and time course of morpho-physiological changes during establishment may depend on what takes place at isolation. Also some lesions from the isolation or other pathological conditions in vivo may only recover in the culture system.

B. THE ISOLATION OF ADULT MYOCYTES

In 1912, Burrows reported that single cells, beating at their own intrinsic rate, migrated from chick embryo heart tissue explants cultured in serum (Burrows, 1910, 1912). This observation, which supported the myogenic and cell theory of myocardium, was superseded by the anatomical syncytium schools of thought (Dow et al., 1981). It was only 40 years later that Moscona (Moscona, 1952, Moscona and Moscona, 1952) and Dulbecco (Dulbecco, 1952, Wollenberger, 1985) reported independently the successful use of trypsin to dissociate chick embryos into single cells. Their work led rapidly to the development of embryonal and newborn cell culture as a model system of myocardium (Sperelakis, 1978, Schanne and Bkaily, 1980, Lieberman et al., 1981).

In 1969, Kono presented one of the first accounts of myocytes isolated from the ventricle of adult rat. Kono noted that perfusion of heart tissue with low- Ca^{++}

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

INTRACELLULAR CONCENTRATION AND MEMBRANE PERMEABILITY RATIO
OF SODIUM AND POTASSIUM ION IN CULTURED CARDIOMYOCYTES OF
THE ADULT RAT

by

ADRIAN SCULPTOREANU, B.Sc. (Hon.)

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements for the
degree of Master of Science.

Department of Biology
Carleton University
Ottawa, Ontario
January, 1988

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-51193-1

The undersigned hereby recommend to the Faculty of Graduate Studies and Research acceptance of this thesis, submitted by Adrian Sculptoreanu, B.Sc.(Hon.), in partial fulfillment of the requirements for the degree of Master of Science.

.....
Chairman, Dept. of Biology

.....
Supervisor

Carleton University

ABSTRACT

Sodium and potassium content of myocytes isolated from adult rat hearts and myocytes at different times following their culturing were analysed by flame spectrophotometric techniques.

Intracellular sodium and potassium ion concentration in newly isolated rod shaped myocytes was (59 ± 12) mM and (111 ± 13) mM when the myocytes were in the isolation saline which had a low concentration of calcium ion. Myocytes in culture medium 199 (Ca^{++} -1.3 mM) had an intracellular sodium ion concentration of (51 ± 20) mM and potassium ion concentration of (98 ± 22) mM. Membrane potential and intracellular sodium and potassium ion concentration of cultures of myocytes followed a time course which suggests a partial recovery of membrane potential towards the potassium equilibrium potential as myocytes establish in culture (Juhasz, 1987). After 15 days in culture, intracellular sodium and potassium ion concentrations reach stable values, respectively, of (28 ± 8) mM and (148 ± 17) mM. At this same time the ratio of sodium to potassium ion membrane permeability is calculated to be 0.17.

Permeability ratios for other myocardial preparations reported in the literature are about one order of magnitude smaller. This suggests the sodium to potassium ion membrane selectivity of the ventricular myocytes in cultures investigated in this study may be unusually low.

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisor, Dr. S.L. Jacobson, and my advisors Dr. D.R. Gardner, and Dr. G.W. Mainwood for their patient guidance during the progress of this project.

Many thanks to Mr. C. Wilson for his expert technical help and suggestions to aid in experimentation. I also thank Mrs. I. Fiser for her advice on scintillation counting techniques.

TABLE OF CONTENTS

	<u>PAGE</u>
I. ABSTRACT	iii
II. ACKNOWLEDGEMENTS	v
III. TABLE OF CONTENTS	vi
IV. LIST OF TABLES	ix
V. LIST OF FIGURES	x
VI. LIST OF APPENDICES	xi
VII. GLOSSARY OF ABBREVIATIONS	xii
.	
VIII. INTRODUCTION	1
A. ISOLATED AND CULTURED ADULT MYOCYTES AS A MODEL SYSTEM OF THE HEART	2
B. THE ISOLATION OF ADULT MYOCYTES	4
C. REQUIREMENTS FOR CELL ISOLATION	
1. Low calcium perfusion.	6
2. Requirement for pro- teolytic enzymes.	9
D. CALCIUM TOLERANCE	11
E. CULTURED CARDYOMYOCYTES: MORE THAN ONE MODEL SYSTEM	12
F. RESTING POTENTIAL: REDIFFERENTIATED CULTURE MODEL	13

IX. MATERIALS AND METHODS

A. ISOLATION OF CELLS AND THEIR CULTURING	19
B. ION ANALYSIS	
1. Calculation of intracellular sodium and potassium concentration.	21
C. THE NYLON MESH METHOD	
1. Equilibration with the extracellular marker and reduction of the extracellular volume.	24
2. Determination of total water.	26
3. Digestion in nitric acid.	27
4. Determination of the extracellular volume.	28
5. Measurement of ion content.	29
D. THE SILICONE OIL METHOD	
1. Equilibration with the extracellular volume marker, tritiated water and reduction of the extracellular volume.	30
2. Digestion in nitric acid.	32
3. Determination of the extracellular volume.	33
4. Determination of total water.	34
5. Measurement of ion content.	35
E. THE CELL CULTURE EXPERIMENTS	37

X. RESULTS

A. ISOLATED MYOCYTES

1. Morphological heterogeneity of the preparation. 42
2. Measurement of intracellular sodium and potassium concentration. 42
3. Effect of changes in extracellular calcium ion concentration on the intracellular sodium and potassium concentrations of the isolated myocytes. 53

B. CULTURED MYOCYTES

1. Measurement of intracellular sodium and potassium ion concentration. 59

XI. DISCUSSION 63

A. ISOLATED MYOCYTES

1. Is the concentration of ion in hypercontracted cells at equilibrium with that of extracellular ions? 63
2. Which extracellular markers freely permeate the intracellular volume of hypercontracted cells? 67
3. Intracellular sodium and potassium ion concentration. 71
4. Effect of changes of the extracellular calcium concentration on intracellular sodium and potassium concentration of isolated myocytes. 74

B. CULTURED MYOCYTES

1. Time course of intracellular sodium and potassium ion concentration change in cultures of adult rat ventricular myocytes.	77
2. Calculation of the sodium to potassium membrane permeability ratio and its time course.	79
3. Range of sodium to potassium membrane permeability ratio in cultured myocytes.	87
4. Activity coefficients are required for a correct estimate of the sodium to potassium membrane permeability ratio.	88
5. E_M versus $[K^+]_e$, is sodium to potassium membrane permeability ratio constant?	90
 XII. CONCLUSIONS	 95
 XIII. APPENDIX I	 99
XIV. APPENDIX II	108
XV. APPENDIX III	109
XVI. APPENDIX IV	111
 XVII. REFERENCES	 113

LIST OF TABLES

	<u>PAGE</u>
TABLE 1. Mean intracellular sodium and potassium concentration in isolated myocytes.	52
TABLE 2. Effect of changes in extracellular calcium concentration on intracellular sodium and potassium concentration in isolated myocytes.	55
TABLE 3. Intracellular concentration and membrane permeability ratio of sodium and potassium in isolated and cultured myocytes.	60
TABLE 4. Intracellular concentration of sodium and potassium in different myocardial preparations.	72
TABLE 5. Time course of intracellular sodium and potassium ion concentration and membrane permeability ratio change in cultures of adult rat ventricular myocytes.	78
TABLE 6. Membrane properties of different myocardial preparations.	82

LIST OF FIGURES

	PAGE
FIG.1 Time course of membrane potential change in cultures of adult rat ventricular myocytes.	17
FIG 2.The nylon mesh method.	25
FIG 3.The silicone oil method.	31
FIG 4.The method of analysis for cell cultures.	38
FIG.5 Sodium fraction of the cell pellet as a function of hypercontracted cells.	44
FIG.6 Extracellular volumes measured with different extracellular markers.	47
FIG.7 Effect of changes in extracellular calcium concentration on intracellular sodium and potassium concentration and percent hypercontracted cells of isolated myocyte preparation.	57
FIG.8 Simulation of constant field equation at different times in culture.	92

LIST OF APPENDICES

	<u>PAGE</u>
APPENDIX I. CALIBRATION OF LS7500 LIQUID SCINTILLATION SYSTEM	99
APPENDIX II. CALCULATION OF THE DILUTION FACTOR	108
APPENDIX III. CALCULATION OF ESTIMATED INTRACELLULAR Na^+ AND K^+ CONCENTRATION	109
APPENDIX IV. CALCULATION OF THE ACTIVITY COEFFICIENT	111

GLOSSARY OF ABBREVIATIONS

1. Numerical parameters:

E_K^+ : potassium equilibrium potential (i.e. described by Nernst equation).

E_M : membrane potential (mV). It includes the resting potential of quiescent cells and the maximum diastolic potential of pacemaking cells.

F : Faraday constant.

F_{ex} : fraction of total pellet volume which is extracellular (i.e. V_{ex}/V_T in $\mu l/\mu l$).

F_{Na^+} : sodium fraction of cell pellet (i.e. $[Na^+]_T/([Na^+]_T + [K^+]_T)$, mM/mM).

F_{RND} : hypercontracted cell fraction of the cell suspension (i.e.

number of hypercontracted cells / (number of hypercontracted + number of rod cells).

F_{ROD} : rod cell fraction of the cell suspension (i.e. $1 - F_{RND}$).

hypercontraction : change of rod shape to round shape (presumably irreversible) in newly isolated myocytes.

hyperpermeability: relatively high permeability to ions resulting in a large influx or efflux of ions along their electrochemical gradients.

n : with means $\pm$ SEM, number of observations, in RT/nF , valence of an ion.

pellet water: the total (intracellular and extracellular) water measured in a centrifuged cell pellet.

P_X : membrane permeability to an ion (i.e. $X = Na^+$ or K^+).

P_{Na^+}/P_{K^+} : sodium to potassium membrane permeability ratio.

R : gas constant.

T : absolute temperature.

V_{ex} : extracellular volume of water in the cell preparation, (μ l).

V_T : total volume of water in the cell preparation (μ l).

$[X]_{i,e}$: intracellular (i) or extracellular (e) concentration (mM) of an ion (i.e. $X = Na^+$, K^+ , or Ca^{++}). Note that the determined intracellular value of intracellular ion concentration (equation 1, page 22) may depend on the extent to which the extracellular marker molecule equilibrates with the intracellular volume of hypercontracted myocytes (equation 6, page 66)

$[X]_T$: total ion content of the cell preparation ($X = Na^+$ or K^+). Units are mM.

2. Chemical and pharmacological agents.

ATP: adenosine triphosphate.

D600: methoxyverapamil.

HEPES: N-2-hydroxyethylpyperazine-N'-2-ethanesulphonic acid.

TTX: tetrodotoxin.

3. Units of measurement.

$^{\circ}C$: degrees Celsius.

cpm: counts per minute.

dpm: decays per minute.

mM : millimolar.

mV : millivolts.

ppm: parts per million.

nM : nanomolar.

μCi : microCurie.

μl : microliters.

μM : micromolar.

INTRODUCTION

Myocytes isolated from ventricles of adult mammalian hearts and myocytes in culture have become an area of investigation only recently.

One area of research that may benefit from the development of these preparations is cardiac muscle membrane physiology. Experimental manipulations during the isolation of myocytes may have short term, and in cultures long term effects on the membrane properties of the myocytes. Therefore, an understanding of possible lesions induced at the time of isolation is crucial in establishing isolated and cultured myocytes as a model system of the intact heart.

This study is concerned with the time course of changes in intracellular sodium and potassium concentration from the time of isolation of myocytes to different stages of establishment of the cells in cultures. The cardiomyocytes were isolated from the ventricles of adult rats three to four months old.

One of the problems encountered and solved in the present approach was the determination of extracellular volume. It was critical to find a molecule that could provide an accurate measure of extracellular volume. An ideal extracellular marker molecule is one that

equilibrates rapidly with all the extracellular volume, including the diffusion limited extracellular spaces, but is not bound to cells or taken up by intracellular compartments. Such a molecule should equilibrate with the intracellular volume of hyperpermeable and nonviable round myocytes yet be excluded from viable myocytes thereby behaving as an ideal marker.

A. ISOLATED AND CULTURED ADULT MYOCYTES AS A MODEL SYSTEM OF THE HEART

The advantage of using isolated myocytes as opposed to tissue preparations is that they are free of influences from systemic hormones, neural activity, vascular and mechanical effects and the effect of the metabolic breakdown of drugs. These interferences make interpretation of data from cells in situ complex or impossible. Isolated and cultured cells lack diffusion limited extracellular spaces, which in situ are due to tight packing of cells and extracellular matrix, making them more suitable for studies of ion fluxes, exchange of metabolites, and for pharmacological studies (Wollenberger, 1985).

The complexities of intracellular connections of the myocardium, and the presence of low resistance

intercellular pathways have made voltage control and application of conventional electrophysiological techniques to myocardial tissue difficult since they result in nonhomogenous spread of current (Johnson and Liberman, 1971, Fozzard et al., 1985).

In the adult heart from 12% to 25% of the total number of cells are myocytes (Dow et al., 1981). Myocytes are, however, considerably larger than other types of cells, making up 80% of the myocardial volume (Dow et al., 1981). A purified suspension of isolated adult myocytes would, however, offer a homogeneous base for biochemical studies, preparations of sarcolemmal vesicles and other subcellular fractions.

Cell cultures can provide an additional tool for long term studies such as, the role of cell adhesion in cellular organization and function, modulation of membrane protein density and turn over, assembly of cytoskeleton proteins and formation of gap junctions.

