.3 ± 0.4 |
| 5 | 7.0 ± 0.4 | 20.3 ± 0.7 | 18.5 ± 0.9 | 1.6 ± 0.6 |

not altered when hearts with or without 5 g tension were homogenized in the presence of 5 mM azide (Table XVII). Furthermore, the observed increment in the reticular calcium due to 5 g stretch tension was found to be time-dependent (Table XVIII).

F. Effect of Substrate-Lack on the Subcellular Distribution of Calcium and Myocardial Contractility:

Perfusion of the isolated rat heart with oxygenated substrate-free medium was found to maintain contractile force for only 30 minutes but thereafter the contractility declined exponentially towards zero during a subsequent 90 minute period of perfusion. The presence of 8.4 mM glucose in the aerobic perfusion medium maintained contractile force of the heart during a period of 2 hours. These results are in agreement with those reported earlier (24 - 26).

Subcellular distribution of calcium in hearts perfused for different intervals with aerobic substrate-free medium is shown in Table XIX. On perfusion for 30 minutes when the contractility started to decline, there was a significant decrease in reticular calcium ($P < 0.05$). No significant change in the homogenate, mitochondrial or supernatant calcium was observed in hearts perfused for 60 minutes when the contractility declined by about 50% of the control value. The mitochondrial and reticular calcium pools were markedly reduced whereas the level of calcium in the supernatant fraction was elevated ($P < 0.005$) in hearts perfused with substrate-free medium for 2 hours, when these hearts failed to generate contractility. The presence of glucose in the aerobic medium was found to partially prevent the changes in the subcellular distribution of calcium in hearts due to substrate-lack (Table XX).

In hearts perfused with aerobic substrate-free medium for 90 minutes the contractility decreased to about 10% of the control value but perfusing these hearts for further 30 minutes with aerobic medium containing glucose was found to restore the contractile force to about 60% of the control value. The results on subcellular distribution of calcium shown in Table XXI reveal that the reduced levels of calcium in both mitochondria and sarcoplasmic reticulum due to substrate-lack for 90 minutes were restored towards the control values on perfusing these hearts for 30 minutes with aerobic medium containing glucose.

TABLE XVII

EFFECT OF 5 GRAMS OF STRETCH TENSION FOR 10 MINUTES ON SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED RAT HEART HOMOGENIZED IN THE PRESENCE OF 5 mM SODIUM AZIDE

| Tension (g) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 7.2 ± 0.3 | 12.2 ± 0.5 | 14.9 ± 0.4 | 13.1 ± 0.9 |
| 5 | 7.0 ± 0.2 | 12.9 ± 0.5 | 18.9 ± 0.6 | 14.7 ± 0.9 |

*Each value is a mean ± S.E. of 4 experiments.

TABLE XVIII

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED PERFUSED RAT HEART SUBJECTED TO 5 GRAMS OF STRETCH TENSION FOR DIFFERENT INTERVALS

| Period of tension (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| 0 | 7.6 ± 0.6 | 20.2 ± 1.2 | 14.8 ± 0.6 | 0.9 ± 0.4 |
| 3 | 7.0 ± 0.6 | 19.2 ± 1.2 | 15.6 ± 0.7 | 1.4 ± 0.6 |
| 7 | 7.9 ± 0.8 | 22.7 ± 1.1 | 17.8 ± 0.9 | 1.6 ± 0.8 |
| 10 | 7.3 ± 0.7 | 19.4 ± 1.0 | 18.2 ± 1.0 | 1.2 ± 0.3 |

*Each value is a mean ± S. E. of 5 experiments.

TABLE XIX

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEARTS PERFUSED WITH SUBSTRATE-FREE MEDIUM FOR VARIOUS INTERVALS

| Time (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.5 ± 0.4 | 21.5 ± 0.5 | 17.3 ± 0.4 | 0.9 ± 0.2 |
| 30 | 7.3 ± 0.3 | 20.1 ± 0.8 | 14.6 ± 1.0 | 1.0 ± 0.5 |
| 60 | 7.4 ± 0.4 | 19.0 ± 0.7 | 12.9 ± 0.8 | 0.9 ± 0.3 |
| 90 | 6.9 ± 0.5 | 14.6 ± 0.8 | 12.4 ± 0.5 | 1.2 ± 0.6 |
| 120 | 6.8 ± 0.3 | 12.0 ± 0.4 | 10.8 ± 0.3 | 3.0 ± 0.6 |

*Each value is a mean ± S.E. of 5 experiments.