The validity of using an isolated or cell culture preparation depends on its validity as a model of the heart. To this end, understanding what factors of the isolation technique change the cells is crucial not only in controlling the viability and yield of cells but also in extrapolating the data to the intact heart. With respect to the cell culture system, long term viability

and time course of morpho-physiological changes during establishment may depend on what takes place at isolation. Also some lesions from the isolation or other pathological conditions in vivo may only recover in the culture system.

B. THE ISOLATION OF ADULT MYOCYTES

In 1912, Burrows reported that single cells, beating at their own intrinsic rate, migrated from chick embryo heart tissue explants cultured in serum (Burrows, 1910, 1912). This observation, which supported the myogenic and cell theory of myocardium, was superseded by the anatomical syncytium schools of thought (Dow et al., 1981). It was only 40 years later that Moscona (Moscona, 1952, Moscona and Moscona, 1952) and Dulbecco (Dulbecco, 1952, Wollenberger, 1985) reported independently the successful use of trypsin to dissociate chick embryos into single cells. Their work led rapidly to the development of embryonal and newborn cell culture as a model system of myocardium (Sperelakis, 1978, Schanne and Bkaily, 1980, Lieberman et al., 1981).

In 1969, Kono presented one of the first accounts of myocytes isolated from the ventricle of adult rat. Kono noted that perfusion of heart tissue with low- Ca^{++}

saline and trypsin, followed by incubation of the partially digested tissue with purified bacterial collagenase resulted in a high yield (20%-40%) of ventricular myocytes 60%-80% of which were rod-shaped as in vivo (the rest were spherical). Since neither of the enzymes alone were effective in disrupting the tissue, Kono proposed that the cells were bound by two types of proteins. This report was soon followed by work from other laboratories (Berry et al., 1970, Bloom, 1970, Vahouny et al., 1970). It is now recognized that these early preparations were not tolerant to physiological concentrations of calcium at normal physiological temperatures (Dow et al., 1981, Farmer et al., 1983). Tsokos and Bloom (1976) proposed that these cells were hyperpermeable or leaky to ions. In 1972, Powell described isolated adult rat myocytes which could survive for a long time in salines containing physiological concentrations of Ca^{++} , but at lower than physiological temperatures (20°C), and later coined the term calcium tolerance (Powell and Twist, 1976, Powell 1985a).

Although at present there is no consensus on the isolation technique (Bkaily et al., 1984), several authors have modified the early procedures in order to make the preparations more tolerant to physiological calcium (Farmer et al., 1983). This work pointed out possible

consequences of various steps in the isolation procedure and may be an indication of the physiological state of the newly isolated myocytes.

C. REQUIREMENTS FOR CELL ISOLATION

1. Low $[Ca^{++}]$ perfusion.

The importance of calcium ion to myocardial function was first recognized by Ringer (1883) who noted that omission of Ca^{++} from saline resulted in uncoupling of excitation from contraction. The role of calcium ion in intercellular adhesion became an area of interest following the observations of Zimmerman et al. (1966, 1967) and Muir (1966) on changes in the ultrastructure and functional integrity of Ca^{++} -depleted hearts, and after their reperfusion with salines containing physiological $[Ca^{++}]$.

Zimmerman et al. (1966, 1967), noted that perfusion of rat hearts with Ca^{++} -free salines resulted in loss of contractile activity and only slight ultrastructural changes, manifested as extracellular edema. When the hearts were reperfused with Ca^{++} -containing medium (2.6mM), contractile activity was not restored, and there was a loss of electrical activity, loss of lactate

dehydrogenase, and loss of myoglobin. The morphological changes included hypercontraction of cells, appearance of damaged empty cells and swollen mitochondria. Zimmerman et al. proposed that these changes were due to an excessive Ca^{++} -influx during the Ca^{++} reperfusion and named these changes the calcium paradox. Muir, 1967, noted that there was a distinction between functional and adhesive roles of Ca^{++} . If calcium ion concentration in the saline perfusing the heart was maintained at $25\mu\text{M}$, contractility and electrical activity (ECG) were maintained for long times (more than 90 min.). Below this concentration intercalated discs between adjacent cells were separated and contractility was lost. Ultrastructurally the cells were similar to the hypercontracted, calcium paradox cells. Separation of basement membrane from the sarcolemma was observed after 32 min. of Ca^{++} -free perfusion.

The picture that emerged in the past decade added some details to the initial observations of Zimmerman et al. (1966, 1967) and Muir (1967). Specifically, changes induced by Ca^{++} -free perfusior make the heart (calcium paradox) and isolated myocytes (terminal rounding) susceptible to damage from reperfusion with physiological Ca^{++} (Farmer et al., 1983, Lambert et al., 1986). Furthermore, changes initiated by low $[\text{Ca}^{++}]$ seem to

require different levels of reduction of extracellular calcium ion concentration ($[Ca^{++}]_e$). Separation of gap junctions may require the greatest reduction in $[Ca^{++}]_e$, one attainable only with calcium ion chelators. Separation of the glycocalyx from the plasma membrane requires an intermediate reduction in $[Ca^{++}]_e$ (below $5\mu M$, Frank et al., 1977, Grinwald and Nayer, 1981), while the macula adherens of the desmosomes may be the easiest to split between adjacent myocytes, though the latter appears to be species specific (e.g. lower for rat than rabbit, Dani et al., 1979, Dow et al., 1981). Only at submicromolar Ca^{++} levels ($0.20-0.50\mu M$) do gap junctions split along the hemichannels (Mazet et al., 1985, Hirokawa and Heuser, 1982).

In the micromolar $[Ca^{++}]_e$ range, proteolytic enzymes are required to dissociate myocytes, and gap junctional membranes of previously connected cells appear not to split along the hemichannels but rather both membranes of each junction are attached to one or the other cell (Berry et al., 1970, Fry et al., 1979, Forbes and Sperelakis, 1985, Mazet et al., 1985, Severs et al., 1985).

During the isolation of myocytes in Ca^{++} -free salines endogenously released calcium and Ca^{++} -contaminant from collagenases and salts may contribute enough calcium to

protect the cells from some of the deleterious effects of $[Ca^{++}]_e$ reduction. Dow et al., 1981, measured 20-40 μ M Ca^{++} at the end of the perfusion of hearts with nominally calcium free saline. Schwarzfeld and Jacobson (1981) measured 5 μ M in the initial isolation saline which increased to 100 μ M at the end of isolation period. The proportion of rod-shaped relative to rounded, hypercontracted cells increases towards the end of isolation (Lundgren et al., 1984) in parallel with the increase in calcium ion concentration originating from endogenous sources (see above).

2. Requirement for proteolytic enzymes.

Two general types of enzymes have been used to release myocytes in suspension: proteolytic enzymes (trypsin, hyaluronidase) and enzymes which hydrolyse the intercellular matrix of the connective tissue (crude bacterial collagenases), (Dow et al., 1981, Farmer et al., 1983, Bkaily et al., 1984). Collagenase is Ca^{++} -activated and some investigators add calcium to the dispersion solution (Wittenberg and Robinson, 1981, Dow et al., 1981, Bkaily et al., 1984).

Bendukidze et al., 1985, reported a preparation of Ca^{++} -tolerant ventricular myocytes isolated from adult

guinea pig by use of pronase in the presence of $250\mu\text{M}$ Ca^{++} . If the tissue was exposed to the enzyme medium without prior perfusion with an enzyme free, Ca^{++} -free saline only rounded cells were dissociated.

Some investigators use crude collagenases alone to isolate cells (Bkaily et al., 1984), and these heterogenous enzyme preparations are very probably contaminated with other types of proteolytic activity, since the use of purified collagenase results in few or no cells being released from the tissue.

The effect of the enzymes used to isolate myocytes (Fry et al., 1979) is to disrupt the capillary endothelium and swell the extracellular space, which produces stretching forces. This, in concert with the effect of low calcium ion concentration on cell adhesion and applied mechanical forces such as trituration, shaking, stirring, etc., promotes the dissociation of cells from tissue.

A recent report (Bkaily et al., 1984) has raised questions as to the necessity of using enzymes to isolate the cells. Bkaily et al., 1984, described a new method in which a high proportion of rod shaped rat ventricular myocytes were mechanically dispersed using Jacobson's finger apparatus (Jacobson, 1977) in an enzyme free, low Ca^{++} medium. Strontium and barium were added with the intention of preserving the glycocalyx. Taurine, which

makes up 50% of the myocardial amino acid pool (Dow et al., 1981) and adenosine triphosphate (ATP) were also added to this medium. However, the exact yield of myocytes was not reported and other laboratories have not been able to reproduce the reported results (S.L. Jacobson, personal communication).

D. CALCIUM TOLERANCE

In summarizing the progress in isolating adult cardiac myocytes, Farmer et al. (1983), pointed out three classes of manipulations in the isolation procedure which were designed to increase calcium tolerance by: preventing sodium loss and potassium gain (also see Goshima et al., 1980, Altsculd et al., 1980, Cheung et al., 1982, Isenberg and Klockner, 1982, Hohl et al., 1983, Lambert et al., 1986); promoting 'healing' of gap junctions (Berry et al., 1970, Fry et al., 1979, Haworth et al., 1980, Mazet et al., 1985, Severs et al., 1985); and, preserving the glycocalyx (Fabiato, 1973, Powell and Twist, 1976, Kao et al., 1980, Hohl et al., 1983, Bkaily et al., 1984, Bendukidze et al., 1985).

If calcium tolerance was defined as the proportion of rod shaped cells to round cells, it was invariably found that when myocytes isolated in Ca^{++} -free saline were

exposed to 1-3mM calcium, the proportion of rod cells decreased with time or with increasing calcium ion concentration (Table 1 of Farmer et al., 1983). The sensitivity of isolated myocytes to calcium ion and the mechanisms that have been proposed to account for this sensitivity are reminiscent of findings in the calcium paradox protocol (i.e. perfusion of the heart with low $[Ca^{++}]$ salines followed by reperfusion with physiological calcium salines) of the intact heart. Several investigators have questioned the relevance of the calcium paradox phenomenon to the isolated cardiomyocyte system (Frank et al., 1977, Hearse et al., 1978, Alto and Dhalla, 1979, Grinwald and Nayer, 1981, Ganote, 1985, Piper et al., 1985, Lambert et al., 1986).

E. CULTURED CARDIOMYOCYTES: MORE THAN ONE MODEL SYSTEM

In 1977, Jacobson described adult rat cardiomyocytes which could be maintained in culture for up to 60 days. Twenty percent of the isolated cells attached to the substratum within a few days after isolation. Soon after attachment, cells spread out and cross-striations appeared, indicating reorganization of the myofibrillar apparatus. Subsequently, this and two other laboratories have described the sequence of morphological and

ultrastructural changes leading to the establishment of cells in 'redifferentiated' model cultures (Jacobson and Piper, 1986, Schwarzfeld and Jacobson, 1981, Nag and Cheng, 1981, Nag et al., 1983, Claycomb and Palazzo, 1980, Moses and Claycomb, 1982a and 1982b).

In 1982, Piper et al., described cultured cardiomyocytes of the adult rat, which attached to the substratum within 4 h of plating ('rapid attachment' model, Piper et al., 1985, Schwartz et al., 1985 a,b, Jacobson and Piper, 1986). These cells took on a 'bone shape' appearance, with cross striations clearly maintained after about one week in culture. However, the method used to maintain cellular homogeneity of these cells in culture, frequent washings with serum-free M199, also progressively reduces the number of cells in culture. Early attachment of myocytes has also been investigated in other laboratories (Lundgren et al., 1984, 1985, Cooper et al., 1986).

F. RESTING POTENTIAL: REDIFFERENTIATED CULTURE MODEL

There are few available data on the electrical properties of the redifferentiated culture model and no data on the rapid attachment model (Jacobson and Piper, 1986).

Jacobson et al., 1983, demonstrated the presence of tetrodotoxin-sensitive ion channels following hyperpolarisation of cells, slow channels blocked by methoxyverapamil (D600) and action potentials resembling the in situ adult rat ventricle in cultures 12 to 28 days old. However, the mean membrane potential (E_M) of these cells was more depolarized ($-53 \pm 1 \text{ mV}$) than either in situ (-76 to -82 mV) or isolated preparation (-70 to -92 mV), (table 1 of Meier et al., 1986).

A later study in the same laboratory (A. Juhasz, MSc Thesis, 1987, fig 1), documented the time course of mean membrane potential (E_M) change in parallel with the morphological sequence of establishment in culture. The results suggest that the E_M depolarizes as cells enter the round stage and then quickly repolarize to a new level (mean of -50 mV , and ranging from -22 to -80 mV), as cells spread out. Subsequently, the mean E_M tends to change slowly to more hyperpolarized values. Meier et al., 1986, reported more negative membrane potentials (-75 mV) for this culture system between 10 to 28 days old. However, the membrane potentials in these experiments were recorded following 30 to 60 min of incubation in high Ca^{++} -saline (5mM) and the cells were impaled in this saline. The results of a control experiment, E_M recorded in saline with physiological levels of Ca^{++} , was not

reported. Also, the distribution of membrane potentials was not reported, and only measurements of a selected population of cells (i.e. having stable resting potentials) was included in the sample.