TABLE XX

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEARTS PERFUSED FOR 2 HOURS IN THE ABSENCE OR PRESENCE OF GLUCOSE

| | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| Medium | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.6 ± 0.3 | 22.3 ± 0.4 | 17.0 ± 0.6 | 0.8 ± 0.6 |
| Substrate-free medium (120 min) | 7.4 ± 0.4 | 12.7 ± 0.7 | 10.7 ± 0.8 | 3.2 ± 0.4 |
| Substrate-containing medium (120 min) | 8.6 ± 0.3 | 19.7 ± 1.0 | 14.5 ± 0.9 | 1.6 ± 0.5 |

*Each value is a mean ± S.E. of 8 experiments.

TABLE XXI

EFFECT OF GLUCOSE ON THE SUBCELLULAR DISTRIBUTION OF CALCIUM IN HEARTS PREPERFUSED WITH SUBSTRATE-FREE MEDIUM FOR 90 MINUTES

| Medium | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.3 ± 0.3 | 22.1 ± 0.6 | 18.6 ± 0.5 | 0.9 ± 0.2 |
| Glucose-free (90 min) | 6.8 ± 0.3 | 14.4 ± 0.7 | 11.7 ± 0.7 | 1.5 ± 0.5 |
| Glucose-free (90 min) + glucose (30 min) | 8.4 ± 0.3 | 21.4 ± 0.6 | 16.6 ± 0.6 | 1.9 ± 0.8 |

*Each value is a mean ± S.E. of 7 experiments.

G. Effect of Oxygen-Lack on the Subcellular Distribution of Calcium and Myocardial Contractility:

In another series of experiments the effects of hypoxia on contractile force and subcellular distribution of calcium in the isolated perfused hearts were investigated. The contractility declined to about 35% within 10 min and to about 25% of the control value within 30 min of perfusion with hypoxic medium containing glucose whereas no alteration in contractile force was apparent in 30 minutes of perfusion with aerobic medium. These results are similar to those described elsewhere from this laboratory (29). It was observed that the levels of calcium in homogenate, mitochondrial and reticular fractions decreased and that in supernatant increased significantly ($P < 0.05$) after 30 minutes of perfusion with hypoxic medium containing glucose (Table XXII). The time-course effect of hypoxia revealed no significant reduction ($P > 0.05$) in the level of mitochondrial calcium in hearts perfused for 10 minutes and no change in the levels of calcium in homogenate and supernatant occurred in hearts during 20 minutes of perfusion with hypoxic medium (Table XXIII).

In hearts perfused for 20 minutes with hypoxic medium containing glucose the contractile force was decreased to about 30% but was restored to about 75% of the control value on further perfusion for 10 minutes with aerobic medium. The changes in mitochondrial and reticular calcium due to 20 minutes of hypoxia were restored towards the control levels on perfusing these hearts for 10 minutes with aerobic medium containing glucose (Table XXIV).

H. Effect of Substrate-Free Hypoxic Medium in the Subcellular Distribution of Calcium and Myocardial Contractility:

The isolated heart perfused with substrate-free hypoxic medium failed to generate contractile force within 10 minutes (28). It was found that on perfusing the hearts with either substrate-free or hypoxic medium alone for 10 minutes decreased the reticular calcium pool ($P < 0.05$) without any significant changes in the levels of calcium in homogenate, mitochondria or supernatant fractions (Table XXV). However, when hearts were perfused for 10 minutes with substrate-free hypoxic medium, the levels of mitochondrial calcium also declined and that in the supernatant fraction increased. The changes in subcellular distribution of calcium in

TABLE XXII

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEARTS PERFUSED FOR 30 MINUTES WITH HYPOXIC OR AEROBIC MEDIUM CONTAINING GLUCOSE

| | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| Medium | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.4 ± 0.3 | 21.3 ± 0.5 | 17.8 ± 0.6 | 1.1 ± 0.4 |
| Hypoxic medium (30 min) | 6.4 ± 0.3 | 15.9 ± 0.7 | 12.9 ± 0.6 | 3.2 ± 0.8 |
| Aerobic medium (30 min) | 7.6 ± 0.2 | 21.5 ± 0.4 | 17.5 ± 0.6 | 1.7 ± 0.5 |

*Each value is a mean ± S. E. of 8 experiments.