The membrane potential is a function of mainly sodium and potassium ion concentration gradients and of the membrane permeability to these ions (the Goldman, Hodgkin and Katz equation, Sperelakis, 1979). Hence, it was reasonable to speculate that any of these parameters may change in parallel with the observed change in membrane potential as cardiac muscle cells establish in culture. To determine which parameters were responsible for the changes of membrane potential difference, experiments were designed to measure intracellular sodium and potassium concentration in isolated and cultured cardiomyocytes. Initially, newly isolated cells in suspension were used, not only to determine the intracellular concentration of sodium ($[Na^+]_i$) and potassium ions ($[K^+]_i$) for cells in the isolation saline (low Na^+ , high K^+ , and nominally Ca^{++} free) and following their resuspension in culture medium (Ca^{++} 1.3mM) but also to assess techniques for measuring these ions. Subsequently, a technique used for newly isolated cells was adopted for determination of the ion content of cells at different times in culture. These values were then used to estimate the

sodium to potassium permeability ratios (P_{Na^+}/P_{K^+}) at membrane potential values measured by A. Juhasz (1987, see figure 1).

Fig.1. Adult ventricular myocytes follow a time course of morphological and membrane potential changes as they establish in culture (E_M is the resting potential in quiescent cells and maximum diastolic potential in beating (culture) or stimulated cells (isolated myocytes)).

The morphological change from rod (elongated, striated) to rounded cells is accompanied by a rapid drop in membrane potential. The membrane potential then slowly returns to more negative values as cells establish in culture.

The triangles are from myocytes in culture medium 199 (Ca^{++} , 1.3mM), for the squares Ca^{++} was adjusted to 5mM, for the rhomb (elongated myocytes) Ca^{++} was added to 10mM. The data are presented as mean $\pm$ SEM, and the number of observations is shown for each point. The hexagons are observed minimum and maximum values of membrane potential for the given morphological stage. Therefore the broken line is the minimum and maximum envelope of the membrane potential time course. The figure is from A.Jabasz, Carleton University, M.Sc thesis, 1987.

MATERIALS AND METHODS

A. ISOLATION OF CELLS AND THEIR CULTURING

Cardiomyocytes were isolated from ventricles of 90-130 day old adult male albino rats (Sprague-Dawley), reared in the vivarium of Carleton University, Biology Department, using a modified version of an earlier procedure (Jacobson, 1977, Schwarzfeld and Jacobson, 1981), to include a low sodium (90mM), high potassium (30mM) isolation saline, as described previously by Altschuld et al. (1980), (Jacobson et al., 1985).

Cell suspensions for ion analysis, were isolated from the lower ventricles of two and occasionally three hearts, using nonsterile technique. The yield of rod-shaped myocytes in such preparations ranged between 2×10^5 and 4×10^5 per piece of tissue used from each heart. The preparations were heterogeneous. In addition to rod-shaped myocytes with clear striations and occasionally branched morphology, they contained variable amounts of cellular debris and rounded, hypercontracted cells. Although centrifugation in 6% bovine serum albumin (BSA), removed most of the cellular debris, the hypercontracted cells were resistant to differential density sedimentation.

Typically, the fraction of rod cells in the low Ca^{++} saline, varied between 50% and 70%, and was stable with time. When the cells were resuspended in Ca^{++} -containing culture medium 199 (1.3mM calcium), the fraction of rod cells was further decreased to 30% to 60% and continued to decrease slowly for the next 20 min when it reached a stable value.

Suspensions of cardiomyocytes for cultures were prepared under sterile conditions. The culture medium (199, Grand Island Biological Co., cat. nr. E12), was supplemented with 20% fetal calf serum (Hyclone Laboratories, Logan, Utah, cat. nr. 319), and $10\mu\text{M}$ cytosine 1-beta-D-arabinofuranoside (ARA-C, Sigma Chemical Co., St. Louis, Mo., cat. nr. C-6645). ARA-C inhibits the growth of nonmyocytes and may slow the rate of establishment of myocytes in culture (Jacobson et al., 1985).

Experiments established that culturing myocytes at a density greater than 2×10^4 cells per 35mm diameter culture dish did not increase the number of cells established in culture. Subsequently, the cells were plated at a density of $2-3 \times 10^4$ per dish on plastic cover slips of about 5cm^2 (Lux, Thermanox, Miles Laboratories, Naperville, Il., cat. nr. 5412) .

When viewed in the inverted phase contrast microscope,

the myocytes in the spread stage seemed to congregate in separate groups of cells, which occasionally were seen to contract spontaneously in synchrony. Most of the cells were quiescent, however. Plastic, instead of glass coverslips, were chosen because of minimal risk of contamination from Na^+ and K^+ in the glass leached into the nitric acid used in ion analysis. Ion analysis of cover slips presoaked in culture medium 199 (130mM Na^+) and then washed in sodium free saline (i.e. see method for cell culture ion analysis, page 37), showed sodium and potassium levels identical to the concentration of these ions in an aliquot of the sodium-free saline. Therefore, the plastic cover slips did not bind or liberate detectable levels of sodium and potassium ions.

B. ION ANALYSIS

1. Calculation of intracellular sodium and potassium concentration.

In these experiments the total sodium and potassium content of acid digestates of newly isolated cells and cultures of myocytes at different times in culture were measured by atomic absorption (Na^+) and flame emission (K^+).

Direct measurements were made of total concentration of an ion in the digestate, the total volume of water and the volume of the extracellular space of the cells dissolved in the digestate. The intracellular ion concentration was calculated by subtracting from the total ion content the contribution of ions in the volume equilibrated by an extracellular marker molecule (^3H -inulin, ^{14}C -sucrose, or $\text{H}_2^{35}\text{SO}_4$), according to the relationship:

$$[\text{X}]_i = ([\text{X}]_T - F_{\text{ex}} \cdot [\text{X}]_e) / (1 - F_{\text{ex}}) \quad (1)$$

where,

$\text{X} = \text{Na}^+$, or K^+

$[\text{X}]_i$ = determined intracellular ion concentration (mM)

$[\text{X}]_T$ = measured total ion concentration (mM).

$[\text{X}]_e$ = measured extracellular ion concentration (mM).

F_{ex} = the ratio of the extracellular volume of a cell sample to total water volume of the sample =

$$V_{\text{ex}} / V_T$$

(where, V_{ex} is the volume equilibrated by an extracellular marker molecule in the cell preparation.

V_T is the total water volume, wet weight - dry weight or the volume equilibrated by tritiated water, see page 26 and 34).

$1 - F_{ex}$ = the denominator of this equation, is the portion of total water which is cellular.

Note that if the extracellular volume approaches the total water volume, F_{ex} approaches one and the denominator of the above equation will approach zero. Since this produces an asymptotic increase of intracellular ion concentration as F_{ex} approaches unity, the intracellular concentration of ion ($[X]_i$) will become very sensitive to small errors in measurements of extracellular and total water volume. Therefore, it is important to keep the extracellular volume of the samples as small as possible.

Two methods were used to reduce the extracellular volume: a) filtration, i.e. the use of suction to remove free water from a cell suspension retained on a nylon mesh, and b) centrifugation of cells through silicone oil. In order to reduce the extracellular volumes in cell cultures, the cover slips with the cultured cells on them were passed through silicone oil. The silicone oils used here had a specific gravity greater than one. This and their viscosity were such that free water in a cell sample would be largely displaced by the oil as the wet samples passed through the oil.

C. THE NYLON MESH METHOD

1. Equilibration with extracellular marker and reduction of the extracellular volume.

Cell suspensions were equilibrated with $10\mu\text{Ci/ml}$ of the extracellular marker (^3H -inulin, Amersham, Can., cat. nr. TRA.324) and were divided into aliquots of at least 10^5 cells in about 1ml of medium. (see fig. 2, page 25) At the end of the equilibration period (2 min.) duplicates of $20\mu\text{l}$ aliquots of the cell suspension were pipetted on a clean, 20mm diameter nylon mesh ($25\mu\text{m}$ pore size, Saatifil, Saati Co, Italy) for measurement of radioactivity and ion content in the extracellular volume. This volume contains a negligible amount of cells (i.e. about 2×10^3). This number of cells comprise only about 0.25% of the total volume of $20\mu\text{l}$, as calculated from the estimate of $2.5\mu\text{l}$ cell water/ 10^5 cells given by Farmer et al., 1983.

Each aliquot of cell suspension was then poured onto a 20mm diameter nylon mesh held in a small filter holder fitted in an Erlenmeyer vacuum flask. A syringe attached to the side arm of the flask provided the suction pressure for gentle filtration of the cell suspension in about 1 second. Filtration was done in a plexiglass box where

Fig 2. THE NYLON MESH METHOD

O/N-over night

humidity was maintained at 90%, in order to minimize the rate of evaporation of water from the cell suspension retained on the mesh. This helped to obtain an accurate measurement of wet weight. Scanning electron microscopy (data not shown), revealed that rod cells retained on the nylon mesh maintained their morphology, and their proportion to round cells was comparable to that in cell suspension (70%).

2. Determination of total water.

The method used to measure the total water in the nylon mesh samples was to subtract the dry weight of the cell preparation from the initial wet weight. To estimate the initial wet weight, the nylon meshes were hung on a Cahn Electrobalance (Millibalance, Model 7500, Cahn Division, Ventron Instr. Corp.) which was connected to a chart recorder (Coleman Recorder, Hitachi 165, Hitachi, Japan).

The nylon meshes were transferred from the humidity-box (section 1, above) to the balance in a sealed chamber designed to contain a small volume (100 μ l) in order to minimize loss of water by evaporation. To account for evaporation during the time it took to transfer the nylon mesh from the chamber to the balance, the time taken to weigh the mesh was measured and the measured wet weight

was extrapolated to time 0 (i.e. the time when the sealed wet chamber was opened) from the initial linear portion of the drying curve. The total volume of water was taken as the difference between the wet weight and dry weight. It was assumed that the cell suspension had a density of 1 as over 80% of the wet weight was water. The cells were dried in vacuum at 37°C, to constant dry weight. Additional drying of the vacuum dried samples in the oven at 80°C did not significantly change the dry weight suggesting that vacuum drying was indeed adequate. The lower temperature (37°C) was used because it was found that drying the preparation at 80°C gave an erroneous estimate of the tritiated inulin activity. Preparations that initially had the same activity would show a variable decrease in activity when dried over night at 80°C. The dried nylon meshes were transferred to nitric acid for extracellular volume measurement (section 4) and ion analysis (section 5).

3. Digestion in nitric acid.

This is a standard step in ion analysis. Its main purpose is to dissolve the cell sample to produce an aqueous, homogeneous matrix. The dried nylon meshes (see section 4, below) were placed in 1ml HNO₃ pipetted into

vydor or quartz containers, with tightly fitted teflon stoppers. The nitric acid was reagent grade (Aristar, BDH Chemicals, Toronto, Ont., cat. nr. 45004) which contained 0.05 ppm or less Na^+ and K^+ contaminant. The contents of the tubes were mixed over night in an oven maintained at 37°C . This was sufficient to dissolve the mesh and cells producing a clear solution by the end of the digestion period. With this method there was no significant loss of isotope activity for up to 90 h of mixing. This was particularly important in the case of tritiated inulin, since any oxidized tritium might have been lost by evaporation.

4. Determination of the extracellular volume.

The extracellular volume, V_{ex} , was taken to be the volume equilibrated by the radioactive extracellular marker molecule in the cell preparation. It was calculated according to the relationship:

$$V_{\text{ex}} = (A_{\text{ex}} / A_{\text{ms}}) \cdot V_{\text{ms}} \quad (2)$$

where,

V_{ex} = extracellular volume in the cell preparation, in microliters.

A_{ex} = radioactivity of the extracellular marker

in 40 μ l of the cell preparation acid digestate (cpm).

A_{ms} = radioactivity of the acid digestate (40 μ l) of a measured sample of the total cell suspension (20 μ l) (cpm).

V_{ms} = measured sample volume (i.e. the volume (20 μ l) pipetted onto a clean nylon mesh from the cell suspension equilibrated with the extracellular marker and prior to filtration on the nylon mesh, which was then dried and digested in nitric acid), in μ l.

Volumes of twenty microliters of cell suspension were pipetted onto clean nylon meshes, vacuum dried, and then digested in 1ml of nitric acid. Subsequently, 5-10 aliquots of 40 μ l from the sample volume and cell preparation acid digestates (section 3, above) were pipetted into 10ml of scintillation fluid (Amersham Co., Oakville, Ont., cat. nr. 196290). The tritium activity on the nylon meshes (^3H -inulin) was counted in a Beckman LS150 liquid scintillation counter for 50 min. each.

5. Measurement of ion content.

The procedure for this step was identical with that in the silicone oil experiments (see page 35, below).

2. THE SILICONE OIL METHOD

1. Equilibration with extracellular volume marker, tritiated water and reduction of the extracellular volume.

This method is similar to that in previous reports (Powell et al., 1980, Altschuld et al., 1980, Silver and Houser, 1985a, and 1985b), see fig.3 page 31. Silicone oil (SF-1250, General Electric, Waterford, NY) was pipetted in 600 μ l aliquots in 1.5ml plastic microcentrifuge tubes (Simport Plastic, Boleuil, Que.). About 4.0ml of the suspension of isolated cells was equilibrated for 20 min. at 37°C with tritiated water. The extracellular marker molecule, ^{35}S (sulfuric acid, ICN Radiochemicals, Irvine, Ca., cat. nr. 64040) was added to the cell suspension just prior to layering 500 μ l aliquots (80,000-100,000 rod cells) on the silicone oil.

The layered cell suspension was centrifuged for 20 sec at 10,000 g. This produced a three phase system with a cell pellet below the oil phase and the clear saline solution that previously suspended the cells, above the oil. Squashes of the pellets which were examined under a phase-contrast microscope, revealed that the rod shape morphology of the cells was preserved.

Fig 3. THE SILICONE OIL METHOD
CELL SUSPENSION

31

Before removing the clear saline supernatant, duplicates of 40 μ l aliquots were pipetted with a Hamilton syringe into 1ml of ice cold nitric acid. This acid digestate was used for measurement of extracellular volume (section 3, page 33), measurement of the total water (section 4, page 34), and measurement of extracellular ion concentration (section 5, page 35). The remaining supernatant was aspirated along with most of the oil, leaving a small layer of silicone oil over of the pellet to prevent evaporation of the pellet water. The plastic centrifuge tube was then cut with a sharp razor blade just above the cell pellet. The portion of the microcentrifuge tube which contained the pellet was allowed to drop into 1ml of ice cold nitric acid. It was important to keep the acid temperature low since the contact of the aqueous cell pellet with concentrated nitric acid is exothermic.

2. Digestion in nitric acid.

The cell pellets were digested in 1ml of nitric acid (Aristar, BDH Chemicals, Toronto, Ont., cat. nr. 45004). Digestion was done over night in an oven maintained at 37°C. Unlike the nylon, which dissolved in HNO₃, the plastic of the microcentrifuge tubes was inert to this treatment. Aliquots of the digestates were used in the

determination of extracellular volume (section 3, below), total water content (section 4, page 34), and ion content analysis (section 5, page 35).

3. Determination of the extracellular volume.

The extracellular volume, V_{ex} , was calculated from the radioactivity carried by the extracellular marker molecule in the cell pellet, and the radioactivity in a measured volume of the incubation saline, according to equation 2, on page 28.

Samples of 20 μ l volume (V_{ms}) of supernatant of the centrifuged cell suspension were pipetted in 1ml of nitric acid and digested. Subsequently, 5-10 aliquots of 40 μ l from the cell pellet (A_{ex}) and sample volume (A_{ms}) digestates were pipetted into separate 10ml volumes of scintillation fluid (Amersham Co., Oakville, Ont., cat. nr. 196290). In the double label silicone oil experiments (3H_2O , and $H_2^{35}SO_4$), the microprocessor controlled counter (Beckman LS7500, Beckman Inst. Co.) was calibrated with quenched standards (tritium and carbon 14, Beckman Instrument Co., Fuller, Ca). Sulfur 35 has an energy spectrum similar to carbon 14 but is short lived (half life of 80 days) and therefore unsuitable for preparation of commercial quenched

standards. The samples were then counted for 20-50 min each and the data were automatically converted into decays per minute (dpm) units (see Appendix I, page 99, for a detailed account of the calibration procedure).

4. Determination of total water.

On the assumption that the tritiated water equilibrated uniformly with the intracellular and extracellular compartments, the total volume of water of the cell pellet was measured using a similar procedure as for the extracellular volume measurement. The water volume was calculated from the ratio of tritium activity present in the cell pellet and the tritium activity in a measured volume of supernatant:

$$V_T = (A_T/A_S) \cdot V_S \quad (3)$$

where,

V_T = total water volume in the cell pellet (μ l).

A_T = tritium radioactivity in the cell pellet acid digestate (dpm).

A_S = tritium activity in a 40 μ l aliquot of the acid digestate of the (20 μ l) sample volume pipetted from the supernatant saline following centrifugation (dpm).

V_S = the measured volume (20 μ l) pipetted from the supernatant into the 1ml. of nitric acid.

5. Ion content measurement.

This step was identical in both the nylon mesh and the the silicone oil methods.

The value of total water volume (see section 4, above) was used to determine the fraction of extracellular space (F_{ex}), as well as to convert the total ion content from ppm into mM units:

$$[X]_T = (X/AW.V_T).D \quad (4).$$

where,

$[X]_T$ = determined total concentration of Na^+ or K^+ ion concentration in mM.

X = measured concentration of sodium or potassium ions in ppm.

AW = atomic weight.

V_T = total water volume of the cell pellet or the cell suspension retained on the nylon mesh (μ l)

D = dilution factor (i.e. calculation of the dilution factor is explained in Appendix II, page 108.

Sodium and potassium ion concentrations in the acid digestates were measured by a Perkin Elmer 703 Atomic

Absorption Spectrophotometer. Sodium was measured on the absorption band (589nm) and potassium on the emission band (767nm). Standards (0.5, 1.0, 1.5, 3.0 ppm) were prepared from 1000ppm standards (Fisher Scientific, Pittsburgh, Pa., cat. nr. 13-639iNa, 15-639iK). The blank accounted for the ion contamination in the acid since it was used as a nominally zero intercept on the internal absorption vs. concentration calibration curve. Nitric acid but no Na^+ or K^+ was added to this blank at the same range of dilution with double distilled deionized water as the cell preparation. Nitric acid was also added to the ion concentration standards at the same range of dilution as the cell preparation and extracellular volume controls. The spectrophotometer was internally calibrated to convert absorption or emission units into ppm units.

800 μl aliquots of the digestates of a preparation and controls were diluted 1:50 with double distilled deionized water. This dilution resulted in concentrations which were within the linear range of the calibration curve (0.1-3.0 ppm) of the spectrophotometer. Duplicates of averages of three consecutive measurements were not different by more than 1% and the measurements did not shift in time. The concentration of intracellular ion was then calculated according to equation 1 on page 22.

Typically, F_{ex} in the nylon mesh experiments ranged

between 0.5-0.8 and was higher for the cell pellets in the silicone oil experiments, i.e. 0.7-0.9.

E. THE CELL CULTURE EXPERIMENTS

The method of ion analysis for cell cultures was a modification of that for the cell suspensions in the silicone oil experiments (fig.4, page 38). It was designed to reduce the higher F_{ex} carried by the cover slips of the cell cultures. The modifications included the use of an oil of a higher density and viscosity than used for the isolated cells and which was more effective in removing the saline from the cover slips. Also, Na^+ -free wash was used to reduce the contribution of the extracellular sodium ion to the total sodium content present on the cover slips. The Na^+ -free wash is rapid (i.e., 10 sec.) relative to the rate of intracellular sodium loss in sodium free saline, which was previously found to be (1.7 ± 0.2) mM per min. in isolated feline myocytes (Silver and Houser, 1985b).

Twenty ml of a sodium-free M199-HEPES (HEPES: N-2-hydroxyethyl- piperazine- N'-2-ethanesulphonic acid, Sigma Chemical, St. Louis, Mo., cat. nr. H3375) buffered saline had tritiated water pipetted at 20 μ l/ml from a stock solution (containing 100 μ Ci/ml of tritiated water)

Fig 4. METHOD FOR CELL CULTURE ANALYSIS

prepared in the same kind of saline. This solution was then divided into two 10ml aliquots. Into one of the aliquots NaCl was pipetted from a 1M stock solution prepared in double distilled water to a final concentration of 130mM. Three to six cultures on coverslips, containing an estimated 10,000 to 30,000 myocytes each, were washed by immersing in a series of three petri-dishes containing the NaCl HEPES-M199 incubation saline warmed to 37°C and then incubated (37°C, 20 min.) in this saline. The other 10ml aliquot of the saline had sucrose added to it instead of NaCl, isotonicity (i.e. to a final osmolarity of 280-290mOsm) and was kept in an oven at 37°C until ready to use.

At the end of the 20 min., ³⁵S (sulfuric acid) was added (10μCi/ml) from a stock solution made in sodium-free M199-HEPES saline to the sucrose-HEPES-M199 saline. Five mls of this saline was layered on 10mls of silicone oil (SF 1265, General Electric, Waterford, NY) in a 15ml plastic test tube. The cover slips were transferred to 2-3 washes of the sodium-free sucrose solution and then passed into the prepared 15ml plastic test tube. The heavy density oil in the test tube reduced the extracellular volume by 2%-10% (i.e. ,F_{ex} was reduced from an initial value of 0.99 to final values of 0.90 to 0.98,.

The incubation saline was removed from above the oil phase along with some of the oil. This was done to avoid any possible contamination with saline of a nitric acid phase which was next injected below the oil phase. Four mls of nitric acid were injected below the oil phase with a plastic syringe. This volume was enough to completely immerse the cover slips. The oil was then removed leaving only a small layer over the acid phase, the test tubes were sealed with a teflon stopper and shaken occasionally for the next 20 min. forming the acid digestate.

The activity of tritium and (^{35}S)-sulfate was measured in eight replicates of 40 μl each, pipetted from the acid digestate into 10ml of aqueous scintillation fluid and counted on a Beckman LS7500 as was done with the cell suspension digestate of the silicone oil method (see page 32).

Two mls of the acid digestate were diluted with 6ml of deionized double distilled water and analysed for sodium and potassium content as described for the silicone oil method (section 5, page 35). The sample volume was pipetted in duplicates of 40 μl per 4ml of nitric acid from the sucrose substituted, sodium-free saline. Four aliquots of 40 μl from these sample volume digestates were pipetted in scintillation fluid and counted for tritium and (^{35}S)-sulfate activity as before (section 3, page 33).

Typically the total water volumes carried into the nitric acid by the cover slips and their cultures were 10-30 μ l of which 0.6-2 μ l was cellular water, therefore giving a value of F_{ex} of 0.93 to 0.98.

Duplicates of 1ml of these calibration digestates were diluted 1:40 with deionized double distilled water and analysed for ion content as previously (section 5, page 35). The intracellular sodium and potassium ion concentration was then calculated according to equation 1 of Materials and Methods (page 22).

RESULTS

A. ISOLATED MYOCYTES

1. Morphological heterogeneity of the preparation.

Preparations of newly isolated cells contained myocytes of two distinct morphological forms. One type was rod shaped with clear cross-striations, the other consisted of round, hypercontracted cells. The rod shaped cells comprised a mean of $60 \pm 3\%$ of the cell population suspended in the isolation saline (Ca^{++} -free). When the cells were resuspended in culture medium 199 (Ca^{++} :1.3mM), the proportion of rod shaped cells was decreased to 50% to 45%. The proportion of rod cells was further reduced if calcium ion concentration in the resuspending media was increased (Fig.7, page 58).

2. Measurement of intracellular sodium and potassium concentration.

Silver and Houser, 1985a, have shown that the sodium fraction of the cell pellet increased with the amount of round, hypercontracted cells present in a preparation of

newly isolated feline ventricle cardiomyocytes. The symbols in fig.5 are data from experiments in this study in which tritiated-inulin was the extracellular marker molecule (nylon mesh method). The broken line is the linear regression for these data. The continuous line is a linear regression for data in Silver and Houser, 1985a. Their data are plotted as the sodium fraction of the cell pellet (F_{Na^+}) i.e., the ratio of total measured sodium concentration ($[Na^+]_T$), to the sum of total ion content (sodium and potassium) in the cell preparation:

$$F_{Na^+} = [Na^+]_T / ([Na^+]_T + [K^+]_T) \quad (5)$$

where,

F_{Na^+} = the sodium fraction of the cell pellet.

$[Na^+]_T$ = total sodium ion concentration measured in the cell pellet (mM).

$[K^+]_T$ = total potassium ion concentration measured in the cell pellet (mM).

The linear relationship between the sodium fraction of the cell pellet and the percent hypercontracted cells (fig.5) suggests that the hypercontracted cells may be at equilibrium with extracellular sodium. In line with this hypothesis, the sodium fraction of the cell pellet extrapolated for 100% hypercontracted cells from the linear regression of data in this study and Silver and

Fig. 5 Sodium fraction of the cell pellet ($\text{Na}^+_{\text{T}}/(\text{Na}^+_{\text{T}} + \text{K}^+_{\text{T}})$) as a function of percent hypercontracted cells. The solid line is the linear regression of the sodium fraction of the cell pellet in a preparation of cat ventricle myocytes (Silver and Houser, 1984a). The symbols are some of the experiments in this study in which ^3H -inulin was the extracellular marker (isolation saline, nylon mesh method). The broken line is the linear regression for this data. The table below gives the sodium fraction of the cell pellet at 0% and 100% hypercontracted cells (i.e. extrapolated from the linear regression). The values in the brackets are the ratio of total sodium to total sodium plus total potassium concentration in the suspending salines (i.e. $[\text{Na}^+]_{\text{e}} : 130\text{mM}$, $[\text{K}^+]_{\text{e}} : 4.5\text{mM}$ for Silver and Houser's data and $[\text{Na}^+]_{\text{e}} : 90\text{mM}$, $[\text{K}^+]_{\text{e}} : 30\text{mM}$, isolation saline, this study).

		SODIUM FRACTION OF THE CELL PELLET	
PERCENT HYPERCONTRACTED CELLS		SILVER AND HOUSER	THIS STUDY
	0	0.14	0.28
100		0.98 (0.96)	0.78 (0.75)

Houser (1985b) data corresponds closely to the ratio of total sodium to total sodium plus total potassium concentration in the salines suspending the myocytes (i.e. numbers in brackets, table below fig.5). A similar estimate for 0% hypercontracted cells is not possible to obtain since it requires knowledge of extracellular volume which varies from preparation to preparation (see equation 1, page 22). If the, presumably nonviable, hypercontracted cells are in equilibrium with ions in the surrounding saline, then for purposes of analysis of intracellular sodium and potassium in the rod cells, the hypercontracted cells internal volume is equivalent to extracellular volume. It is then of utmost importance that the molecule used to measure extracellular volume be able to freely permeate the membrane of hypercontracted cells but not the membrane of rod cells. Since the experiments in figure 2 suggested that inulin may not permeate the hypercontracted cells, two smaller molecular weight extracellular volume markers were tested, ^{14}C -sucrose and ^{35}S -sulfate.