TABLE XXIII

SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEART PERFUSED WITH HYPOXIC MEDIUM CONTAINING GLUCOSE FOR DIFFERENT INTERVALS

| Time (min) | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.4 ± 0.2 | 21.7 ± 0.5 | 17.0 ± 0.7 | 1.4 ± 0.5 |
| 10 | 7.2 ± 0.4 | 19.4 ± 0.8 | 14.0 ± 0.5 | 1.8 ± 0.4 |
| 20 | 7.2 ± 0.3 | 16.6 ± 0.6 | 12.6 ± 0.8 | 1.8 ± 0.7 |
| 30 | 6.1 ± 0.3 | 14.3 ± 0.5 | 12.7 ± 0.5 | 3.6 ± 0.8 |

*Each value is a mean ± S. E. of 6 experiments.

TABLE XXIV

EFFECT OF AEROBIC MEDIUM ON THE SUBCELLULAR DISTRIBUTION OF CALCIUM IN HEARTS PREPERFUSED WITH HYPOXIC MEDIUM CONTAINING GLUCOSE

| Medium | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.4 ± 0.3 | 21.8 ± 0.5 | 17.9 ± 0.7 | 1.2 ± 0.3 |
| Hypoxic medium (20 min) | 7.2 ± 0.1 | 15.3 ± 0.5 | 13.2 ± 0.8 | 1.5 ± 0.4 |
| Hypoxic medium (20 min) + Aerobic medium (10 min) | 8.5 ± 0.3 | 20.8 ± 0.6 | 18.2 ± 0.5 | 2.4 ± 0.5 |

*Each value is a mean ± S. E. of 6 experiments.

TABLE XXV

EFFECTS OF LACK OF SUBSTRATE AND OXYGEN ON THE SUBCELLULAR DISTRIBUTION OF CALCIUM IN ISOLATED RAT HEARTS

| | Calcium content (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| Medium | Homogenate | Mitochondria | Reticulum | Supernatant |
| Control | 7.4 ± 0.4 | 21.1 ± 0.5 | 18.1 ± 0.4 | 0.8 ± 0.1 |
| Substrate-free medium (10 min) | 7.3 ± 0.3 | 20.9 ± 0.6 | 15.1 ± 0.8 | 1.2 ± 0.4 |
| Hypoxic medium (10 min) | 7.1 ± 0.3 | 18.9 ± 0.8 | 12.8 ± 0.7 | 1.8 ± 0.2 |
| Substrate-free hypoxic medium (10 min) | 6.7 ± 0.3 | 16.1 ± 0.3 | 11.2 ± 0.6 | 3.1 ± 0.2 |

*Each value is a mean ± S. E. of 4 to 6 experiments.

hearts perfused for 10 minutes with substrate-free medium were comparable to those seen in hearts either perfused with substrate-free medium for 2 hours or with hypoxic medium for 30 minutes.

# IV. DISCUSSION

## A. Subcellular Distribution of Calcium in Hearts Perfused with Different Concentrations of Calcium:

In this study we have demonstrated an increase in the calcium content of homogenate of the isolated rat heart on increasing the concentration of calcium in the perfusion medium. This is in agreement with earlier reports on cardiac tissues from guinea pigs and rabbits (5, 6, 16, 38, 39).

The experiments on subcellular distribution of calcium revealed that the levels of calcium in both mitochondrial and reticular fractions were higher in rat hearts perfused with 5 mM calcium for 40 minutes than those in hearts perfused with 1.25 mM calcium. Similar results were observed by Ueba et al (16) in the isolated perfused rabbit hearts; however, in their study the changes in the calcium content of the reticular fraction were only apparent at higher concentrations of calcium in the perfusion medium. This may be due to differences in species or experimental design.