The extracellular marker molecules: ^3H -inulin, ^{14}C -sucrose and ^{35}S -sulfate, equilibrate different amounts of extracellular space. Data in fig.6 are from 2 experiments in which extracellular volumes were measured in the same pellet (silicone oil method) by ^{14}C -sucrose and ^3H -inulin molecules, or by ^{35}S -sulfate and

Fig. 6. Extracellular volume ratios in two experiments (values shown in brackets) in which two extracellular markers were used simultaneously (silicone oil method). The results indicate that extracellular markers may equilibrate different amounts of extracellular volumes. The largest fraction of the extracellular volume is equilibrated by sulfate followed by sucrose and inulin. Note that of the three compounds, inulin has the largest molecular weight (i.e. approximately 5,000).

^3H -inulin molecules. Similar results (not shown) were obtained comparing ^3H -inulin and ^{14}C -sucrose volumes by the nylon mesh methods. Relative to inulin, sulfate measures 40% more extracellular volume, while the extracellular volume indicated by sucrose is about 24% larger than that with inulin. The molecular diameter of these compounds is inulin:sucrose:sulfate = 30:4.5:3.8 Ångströms (Page, 1968).

To test whether sulfate permeates the intracellular volume of hypercontracted cells, intracellular sodium and potassium concentrations were measured in preparations having different proportions of hypercontracted cells (silicone oil method, $\text{H}_2^{35}\text{SO}_4$) and compared with similar experiments using ^3H -inulin to measure the extracellular volume (nylon mesh method).

Table 1 compares the mean determined intracellular sodium and potassium ion concentration in experiments in which either ^3H -inulin (nylon mesh method) or ^{35}S -sulfate (silicone oil method) were used as extracellular markers. The myocytes were analysed for ion content either after isolation (isolation saline) or following equilibration in medium 199.

When sulfate was the extracellular marker molecule, the intracellular sodium ion concentration was independent of

the percentage of hypercontracted cells (see Appendix III, page 109, and Discussion, page 66). For the experiments in which inulin was the extracellular marker, myocytes showed a statistically significant increase (t-test, $p < 0.01$) in the estimated mean intracellular sodium ion concentration relative to myocytes equilibrated in low sodium, isolation saline. The myocytes equilibrated in M199, in experiments which used sulfate to measure the extracellular volume, showed a small but statistically significant decrease (t-test, $p < 0.05$) in intracellular sodium ion concentration relative to myocytes equilibrated in the isolation saline. Note, also, that there is a large difference between the intracellular sodium concentration determined with sulfate and inulin for myocytes suspended in medium 199, which has 40mM more sodium than the isolation saline. This is consistent with the hypothesis that, unlike ^{35}S -sulfate, ^3H -inulin may not permeate a population of myocytes which have a high intracellular sodium concentration. Also shown in this table are estimated values of change in intracellular sodium and potassium concentration (see Appendix III, page 109, and Discussion, page 66).

It was previously reported and also observed in the present study that increasing extracellular calcium induced an increasing proportion of hypercontracted cells (Farmer et al., 1983). It was also reported that

Table 1. Determined mean intracellular sodium and potassium ion concentration in isolated myocytes. Also shown is the change in intracellular sodium and potassium ion concentration calculated by assuming that experiments using sulfate as extracellular volume marker represent the intracellular concentration of rod shaped myocytes but an additional 20%, 30%, 50% of extracellular volume would not be measured by the extracellular marker molecule. Note that the values of estimated sodium concentration calculated for 30% (isolation saline) and 50% (MI99) correspond closely to intracellular sodium ion concentration determined when inulin was the extracellular volume marker. In the case of potassium ion a value of 20% ⁹ AND is required for the estimate to be close to the intracellular potassium ion concentration determined by inulin. For a sample of this calculation see Appendix III.

Extracellular volume marker	Intracellular ion concentration (mM)	Mean SEM	Percent hypercontracted cells (range)	(A) Calculated change in Na ⁺ and K ⁺ (range)	
				Na ⁺	K ⁺
Isolation solution	³⁵ S-SULFATE n=21 N=8	Na ⁺ 59 ± 12	30 (20 to 50)	68 ± 14 (65 to 75)	8
		K ⁺ 111 ± 13	30 (20 to 50)	87 ± 32 (95 to 71)	
	³ H-INULIN n=11 N=7	Na ⁺ 68 ± 12			
		K ⁺ 98 ± 16			
Medium 199	³⁵ S-SULFATE n=32 N=6	Na ⁺ 51 ± 20	35 (20 to 50)	80 ± 32 (70 to 91)	
		K ⁺ 98 ± 22	35 (20 to 50)	67 ± 15 (79 to 52)	
	³ H-INULIN n=12 N=7	Na ⁺ 96 ± 15			
		K ⁺ 81 ± 20			
A- I _{calc} = F _{rad} I _{SO4} + F _{round} I _{ex} n- number of experiments					
B- estimated SEM N- number of cardiac preparations three (b) and two (1) preparations were common.					

intracellular sodium concentration decreases in response to increasing calcium concentration in the absence of a large change, i.e. only 5%, in the population of hypercontracted cells (Desilets and Horackova, 1982). Therefore, changing extracellular calcium concentrations may be used in the following way to further test the hypothesis that sulfate fully permeates the hypercontracted cells:

If sulfate would not fully permeate the hypercontracted cells, the expected result would be an increase in the determined intracellular sodium concentration. Due to the high extracellular sodium concentration and the increase in the hyperpermeable myocytes with increasing extracellular calcium concentrations. Alternatively, if sulfate does fully permeate with the hypercontracted myocytes, the results would show a decrease in intracellular sodium concentration. Similarly, if calcium induced hypercontraction selectively eliminates sodium loaded myocytes a reduction in intracellular sodium ion concentration would be observed upon resuspension in calcium containing salines.

3. Effect of changes in extracellular calcium ion concentration on the intracellular sodium and potassium concentrations of isolated myocytes.

The determined intracellular sodium concentration in the sulfate experiments, in which cells were resuspended in M199 was 14%-40% lower than that of cells incubated in the isolation saline (Table 2, A). Potassium was also decreased, but to a lesser extent than intracellular sodium concentration. The calcium ion concentration is increased from nominally calcium free in isolation saline to 1.3mM in the M199.

Data in table 2,B and figure 7,A are for experiments in which calcium was added to the cell preparations suspended in isolation saline, at different concentrations, for less than one minute (10 sec.) prior to centrifugation through silicone oil. This procedure resulted in a decrease in the intracellular sodium at a rate of 16% per mM $[Ca^{++}]$ added (i.e. the slope of change in intracellular sodium concentration as a function of extracellular calcium, assuming a linear decrease in intracellular sodium ion concentration in Fig.7 A). The drop in intracellular sodium correlated with an increase of up to 40% in the hypercontracted cells (at 5mM $[Ca^{++}]$, Fig.7 B). The intracellular potassium was also decreased. However, this decrease in intracellular potassium concentration was less clearly dependent on changes in extracellular calcium ion concentration. Two of the changes in intracellular

Table 2. Intracellular sodium and potassium concentration and change of intracellular sodium and potassium concentration relative to nominally zero calcium isolation saline (silicone oil method, $H_2^{35}SO_4$).

A. Intracellular sodium and potassium ion concentration in three experiments (quadruplicates each) in which myocytes in isolation saline ($[Na^+]_e$: 90mM, $[K^+]_e$: 30mM, $[Ca^{++}]_e$: 0mM) were resuspended in medium 199 ($[Na^+]_e$: 130mM, $[K^+]_e$: 4.5mM, $[Ca^{++}]_e$: 1.3mM; and allowed to equilibrate in this medium for 40 min. Note that relative to isolation saline $[Na^+]_i$ showed a significant decrease in all experiments while potassium was significantly decreased in only one experiment when myocytes were resuspended in M199.

B. Intracellular sodium and potassium concentration in three experiments (labeled 1,2,3, duplicates each.) in which preparations of myocytes in isolation saline were exposed for a brief time to different concentrations of calcium. Calcium was pipetted from a stock solution shortly before centrifugation through silicone oil. Note that intracellular sodium concentration is decreasing with increasing extracellular calcium concentrations. The change in intracellular potassium concentration seems to be less clearly dependent on extracellular calcium concentration. These data are also plotted in figure 4.

**CHANGE IN INTRACELLULAR SODIUM AND POTASSIUM ION
CONCENTRATION UPON RESUSPENDING IN MEDIUM 199**

ISOLATION SALINE			MEDIUM 199		PERCENT CHANGE IN INTRACELLULAR SODIUM AND POTASSIUM RELATIVE TO ISOLATION SALINE	
	[Na ⁺] _i (mM)	[K ⁺] _i (mM)	[Na ⁺] _i (mM)	[K ⁺] _i (mM)	SODIUM	POTASSIUM
1	56±7	98±4	29±9	101±10	48**	3 ns
2	76±1	123±4	67±11	117±6	12*	5 ns
3	62±9	115±13	48±4	87±12	23**	29**

MEANS±SEM

n=4 for all means

** p<0.01

* p<0.05

ns—not significant

**CHANGE IN INTRACELLULAR SODIUM AND POTASSIUM ION CONCENTRATION
UPON INCREASING EXTRACELLULAR CALCIUM ION CONCENTRATION**

Ca ⁺⁺ _e (mM)		[Na ⁺] _i (mM)	[K ⁺] _i (mM)	PERCENT CHANGE IN INTRACELLULAR (Na) & (K) RELATIVE TO ZERO-CALCIUM SALINE	
				SODIUM	POTASSIUM
0	1	38±5	85±7	0	0
	2	46±2	120±10		
	3	60±6	95±4		
1.5	1	35±7	83±1	8	26
	2	42±4	105±7	16±7*	12 17±8*
	3	41±5	90±6	32	12
3.0	1	33±6	68±1	13	29
	2	27±5	115±12	41 30±18**	4 12±10ns
	3	15±2	90±6	75	6
5.0	1	16±3	70±3	56	18
	2	14±2	96±6	70 67±13**	25 24±5*
	3	10±3	68±4	83	28

MEANS±SEM

n=2 for all means

** p<0.01

*p<0.05

ns—not significant

Fig. 7 A. Mean intracellular sodium and potassium ion concentration as a function of extracellular calcium concentration for data shown in table 2B (silicone oil method, $\text{H}_2^{35}\text{SO}_4$). Note that intracellular sodium ion concentration decreases with increasing extracellular calcium concentration. The relationship between $[\text{K}^+]_i$ and $[\text{Ca}^{++}]_e$ is less clear (the value at 3mM is not significant from the control value at nominally zero $[\text{Ca}^{++}]_e$).

B. Percent hypercontracted cells as a function of extracellular calcium concentration for the data shown in fig. 4A. The percentage of hypercontracted cells increases with increasing extracellular calcium concentrations.

**INTRACELLULAR SODIUM AND POTASSIUM ION CONCENTRATION
AS A FUNCTION OF EXTRACELLULAR CALCIUM ION CONCENTRATION**

**PERCENT HYPERCONTRACTED CELLS AS A
FUNCTION OF EXTRACELLULAR CALCIUM ION CONCENTRATION**

potassium concentrations in table 2A and the change at 3.0mM in table 2B are not significantly different (t-test, $p > 0.05$).

B. CULTURED CARDIOMYOCYTES

1. Measurement of intracellular sodium and potassium ion concentration.

Having a method that gave consistent intracellular sodium and potassium concentrations (silicone oil, ^{35}S -sulfate) in isolated myocytes, it was possible to extend it to measurements of the intracellular concentration of these ions in cultured myocytes.

Measurements of intracellular sodium and potassium concentration in cultured cardiomyocytes indicate that the concentration of these ions changes along with membrane potential (Table 3).

Initially, for cultures with cells that are primarily in the round morphological stage (5-7 days old, Schwarzfeld and Jacobson, 1981) there is a 20% drop in the intracellular potassium concentration relative to the newly isolated cells, resuspended in M199 (table 3).

The value for intracellular sodium concentration is lower than, but not significantly different from that of

Table 3. Intracellular sodium and potassium concentration, membrane potential (E_m , data from fig.1) and calculated P_{Na^+}/P_{K^+} ratios in isolated myocytes (elongated)^a and at different times in culture.^b

Data from Meiers, Briggs and Claycomb, 1986, are for the 'redifferentiated model' of adult rat ventricular myocytes in culture 12 to 28 days old. Note that their preparation was incubated in 5mM Ca^{++} -containing saline prior to recording the membrane potential, which was used to calculate the value of P_{Na^+}/P_{K^+} . For the method of calculation of P_{Na^+}/P_{K^+} see Discussion, page 80.

a. Data for ion analysis (silicone oil method, 35S) also shown in table 1 and table 4.
b. Data for mean values also shown in table 5.