Although the mechanisms by which these changes in calcium pools of subcellular particles occur are difficult to explain, these alterations do not appear to be the effect of increased myocardial contraction due to an increase in the concentration of extracellular calcium. This is borne by our observation that these changes take place in hearts preperfused with azide under which condition no contractile activity was apparent. Since both azide and dinitrophenol do not block the role in mitochondrial calcium when the hearts were exposed to higher calcium concentrations, it can be argued that the increment in the mitochondrial calcium pool is due to contaminating fragments of sarcoplasmic reticulum. However, such a possibility seems unlikely in view of the distribution of marker enzymes in these fractions and the magnitude of changes in mitochondrial calcium. It may be pointed out that calcium transport in mitochondria is not only ATP-dependent but also is supported by respiration (3) and the possibility cannot be ruled out that these mechanisms were not completely inhibited under our experimental conditions. Alternatively, the observed changes in mitochondrial calcium could be due to a concentration gradient in the whole heart and support the contention that mitochondria represent a buffer mechanism for the regulation of calcium in the myocardial cell.

It was interesting to observe that the increments in the mitochondrial calcium

pools were not only of greater magnitude but also occurred more rapidly than changes in the reticular fraction on perfusing the hearts with 5 mM calcium. Such changes do not appear to take place during the isolation process since these were also apparent when the hearts were homogenized in the presence of sodium azide. In addition, the mitochondrial calcium pool was decreased considerably while reticular calcium did not change significantly on perfusing the hearts with calcium-deficient medium. These observations support earlier claims that mitochondria play an important role in the regulation of intracellular calcium (8, 9, 13 - 16). It should be recognized that our inability of detecting an increase in reticular calcium comparable to that in the mitochondrial fraction upon perfusing hearts with 5 mM calcium could be due to leakage of calcium since the sarcotubular system is ruptured during homogenization of the heart. If this were the case, the level of reticular calcium should have decreased more than that of mitochondria on perfusing the hearts with calcium-deficient medium, but this is contrary to our observations. In view of the limitations of the technique employed in this study, it must be pointed out that the results, particularly concerning the reticular calcium pool, should be interpreted with certain caution. Clearly further experiments are needed to establish the lability of reticular calcium in the heart.

In contrast to the report of Ueba et al (16) on isolated rabbit heart, we have been able to demonstrate well-known changes in contractile force of the rat myocardium during perfusion with a medium in the absence or presence of 5 mM calcium. Their inability to demonstrate an increase in contractility without conduction abnormalities may be due to a high concentration of calcium (2.25 mM) which these workers have employed for the initial equilibration for 30 minutes. At any rate, it was interesting to find that initial rapid changes in contractility and the levels of calcium in homogenate and mitochondria occur simultaneously on perfusing the heart in the absence or presence of 5 mM calcium. However, we have failed to find a point to point relation between increments in contractility and the levels of calcium in homogenate, mitochondria and reticulum during the course of perfusing the hearts with 5 mM calcium.

B. Subcellular Distribution of Calcium in Hearts at Different Contractile States:

In this study the level of calcium in the reticular fraction was found to increase without any changes in homogenate or mitochondrial calcium upon stretching the heart. Such an increase in the reticular calcium pool was not due to contaminating mitochondria since this change was also apparent when the tissue was homogenized in the presence of azide. Although the increments in the reticular calcium were time-dependent, the significance of the results is not clear at present. This statement is based on our observations that the level of reticular calcium in hearts perfused under 5 g resting tension was not different from that in hearts perfused under 1 g resting tension whereas the contractile force generated by hearts under 5 g resting tension was markedly greater than that by hearts under 1 g stretch tension. Furthermore, we were unable to show any changes in the level of calcium in the heart homogenate or in the subcellular distribution of calcium when the contractile force was markedly increased on perfusing the hearts with medium at 25°C. Guthrie and Nayler (40) have also reported that a reduction in perfusion temperature from 25° to 4°C did not cause any significant changes in the total amount of calcium present in toad cardiac muscle. An abrupt decrease in temperature or stretching the myocardium also revealed no alteration in the calcium content of the papillary muscle (41, 42). It is possible that stretching the heart may have increased the number of sites for binding calcium on the sarcotubular system and this may account for the rise in the reticular calcium pool.

The results reported here reveal that increasing the frequency of stimulation from 80 to 320 pulses per minute produced a biphasic change in the mitochondrial calcium pool without any appreciable change in the level of calcium in the homogenate, reticular or supernatant fraction. The higher level of mitochondrial calcium in hearts stimulated at 120 pulses per minute did not seem to be an artifact due to the homogenization procedure since it was not affected by the presence of azide in the homogenizing medium. In addition, increments in mitochondrial calcium in hearts stimulated at 120 pulses per minute were time-dependent. Since electrical stimulation at 120 pulses per minute did not produce an increase in mitochondrial calcium in hearts perfused with azide, it appears that this change is due to some mechanism which involved ATP-dependent transport of calcium in mitochondria. It

was interesting to note that Bailey and Downie (35) have also observed an increase in calcium content of a specific pool in the cat heart on increasing the stimulation from 60 to 120 pulses per minute. It has also been reported that the total amount of calcium in guinea pig atria and dog papillary muscle did not change on increasing the frequency of stimulation (6, 38, 43). The decrease in mitochondrial calcium on stimulating the hearts from 120 to 320 pulses per minute may be due to a greater efflux than influx of calcium into this pool due to dramatic alterations in these membranes. However, such a suggestion should be considered cautiously.

Although we have observed resistance of mitochondrial and/or reticular calcium pools under particular experimental conditions employed in this study, we do not exclude the possibility that perfusion at different temperatures, application of varying amounts of stretch or stimulation at various frequencies may have altered the turnover rates in the mitochondria and sarcoplasmic reticulum and these may account for the changes in contractile activity. This view is consistent with observations that epinephrine, a well-known inotropic agent, has been shown to accelerate the processs of mitochondrial calcium exchange (15) whereas increased contractility by paired stimulation has been found to be associated with an increase in the exchangeable calcium in the myocardium (44). Although an increase in frequency of stimulation has been shown to produce alteration in the exchangeable calcium (6, 38, 43), no changes in myocardial calcium exchange have been observed upon stretching the myocardium to different extents (42). Likewise, Langer and Brady (41) who have failed to show a consistent alteration in calcium exchange due to abrupt cooling of the myocardium have provided some evidence of an increase in the membrane calcium concentration which occurs secondary to the inhibition of active sodium transport. Thus, it appears the alterations in mechanical activity due to interventions employed in this study are manifested through different mechanisms.

An association of increments in myocardial contractility and the mitochondrial calcium pool on increasing the concentration of extracellular calcium was shown in this study while changes in reticular calcium were delayed. A point to point relationship between these processed was not obtained however. The results also revealed no change in the subcellular distribution of calcium on perfusing the hearts at low temperatures when contractile force was markedly increased and the heart rate was

decreased. Increasing the frequency of stimulation produced a negative inotropic effect whereas changes in mitochondrial calcium were biphasic without any alteration in the reticular calcium pool. Furthermore, stretching the hearts markedly increased the contractile force and induced a slight increase in reticular calcium without any changes in heart rate or mitochondrial calcium. These experiments provide support for the contention that mitochondria in addition to sarcoplasmic reticulum may be involved in the regulation of calcium in the heart; however, on the basis of these observations we do not consider that changes in subcellular distribution of calcium as seen under the present experimental conditions have any cause-effect relationship with changes in myocardial function.

C. Subcellular Distribution of Calcium in Hearts Failing Due to Substrate and Oxygen Lacks:

A decrease in the mitochondrial and reticular calcium pools of the isolated rat heart perfused with substrate-free or hypoxic medium was observed in this study. The reduced levels of calcium in the subcellular fractions obtained from substrate-depleted and hypoxic hearts are not due to the presence of contaminating inert proteins since the yields of these particles from the failing hearts were identical to those obtained from the control hearts. Since the levels of calcium in the subcellular particles obtained by homogenizing the substrate-depleted and hypoxic hearts for 1 minute were similar to those homogenized for 20 seconds, it is unlikely that more calcium is lost from these particles during the process of homogenization in comparison to the control preparations. In view of our observation that the reduction in mitochondrial and reticular calcium was partially prevented on perfusing the hearts for 2 hours with aerobic medium containing glucose, it is suggested that the supply of both substrate and oxygen may be necessary for the maintenance of different calcium pools in the myocardial cell.