TABLE 3. INTRACELLULAR SODIUM AND POTASSIUM CONCENTRATION, MEMBRANE POTENTIAL, AND SODIUM TO POTASSIUM PERMEABILITY RATIO IN ISOLATED MYOCYTES AND AT DIFFERENT TIMES IN CULTURE

STAGE (days)	n, N ION ANALYSIS	$[Na^+]_i$ (mM)	$[K^+]_i$ (mM)	n MEMBRANE POTENTIAL	E_m (range) ¹ (mV)	P_{Na}/P_K (range) ²
ELONGATED isolation saline TS (0)	21, 8	59 ± 12	111 ± 13	21 ^①	-25 ± 10 (-80 to -20)	0.22 (0.20-0.38)
ELONGATED medium 199 TS (0 - 0.16)	31, 6	51 ± 20	36 ± 22	30	-47 ± 4 -75 ± 2 ^② (-100 to -20)	0.11 0.02 (0.01-0.40)
Meier et al., 1986 (12-29)	2, 12	30 ^③	140 ^④	11	-78	0.02
ROUND (5-7) TS	4, 15	44 ± 7	72 ± 4	16	-22 ± 14 (-50 to -10)	0.24 ± 0.03 (0.05-0.45)
SPREADING (8-10) TS	2, 14	14 ± 1	144 ± 14	5	-36 ± 7 (-58 to -20)	0.28 ± 0.02 (0.09-0.51)
SPREAD (15-35) TS	6, 34	28 ± 6	146 ± 17	126	47 ± 1 (-80 to -22)	0.17 ± 0.03 (0.02-0.31)

¹ POWELL et al. 1982
² RESULTS FROM GRACEOVA 1982
³ THIS VALUE IS ASSUMED
⁴ DETERMINED FROM ION ANALYSIS
 ①-④ MINIMUM AND MAXIMUM VALUES
 n=NUMBER OF EXPERIMENTS
 N=NUMBER OF CARDIAC PREPARATIONS
 ON COVER SLIPS

the isolated preparation equilibrated in the culture medium 199.

In the next stage of establishment (8-10 days in culture), myocytes, most of which are attached to the culture substratum and are spreading, have their intracellular potassium increased by 40% and intracellular sodium decreased by 60% relative to the newly isolated cells in M199. The value of intracellular potassium ion was not different from that of spread myocytes (15 to 35 days in culture). However, intracellular sodium was increased at this stage.

The time course of sodium to potassium membrane permeability ratio in cultured cardiomyocytes can be estimated from the time course of intracellular sodium and potassium concentration and the observed time course of mean membrane potential. This was done and the results are shown in table 3 (also see Discussion, page 79).

DISCUSSION

One question addressed by this study was how the intracellular sodium and potassium concentrations are changed from the time of cell isolation to increasing times in cultures of adult rat ventricular myocytes. Since the electrochemical gradients of these two ions across the sarcolemma can make a considerable contribution to the membrane potential of myocytes (Goldman, Hodgkin and Katz equation, Sperelakis, 1979) knowledge of intracellular sodium and potassium ion concentration may give an indication of the physiological integrity of the myocyte preparations (e.g. whether active transport or membrane permeability properties have changed). Furthermore, along with the values of membrane potential, intracellular sodium and potassium ion concentrations can be used to estimate the time course of change in sodium to potassium permeability ratio.

A. ISOLATED MYOCYTES

1. Is the concentration of ions in hypercontracted cells at equilibrium with that of extracellular ions ?

Silver and Houser, 1985a, concluded that the hypercontracted cells in a preparation of enzymatically isolated adult feline ventricular myocytes were at equilibrium with the ions in the extracellular saline. Their conclusion was based on three lines of evidence:

i. The sodium fraction of the cell pellet increased in proportion with the percent of hypercontracted cells in the preparation (F_{Na^+} , Fig. 5, page 54). On the other hand, the potassium fraction of the cell pellet was inversely related to the percent of hypercontracted cells (Silver and Houser, 1985a).

Silver and Houser argued that this was consistent with a model in which the hypercontracted cells are at equilibrium with the extracellular sodium concentration, i.e., higher than the intracellular sodium concentration of the rod shaped myocytes, and also in equilibrium with the extracellular potassium ion concentration, i.e., lower than the intracellular potassium concentration in the rod-shaped myocytes.

ii. Experiments which compared internal concentration of sodium and potassium in mixed populations of rod and hypercontracted cells before and subsequent to their resuspension in sodium free saline, showed that the total

sodium was significantly reduced while potassium remained unchanged. The reduction in sodium content was proportional to the number of hypercontracted cells present. This suggested that extracellular sodium was at equilibrium with the hypercontracted cells .

iii. In experiments which measured $^{42}\text{K}^+$ uptake, the offset of $^{42}\text{K}^+$ uptake (i.e. the uptake at time zero) increased with the percent of hypercontracted cells present. Silver and Houser argued that this was consistent with an instantaneous equilibration of the hypercontracted cells with the isotopic potassium (Silver and Houser, 1985a).

iv. Consistent with the hyperpermeability hypothesis is the observation that the membrane potential of hypercontracted cells is close to zero mV (Isenberg and Klockner, 1982). Isenberg and Klockner (1982) noted that as myocytes rounded up, the membrane potential transiently hyperpolarized (i.e. consistent with stimulation of an electrogenic transport) and finally jumped to zero. During the transient hyperpolarization the input resistance was 1 to 2 megohms and later dropped to values less than 200 kilohms.

If the hypercontracted cells are at equilibrium with the extracellular sodium and potassium then, what is estimated as intracellular ion (i.e. see equation 1, page 22) depends on the extent to which the extracellular marker equilibrates the volume of hypercontracted cells present in the cell suspension.

a) At one extreme, the entire fraction of hypercontracted cells may equilibrate with the extracellular marker. In this case, the inside of the hypercontracted cells is measured as extracellular space, and what is estimated as intracellular ion (sodium or potassium), represents the intracellular ion concentration of the rod shaped myocytes.

b). At the other extreme, if the extracellular marker does not permeate the hypercontracted cells at all, their volume is included as part of the intracellular volume. Then, the determined intracellular ion concentration ($[X]_i$, equation 1, page 22) can be approximated by the estimated intracellular ion concentration ($[I]_{EST}$) which is a linear function of the fraction of hypercontracted cells (F_{RND}) which are at equilibrium with the extracellular ions and, the concentration of that ion in the fraction of rod shaped myocytes (F_{ROD}):

$$[I]_{EST} = F_{RND}[I]_e + F_{ROD}[I]_i \quad (6)$$

where,

$I = Na^+$ or K^+ ,

F_{ROD} = number of rod cells / (number of rod cells + number of hypercontracted cells)

F_{RND} = number of hypercontracted cells / (number of rod cells + number of hypercontracted cells)

$F_{ROD} + F_{RND} = 1$;

i = intracellular (i.e. only in rod cells, such as determined by an ideal extracellular marker which fully permeates the hypercontracted cells).

e = extracellular.

The exact form of this function depends on the volume to volume ratio of the rod and hypercontracted cells, which for the purpose of this discussion will be assumed to be one. Note that this relationship predicts an increase in the estimated intracellular sodium concentration and a decrease in the estimated intracellular potassium concentration with increasing fractions of hypercontracted cells.

2. Which extracellular markers freely permeate the intracellular volume of hypercontracted cells ?

An observation that may, at first, seem discrepant is the large variability of sodium and potassium concentration in both the sulfate and inulin experiments (table 1). However, there are three independent lines of evidence which suggest that variable $[Na^+]_i$ and $[K^+]_i$ and variable membrane permeability to these ions may be genuine features of this myocardial preparation rather than variability due to experimental error.

i. Several authors have reported a wide distribution of membrane potential values in newly isolated myocytes:

Powell et al., 1980, isolated adult rat ventricular myocytes which had membrane potentials ranging between -20 and -80mV. Pelzer et al., 1984, isolated adult rat ventricular myocytes which had membrane potentials values ranging between 0 and -89mV. Desilets and Horackova, 1982, recorded membrane potentials in adult rat ventricular myocytes which had values between -70 and -90mV. However, the cells were selected from suspensions of myocytes in which not more than 5% of the cells hypercontracted when resuspended in salines with a physiological concentration of Ca^{++} . Juhasz, 1987, measured membrane potentials in newly isolated ventricular myocytes which ranged between -20 and -100mV.

ii. When inulin is the extracellular volume marker, the estimated concentration of intracellular sodium increases with the percent hypercontracted cells present (Appendix III page 109). This is consistent with a system in which the extracellular volume marker does not penetrate a fraction of the cellular volume at equilibrium with the extracellular sodium, and in line with the prediction of increase in the value of estimated intracellular sodium by equation 6 (presented in the previous section).

iii. The change in the determined mean intracellular sodium ion concentration in the experiments where inulin was used to measure the extracellular volume (table 1, page 51, last column) relative to that in the experiments where sulfate was used (table 1), corresponds to a change that can be predicted by assuming that a fraction of the intracellular volume (30%, isolation saline, 50%, medium 199) is not equilibrated by inulin but is at equilibrium with the extracellular sodium. The estimate for potassium ion concentration requires an assumption of 20% hypercontracted myocytes in both salines in order to approach the determined value of intracellular potassium concentration when inulin was the extracellular volume marker. One possible explanation for this discrepancy is that, unlike sodium ion, intracellular potassium ion may not freely and completely equilibrate with the

extracellular potassium ion. Experiments in which preparations of myocytes retained on nylon mesh were studied under scanning electron microscope (not shown) suggested that about 30% of hypercontracted cells in isolation saline were retained on the nylon mesh. The values of $[Na^+]_i$ and $[K^+]_i$ shown in the last column of table 1 was calculated using equation 6 (page 66). For the calculation it was assumed that the mean values of intracellular sodium and potassium, in the experiments where the extracellular volume was estimated using sulfate represented the true intracellular concentration ($[I]_i$) in the fraction of rod shaped myocytes (F_{ROD}). Then, taking the fraction of hypercontracted cells (F_{RND}) to be 30% (isolation saline) or 35% (medium 199), the predicted shift in intracellular sodium and potassium concentration ($[I]_{CALC}$), due to the contribution of hypercontracted cells was calculated. Table 1 also shows calculated changes for 20% and 50% hypercontracted cells. A sample of this calculation is shown in Appendix III (page 109).

iv. When the percent of hypercontracted cells (Fig. 7 A,B, page 57) was increased by exposing the suspension of myocytes to increasing concentrations of extracellular calcium, the intracellular sodium decreased. If the

additional fraction of hypercontracted cells induced by increasing concentrations of calcium were not permeable to the extracellular volume marker (sulfate) equation 6 (page 66) would predict an increase in the measured intracellular sodium content. Therefore, the results in fig.7 are consistent with the hypothesis that the sulfate ion permeated the additional volume created if the relatively impermeant rod cells were converted to hyperpermeable and hypercontracted cells.

Taken together, the above observations suggest that sulfate, but not inulin, permeates, and therefore may indeed equilibrate with the intracellular space of hypercontracted cells.

3. Intracellular $[Na^+]$ and $[K^+]$.

Table 4 compares measurements of intracellular Na^+ and K^+ concentrations in other mammalian ventricular preparations with data from this study. With the exception of the data of Isenberg and Klockner (1982, $1\mu M$ Ca^{++} saline, $E_M = -4mV$), $[Na^+]_i$ is higher and $[K^+]_i$ is lower in the newly isolated cells of this study than in either intact ventricles of rat and cat (Page, 1962, Page and Page, 1968) or other isolated preparations (Frangakis, 1982, Silver and Houser, 1985a, Powell et al., 1980).

Table 4
Intracellular Concentration of Sodium and Potassium

Preparation	Measurement		
	[Na] _i (mM)	[K] _i (mM)	[Na] _i /[K] _i
Tissue			
Cat papillary FS			
inulin space	43±5	162±3	0.25
manitol space	5±2	208±6	0.02
[Page, 1962]			
Perfused left ventricle of rat FS; H ₂ ³⁵ SO ₄	30±5	151±2	0.29
n = 45			
[Page & Page, 1968]			
Isolated Adult Myocytes			
Rat ventricular myocytes FS, ¹⁴ C - inulin	47	138	0.29
[Frangakis et al, 1982]			
Cat ventricle myocytes FS, n = 18	17±1	134±1	0.13
isotopic equilibrium, n = 15		135±4	
[Silver & Houser, 1985]			
Rat ventricular myocytes FS, ¹⁴ C - manitol, n = 24		122±2	
[Powell et al, 1980]			
Rat ventricular myocytes low Ca ⁺⁺ (1 μM)	65	90	0.72
ion selective electrode (assume act. coeff. of .72)			
[Isenberg & Klockner, 1982]			
Rat ventricular myocytes FS; H ₂ ³⁵ SO ₄			
low Ca ⁺⁺ , n = 21	59±12	111±13	0.53
M199, n = 32	51±20	98±22	0.52
(this study)			

FS is flame spectrometry (extracellular markers are also shown). n is the number of experiments. [Na]_i and [K]_i are intracellular sodium and potassium concentrations and [Na]_i / [K]_i is the ratio of these concentrations.

An observation that may, at first, seem discrepant is the large variability of sodium and potassium concentration in both the sulfate and inulin experiments (table 1). However, there are three independent lines of evidence which suggest that variable $[Na^+]_i$ and $[K^+]_i$ and variable membrane permeability to these ions may be genuine features of this myocardial preparation rather than variability due to experimental error.

i. Several authors have reported a wide distribution of membrane potential values in newly isolated myocytes:

Powell et al., 1980, isolated adult rat ventricular myocytes which had membrane potentials ranging between -20 and -80mV. Pelzer et al., 1984, isolated adult rat ventricular myocytes which had membrane potentials values ranging between 0 and -89mV. Desilets and Horackova, 1982, recorded membrane potentials in adult rat ventricular myocytes which had values between -70 and -90mV. However, the cells were selected from suspensions of myocytes in which not more than 5% of the cells hypercontracted when resuspended in salines with a physiological concentration of Ca^{++} . Juhasz, 1987, measured membrane potentials in newly isolated ventricular myocytes which ranged between -20 and -100mV.

ii. The large variability of percent hypercontracted cells in preparations of newly isolated cardiomyocytes in this study (Fig.7) suggests that there may be a heterogeneity in the degree of 0-Ca^{++} and other isolation induced lesions. This might be expressed as a variation in the distribution of $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ in different populations of rod shaped myocytes.

iii. $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ in preparations of cultured myocytes older than 7 days are less variable than in ii (table 3, page 60). This indicates that the intracellular sodium and potassium as well as the permeability to these ions may take a long time after isolation of cells from the heart to reach steady state.