Both mitochondria and sarcoplasmic reticulum isolated from the substrate-depleted hearts have been reported to accumulate a decreased amount of calcium in vitro (8, 11). Although no report concerning the ability of mitochondria obtained from hypoxic myocardium to accumulate calcium in vitro is available at present, the sarcoplasmic reticulum isolated from hearts after coronary occlusion has been reported to exhibit an impaired calcium uptake (20). Since calcium uptake by these sub-

cellular particles has been shown to be an energy dependent mechanism (8), a reduction in supply of energy in the myocardium would decrease the ability of mitochondria and sarcoplasmic reticulum to accumulate calcium. A marked reduction in the high energy phosphate stores has been observed in both hypoxic and substrate-depleted hearts (24, 25, 27, 28). The decreased calcium uptake by the subcellular fractions in the myocardial cell could also be due to the higher levels of ATP and AMP in the hypoxic and substrate-depleted hearts (24, 25, 27, 28), because these nucleotides have been shown to reduce calcium accumulating abilities of heart subcellular particles (45). Furthermore, a damage to the ultrastructure of both mitochondria and sarcotubular system of the hypoxic and substrate-depleted hearts has been reported in the literature (25, 46). Such a damage in the integrity of these membranes would impair their ability to retain calcium and thus would result in an increased efflux of calcium. It is therefore our view that decreased uptake and increased release of calcium from mitochondria and sarcoplasmic reticulum result in reducing the levels of calcium in these subcellular fractions of the hypoxic and substrate-depleted hearts.

It was interesting to observe that the level of mitochondrial calcium in hearts perfused for 60 minutes with substrate-free medium or in hearts perfused with hypoxic medium for 10 minutes was not significantly different from that in the control hearts. On the other hand, the level of reticular calcium was significantly reduced in hearts perfused with substrate-free or hypoxic medium for 10 minutes. This suggests some differences in the sensitivities of mitochondrial and reticular calcium pools in the heart to substrate or oxygen lack. At any rate, it is quite apparent from this study that both hypoxia and substrate-lack produce essentially similar changes in the subcellular distribution of calcium. This is in agreement with our earlier conclusion based on studies concerning changes in the high energy phosphate stores and ultrastructure of the myocardium that substrate-lack imitates the effects of hypoxia on a longer time scale (47).

The failure to generate contractile force in both hypoxic hearts and substrate-depleted hearts has been considered to be due to an insufficiency in the process of energy generation (24, 25, 28). Therefore, it is difficult to state the exact significance of changes in the subcellular distribution of calcium in these experimental conditions. It is however noteworthy that the substrate-free hypoxic medium pro-

duced effects on contractility and on the subcellular distribution of calcium more rapidly than either substrate or oxygen lack. Furthermore, the effects of hypoxia and substrate-lack on the calcium pools and contractility were partially or fully reversible. These observations, in addition to the experiments on time-course effects of hypoxia and substrate, suggest an association between changes in contractility and subcellular calcium pools in the failing hearts. It may be noted that due to a reduction in the bound stores of calcium a lesser amount of calcium will be released during the process of excitation-contraction coupling and this may result in an impairment of myocardial contractility.

# V. CONCLUSIONS

In this study we have examined the subcellular distribution of calcium in the isolated perfused rat heart under various experimental conditions which are known to alter myocardial function. From the data obtained in this study, the following conclusions are drawn:

1. The results support the contention that both mitochondria and sarcoplasmic reticulum play an important role in the regulation of intracellular calcium in the heart.

2. Under certain conditions it is possible to show changes in the mitochondrial calcium pool which are greater and more rapid than those in the reticular calcium pool.

3. The changes in myocardial contractility may not necessarily be related to changes in the subcellular distribution of calcium, although some association between changes in contractility and subcellular calcium pools have been suggested on perfusing the hearts with calcium-free, substrate-free and hypoxic media.

4. The supply of oxygen and substrate seems essential for the maintenance of the mitochondrial and reticular calcium pools in the heart.

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