It is, therefore, quite possible that variable $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ are an expression of a defect of the cells of the newly isolated preparation which is rectified with increasing time in culture.

4. Effect of changes of the extracellular calcium concentration on intracellular sodium and potassium concentration of isolated myocytes.

Transferring newly isolated myocytes from the Ca^{++} -free isolation saline into the culture medium

(1.3mM Ca^{++} , M199), or into saline containing increasing amounts of Ca^{++} (table 2 and Fig.7), significantly decreased the intracellular sodium in a concentration-dependent manner. This decrease may, however, be only apparent (see below). This change in intracellular sodium was paralleled by an increase in the percent of hypercontracted cells. Hypercontraction of the myocytes has been previously associated with an increase in the cytosolic Ca^{++} -content (Lambert et al., 1986) of isolated myocytes above a threshold value of 400nM. One possible explanation for this phenomenon is a reversed sodium-calcium exchange mechanism (Desilets and Horackova, 1982). In this mechanism one extracellular Ca^{++} is exchanged for 3 intracellular sodium ions (Mullins, 1979, Alexen and Bridge, 1985, Sheu and Fozzard, 1985) and therefore, directly reduces intracellular sodium content. The reversal of the sodium-calcium exchange may be directly related to the extent of sodium loading during preincubation in Ca^{++} -free saline (Wakabayashi and Goshima, 1981) since it is directly dependent on the electrochemical gradient for sodium. Therefore, myocytes that have high intracellular sodium concentrations when exposed to calcium-containing saline may be more prone to hypercontraction. Hypercontraction would then be expected to selectively eliminate myocytes that have a high

intracellular sodium concentration. Hence, this mechanism could also account for the observed reduction in $[Na^+]_i$ induced by the presence of calcium ion. Further experiments would be required to identify which of these mechanisms, or others, are responsible for the observed reduction of sodium concentration.

Intracellular potassium concentrations in the experiments of figure 7 are decreased when calcium ion in the resuspending medium is increased. However, this decrease in $[K^+]_i$ seems to be less clearly dependent on increasing calcium ion concentrations. It could be that membrane permeability to potassium is still relatively high in the calcium containing salines and that the Na^+, K^+ -ATP-ase may not be fully functional. An inhibition of Na^+, K^+ -ATP-ase activity by low $[Ca^{++}]$ salines has been reported previously (Lamers et al., 1984, Lelievre et al., 1984). The results of these studies also suggest that the recovery from this inhibition may take over 60 min.

In view of the drastic effects of unphysiological extracellular concentrations of calcium ion, observed here, on the intracellular sodium and potassium concentrations and the membrane permeability to these ions, caution must be exerted when interpreting membrane

potential values in preparations exposed to high or low concentrations of extracellular calcium ion.

B. CULTURED MYOCYTES

1. Time course of $[Na^+]_i$ and $[K^+]_i$ change in cultures of adult rat ventricular myocytes.

Concentrations of intracellular sodium and potassium (table 3 and table 5) change in parallel with the morphological and membrane potential changes of ventricular cardiomyocytes (Fig.1). As the myocytes round up in culture, intracellular potassium falls below that in rod shaped myocytes equilibrated in culture medium 199. The intracellular potassium ion concentration in 5 to 7 day old rounded myocytes was 72 ± 4 mM, as opposed to newly isolated rod shaped myocytes in M199, which had an intracellular potassium ion concentration of 98 ± 22 mM. Intracellular sodium ion concentration does not change significantly during this time.

Subsequent to 9 days in culture, $[K^+]_i$ increases and $[Na^+]_i$ decreases to a steady level ($[Na^+]_i$: 20 mM; $[K^+]_i$: 145 mM). However, the membrane potential is still changing at that time (Fig.1). This suggests that, initially, changes in membrane potential could be

Table 5

Intracellular sodium and potassium ion concentrations, membrane potential, ratio of sodium permeability to potassium permeability, and potassium equilibrium potential ($61 \log([K]_e/[K]_i)$) of cardiomyocytes at different times in culture (see text).

Days in culture	n	[K] _i (mM)	[Na] _i (mM)	E _m (mV)	P _{Na} /P _K	E _K (mV)	E _m /E _K
5 - 7	4	72±4	44±7	-22	.24±.03	-74	.30
9 - 10	2	144±14	14±1	-36	.26±.02	-92	.39
15 - 35	6	148±17	28±8	-47	.17±.03	-93	.51

due to changes in both ionic content and membrane permeability, while later (after nine days in culture) the changes are likely to be due primarily to changes in membrane permeability. This conclusion rests on the assumption that cell cultures used in this study follow the time course of membrane potential change described in the cultures studied by Juhasz, 1987, of figure 1.

Myocytes in the present study were cultured at densities higher than the standard practice with cultures used by Juhasz (greater than 10,000 myocytes compared to 5,000-8,000 myocytes per culture dish). The consequence of higher myocyte density was a frequent occurrence of foci of three or more adjacent cells which, when spontaneously active, appeared to contract synchronously. Nevertheless, as a first approximation it seems reasonable to assume that cells in the cultures of differing cell density were not significantly different. Preliminary measurements of membrane potential in cultured myocytes that are electrically connected suggest that the values of membrane potential are not significantly different from isolated cells in culture (Juhasz, personal communication).

2. Calculation of P_{Na^+}/P_{K^+} and its time course.

The sodium to potassium membrane permeability ratio

(P_{Na^+}/P_{K^+}) ratio was calculated using a simplified form of the Goldman, Hodgkin and Katz equation (equation 7, below, Sperelakis, 1979) which considers only the role of sodium and potassium ion concentration in production of the membrane potential. Contribution to the membrane potential by electrogenic transport (e.g. Na^+ , K^+ , ATP-ase) and by other cations and anions are ignored:

$$P_{Na^+}/P_{K^+} = \frac{([K^+]_i - [K^+]_e e^{-Em/26})}{([Na^+]_e e^{-Em/26} - [Na^+]_i)} \quad (7)$$

P_{Na^+}/P_{K^+} is sodium to potassium membrane permeability ratio.

Em is the membrane potential

the constant (RT/nF) of 26mV was calculated assuming 37°C and n , the valence of the ions, as 1, where R is the gas constant, T is the absolute temperature, and F is the Faraday constant.

This is a useful approximation (Sperelakis, 1979) since it allows for direct calculation of P_{Na^+}/P_{K^+} .

The evidence which justifies the assumptions used in deriving this simple form of constant field equation was summarized by Sperelakis, 1979:

i. The available evidence suggests that the contribution

- of the electrogenic sodium pump to the membrane potential is only a few mV and the ionic distribution is fixed.
- ii. The key factor controlling the membrane potential is the relative permeability to ions, particularly Na^+ and K^+ (i.e. $P_{\text{Na}^+}/P_{\text{K}^+}$). Other ions that have an effect on membrane potential or action potential are Ca^{++} and Cl^- .
- iii. The electrochemical gradient of Ca^{++} has only a small direct effect on the membrane potential and the resting membrane permeability to Ca^{++} is low.
- iv. The present available evidence suggests that Cl^- is passively distributed according to the membrane potential.

Table 3 and table 5 show the time course of $P_{\text{Na}^+}/P_{\text{K}^+}$ in cultures of myocytes 5-35 days old. These values were calculated using values of E_m from measurements made by Juhasz, 1987, (see figure 1).

Cultured cells 5-10 days old have high $P_{\text{Na}^+}/P_{\text{K}^+}$ ratios, ranging from 0.22-0.28. The permeability ratio drops to approximately half the initial value i.e., 0.13-0.19, after 15 days. These values of permeability ratios are 10-20 times higher than values described previously in other mammalian heart preparations (table 6), and in the newly isolated myocytes of this study after resuspension in M199.

Table 6
Membrane Properties of Different Myocardial Preparations

Preparation	Measurement									
	aNa _i (mM)	A _{cat} Na (mM)	aK _i (mM)	A _{cat} K (mM)	I _{Na} (A.cm ⁻²)	E _m or HPS (mV)	MiK (mol.cm ⁻² .s ⁻¹)	MeK (cm.s ⁻¹)	P _{Na} (cm.s ⁻¹)	P _{Na} /P _K
Cow Purkinje fibers [Carmeliet & Veronck, 1977]						-75			1.76x10 ⁻⁷	
Rabbit papillary [Lee & Fozzard, 1975]	6.4	0.21	83	0.61		-78				.005
Cat ventricle myocytes [Silver & Houser, 1985]	[17	-	135	-		-81	18±2	22±2	3.6x10 ⁻⁸	1.89x10 ⁻⁶ .02
Sarcolemmal vesicles from canine ventricle [Schilling et al, 1984]									1.8x10 ⁻⁹	1.1x10 ⁻⁸ .16
Rat ventricular myocytes low Ca ⁺⁺ (1 μM) [Isenberg & Klockner, 1982]	12	0.2	72	0.70		-4				.78
Cat papillary inulin space manitol space [Page, 1962]	[43 [5	- -	162 208			-75				.01 .02
Rat ventricle myocytes [NMR] 1 mM Ca ⁺⁺ 10 - 300 μM Ca ⁺⁺ [Wittenberg & Gupta, 1984]	*6.9 23	0.14 0.38				-80				
Neonatal rat, whole cell clamp [extrapolated to surface area of adult myocytes] [Wilson et al, 1985]					3.06x10 ⁻⁴	-55§			5.9x10 ⁻⁸	
Adult rat ventricular myocytes [Patlak & Ortiz, 1985]					2.86x10 ⁻⁴	-40§			4.7x10 ⁻¹¹	

aNa_i and aK_i are the intracellular activities of sodium and potassium and A_{cat} is the activity coefficient for these ions. I_{Na} is sodium current density. MiK and MeK are potassium influx and efflux respectively. P_{Na} and P_K are sodium and potassium permeabilities and P_{Na}/P_K is their ratio.

[This data is in concentration units.

* Activity coefficients were calculated assuming intracellular concentrations as per experiments in this study.

§ Holding potential for sodium current data

Meier et al., 1986, reported a preparation of adult rat ventricular myocytes in the 'redifferentiated model' of culture, 12-28 days old, which had an apparent P_{Na^+}/P_{K^+} of 0.02, for selected cells from the myocyte population (see below). In this case intracellular potassium concentration was determined by extrapolation to zero-potential of E_m vs. $\log[K^+]_e$. The potassium ion concentration was 140mM, and for lack of measurements, a $[Na^+]_i$ of 30mM (table 4) is assumed, here, in the calculation of sodium to potassium permeability ratio. However, the preparation of Meier et al., 1986, was equilibrated in high Ca^{++} saline (5mM) 40 min. prior to recording the membrane potential. The control experiment, membrane potentials recorded in physiological $[Ca^{++}]$ -saline, was not mentioned. Furthermore, the membrane potentials in Meier et al. were selected from a larger number of measurements. The criteria of selection may bias the calculation of permeability ratios towards lower values (this point is further discussed in the next section). Therefore, it is questionable whether the low P_{Na^+}/P_{K^+} calculated from the data of Meier et al., 1986, was the physiological value before the calcium treatment.

Changing P_{Na^+}/P_{K^+} values in cell culture have been previously observed in cultures of embryonic chick hearts

of different ages (Pappano and Sperelakis, 1969, McDonald and DeHaan, 1973, Sperelakis, 1979). The data were consistent with an increase in P_K^+ in the older embryos, resulting in a decrease of P_{Na^+}/P_K^+ ratio.

The activity and turn over of the Na^+,K^+ -ATP-ase (sodium pump) in cultures of chick embryo hearts (Kim et al., 1984, Kim and Smith, 1986) could be altered by changes in the intracellular and extracellular sodium and potassium ion concentration. Chronic reduction of the extracellular potassium below the half maximal value for the Na^+,K^+ -ATP-ase activation, resulted in an increase in the number of the sodium pump sites (Kim et al., 1984). This procedure has the effect of initially raising the intracellular Na^+ concentration, presumably by inhibiting the ATP-ase.

Exposure of cultured chick ventricular myocytes to decreasing concentrations of external sodium, decreased the intracellular sodium within 30 min. and resulted in a significant decrease in the number of sodium pump sites at two days in culture (Kim and Smith, 1986). It seems possible that such a modulation of sodium pump activity may take place in the cultures of the present study given the marked alterations in the intracellular sodium and potassium concentration with time in culture. This

requires further investigation.

High P_{Na^+}/P_{K^+} ratio and channels of low sodium to potassium selectivity have been previously described in mammalian preparations. For example, Shilling et al., 1984, isolated sarcolemmal vesicles, from canine ventricle, which had a P_{Na^+}/P_{K^+} ratio between 0.1 and 0.2 (table 6).

Matsuda, 1983, described a Ca^{++} -activated inward current in enzymatically isolated guinea-pig ventricular myocytes which had a reversal potential around -20mV, far from the Nernst potential of any of the major ions. If such channels contribute to the membrane potential of cultured myocytes, they may offer an explanation for the low sodium to potassium selectivity of the membranes of these cells.

Coronado and Latorre, 1982, described calf sarcolemmal channels which, subsequent to their incorporation in planar lipid bilayer, exhibited three types of behavior. One type of channel did not discriminate between Na^+ and K^+ ions, a second had a P_{Na^+}/P_{K^+} ratio of 0.33, while a third channel had a P_{Na^+}/P_{K^+} ratio of 0.2.

Whether the myocytes described in this study exhibit an increase in the density of low permselectivity channels, a modification of the usual Na^+ and K^+ channels, or

whether some other phenomenon is responsible for the high value of P_{Na^+}/P_{K^+} ratio, cannot be established by the available data.

To summarize, it appears that isolated myocytes in this study exhibit two types of permeability changes. Initially, it is probable that the low calcium concentration in the isolation saline and other isolation induced lesions, increase the membrane permeability to both Na^+ and K^+ (i.e. P_{Na^+}/P_{K^+} of 0.4 assuming a resting potential of -20mV, as argued by Powell et al., 1980), since the intracellular sodium concentration is increased and intracellular potassium concentration is decreased relative to in situ preparations. The membrane permeability ratio partially recovers when the isolated myocytes are resuspended in physiological saline (M199, Ca^{++} -1.3mM). The P_{Na^+}/P_{K^+} ratio approaches in situ values at this time (0.02, using a value of E_m of -75mV). Note that the mean membrane potential of newly isolated myocytes in fig.1 is more depolarized than -75mV, i.e. -47mV. However, one third of the newly isolated myocytes had membrane potentials ranging between -73mV and -100mV.

A second drop in the permeability ratio takes place when the cultured myocytes are entering the round stage morphology. Values of membrane permeability ratio

(P_{Na^+}/P_{K^+}) at this stage range from 0.23 to 0.28 (calculated for mean membrane potential of -22mV). The P_{Na^+}/P_{K^+} ratio then recovers to a steady level of 0.13 to 0.18 (beyond 10 days, when the value of mean E_M is -50mV) which is larger than the value of P_{Na^+}/P_{K^+} for cells in the intact heart (see table 6).

3. Range of P_{Na^+}/P_{K^+} ratios in cultured myocytes.

The values of sodium to potassium permeability ratios in the previous section (also see table 3 and table 5) were calculated using mean values of membrane potential and intracellular sodium and potassium concentration. It is very likely, however, that the permeability ratios in individual myocytes may differ. This question is particularly relevant when interpreting data such as those of Meier, Briggs and Claycomb (1986). The criterion of selection of membrane potential values in that study (i.e. membrane potentials which were stable for more than five minutes) may bias the mean resting potential towards more negative values.

An estimate of the range of sodium to potassium permeability ratios in the cultured myocytes can be obtained by calculating the P_{Na^+}/P_{K^+} ratios for the maximum and minimum values of membrane potentials in

individual myocytes at given stages in culture (table 5), and using measured values of mean $[K^+]_i$ and $[Na^+]_i$. Measurements of intracellular sodium and potassium ion concentration in individual myocytes are not available. Cultures of myocytes in the round morphological stage (5-7 days old) had membrane potentials ranging from -10 to -50mV (n=16). Calculation of sodium to potassium permeability ratios for intracellular concentrations of sodium and potassium in table 3 gives sodium to potassium permeability ratios ranging from 0.05 to 0.45 (table 3, range). Similarly, calculated values of sodium to potassium permeability ratios for the range of membrane potentials in spreading myocytes (9 to 10 days, -20 to -58mV, n=5) are between 0.09 and 0.51 and that of spread cells (15 to 35 days, -22 to -80mV, n=155) may vary from 0.02 to 0.51. Therefore, low permeability ratios are a possible occurrence in those individual cultured myocytes which have high resting potentials.

4. Activity coefficients are required for a correct estimate of P_{Na^+}/P_{K^+} ratio.

The P_{Na^+}/P_{K^+} ratios in table 3 were calculated using the intracellular concentration of Na^+ and K^+ and assuming that the activity coefficients for these ions

are one, in the cytosol and saline. However, this may not be the case, as has been demonstrated by Lee and Fozzard, 1975, in rabbit papillary muscle preparation (Table 3). Notably, the intracellular Na^+ activity coefficient was 0.21, indicating that a large fraction of $[\text{Na}^+]_i$ may be sequestered by intracellular organelles (e.g. sarcoplasmic reticulum, mitochondria). Therefore, as pointed out by Lee and Fozzard (1975) this is an "apparent activity coefficient" and is much lower than would be expected if all of the measured intracellular sodium concentration was in a homogenous solution (i.e. the activity coefficient for sodium in a homogeneous saline solution is 0.745, Lee and Fozzard, 1975). Substitution of ion activity for concentration, using the Lee and Fozzard data (equation 7, page 80) reduces the $P_{\text{Na}^+}/P_{\text{K}^+}$ ratio from 0.01 to 0.005.

Wittenberg and Gupta, 1984, measured intracellular sodium in enzymatically isolated adult rat myocytes using a $^{22}\text{Na}^+$ -NMR technique (Table.3). Since this measurement may represent the cytosolic free Na^+ content, it is possible to extrapolate an estimate of intracellular activity coefficient in conjunction with the ion content of the present study (Dr. S. Bharti, visiting researcher, Carleton University, personal communication, also see Appendix IV, page 111). The Na^+ -content in

their preparation of adult rat ventricular myocytes in standard saline (Ca^{++} 1-3mM) was 8.8 ± 1.2 mmol/L and decreased to 6.9 ± 0.5 mM after several hours in this medium. Incubation of myocytes in Ca^{++} -free medium caused a three-fold increase in the intracellular sodium activity to 23 ± 3 mM. Using the Na^+ -concentrations of 59 mM (0-Ca^{++}) and 51 mM (M199) obtained in this study, the extrapolated activity coefficients are: a) in calcium containing saline: 0.14-0.17, and b) in Ca^{++} -free saline: 0.39 (i.e., activity = concentration times activity coefficient, see Appendix IV, page 111, for example calculation).

It is clear from the above observations that a knowledge of the intracellular Na^+ and K^+ activity coefficients is essential for a complete understanding of the recovery time course of the $P_{\text{Na}}^+/P_{\text{K}}^+$ ratio.

5. E_M versus $\log[\text{K}^+]_e$, is $P_{\text{Na}}^+/P_{\text{K}}^+$ constant?

Fig. 8 is a simulation of membrane potential (E_M) vs. $\log([\text{K}^+]_e)$, assuming that $P_{\text{Na}}^+/P_{\text{K}}^+$ ratio remains constant regardless of the extracellular potassium concentration. One prediction of the abnormally high sodium to potassium permeability ratios in cultured cardiomyocytes is a relative insensitivity of membrane

Fig.8 Simulation of dependence of the resting membrane potential (E_M) on extracellular potassium ion concentration (Goldman-Hodgkin-and Katz equation) for the data in table 3. The symbols (black rhombs) are data from Fig.1 of Meleis, Briggs and Claycomb (two cells which maintained stable resting potential while $[K^+]$ was changed in the superfusate).

Note that there is a tendency of E_M to increase towards E_K with time in culture. In the last group (15-35 days old), the P_{Na^+}/P_{K^+} ratio is still 10 fold larger than values reported for this ratio in other myocardial preparations (Table 6).

Simulation Of Constant Field Equation For Different Times In Culture

potential to changes in extracellular potassium concentration (i.e. note the plateau of this simulated relationship in figure 8). Preliminary experiments in cultures 15 to 30 days old, in which the membrane potential was recorded while extracellular potassium in the perfusing saline was changed from 5 to 10 and 20mM indicated that there was only a small change in the membrane potential for these changes in $[K]_e$ (e.g. 10mV or less).

An observation which should be verified by future experiments is whether the sodium to potassium permeability ratio is constant for changing extracellular potassium concentrations. A change in the P_{Na^+}/P_{K^+} ratio and P_{K^+} which was dependent on extracellular potassium concentration, has been shown in preparations of intact myocardium (Page, 1962) and isolated myocytes (Bustamante et al., 1981, Houser et al., 1985). Page, 1962, measured the membrane potential in quiescent papillary muscle while changing the extracellular potassium concentration. The P_{Na^+}/P_{K^+} ratio changed as follows: $[K^+]_e : P_{Na^+}/P_{K^+}$, 5mM: 0.02, 25mM: 0.05, 50mM: 0.17. Houser et al., 1985, noted a reduction in P_{K^+} at high extracellular potassium concentrations in enzymatically isolated feline ventricular myocytes. Consequently, the P_{Na^+}/P_{K^+} ratio increased as follows:

$([K^+]_e : P_{Na^+}/P_{K^+})$, 5mM: 0.01, 10mM: 0.02, 20mM: 0.04, 50mM: 0.06. One possible explanation is that potassium ions may interact as they cross the sarcolemma. This hypothesis was tested by showing that Ussing's flux ratio of potassium passive fluxes required an additional exponential term, for a better fit of the data. It was concluded that this behavior may have been a result of ion to ion interaction (single file diffusion of potassium ions in potassium channel).

To summarize, the data in Fig.8, table 3 and table 5 can be broken down into three groups:

- i. Initially, the membrane potential is 70% lower than E_K (round morphological stage).
- ii. An intermediary stage, in which, myocytes in the spreading morphological stage (8 to 10 days old) show a partial recovery of E_M towards E_K .
- iii. Subsequently (10-35 days) P_{Na^+}/P_{K^+} decreases and E_K increases in such a way that the membrane potential approaches the potassium equilibrium potential. However, the mean membrane potential is still 50% more positive than E_K at the end of 35 days in culture. Whether this behavior is due to a decrease in P_{K^+} , an increase in P_{Na^+} , or both, cannot be resolved by the present study.

CONCLUSIONS:

The intracellular sodium and potassium concentrations measured in isolated and cultured adult rat ventricular myocytes in this study has been found to exhibit three types of changes:

i. Initially, during isolation of cells, intracellular sodium concentration is increased and potassium decreased relative to ventricular myocytes in situ. When the myocytes in isolation saline are transferred to the culture medium 199 intracellular sodium concentration is decreased by 14% to 20%. However, potassium does not show any sign of recovery at this time. One possible explanation is that there may be an isolation induced decrease in Na^+, K^+ -ATP-ase activity which may take a longer time to reestablish physiological levels of activity. A low calcium induced decrease in sodium pump activity in isolated myocytes has been previously reported (Lamers et al., 1984, Lelievre et al., 1984). The decrease in intracellular sodium which took place when the myocytes were transferred from the isolation saline to the culture medium 199 was accompanied by an increase in the fraction of rounded, hypercontracted cells of the isolated preparation.

The latter has been previously correlated with an increase in intracellular calcium concentration of isolated myocytes above a threshold value of 400nM (Lambert et al., 1986). This suggests that one of the possible mechanisms responsible for the initial recovery of intracellular sodium concentration may be a reversed sodium-calcium exchange mechanism, which moves three intracellular sodium ions out of the cell for each calcium ion entering the cell (Alexen and Bridge, 1985). The observed, possibly apparent, reduction in intracellular sodium may be accomplished by two routes:

- 1) elimination of sodium-loaded myocytes by hypercontraction, from the population of cells in which intracellular sodium ion concentration is measured.
- 2) a direct reduction in intracellular sodium ion concentration in the myocytes which retain rod shape morphology.

The available data does not distinguish between these two possibilities.

ii. The second change in intracellular ion content takes place as myocytes enter the round morphological stage in cultures 5 to 8 days old. At this time, intracellular potassium concentration falls by as much as 30% relative to newly isolated myocytes. Intracellular sodium concentration does not change significantly at this stage.

iii. In the next stages of morphological establishment, spreading and spread myocytes in cultures 9 days old or older, intracellular sodium concentration decreases and potassium increases to values which are similar to intracellular ionic concentrations in tissue preparations (Page and Page, 1968). These observations suggest that abnormal levels of membrane potential in the later stages of establishment in culture could be due to changes in membrane permeability.

Calculation of sodium to potassium ion membrane permeability ratios from the ion measurements in this study and mean membrane potentials (Juhasz, 1987) by a simplified form of the constant field equation (Sperelakis, 1979) suggest values of sodium to potassium permeability in cultured myocytes of 0.17 to 0.26. These are at least tenfold larger than sodium to potassium permeability ratios in myocardial tissue preparations, which typically range between 0.005 and 0.02. However, estimates of sodium to potassium membrane permeability ratios for the range of maximum and minimum values of membrane potential, suggest that lower values of permeability ratios (0.02-0.05) are a possible occurrence in those individual cultured myocytes which have more negative membrane potentials.

Measurements of this study do not allow separate

estimation of sodium or potassium ion membrane permeability. Therefore, it was not possible to determine whether the increase in mean P_{Na^+}/P_{K^+} was due mainly to an increase in P_{Na^+} or a decrease in P_{K^+} , or both.

APPENDIX I

CALIBRATION OF LS7500 LIQUID SCINTILLATION SYSTEM.

H-NUMBER AND AUTOMATIC QUENCH CORRECTION

Liquid scintillation counting involves conversion of the kinetic energy of the radioactive decay (beta-particles) into photons. Special electronic components (photomultipliers) of the liquid scintillation counter convert these photons into a voltage pulse whose height (amplitude) is proportional to the number of photons detected. Counting channels monitor the pulse amplitudes and register as counts only pulse amplitudes which are within preset lower and upper limits of pulse height for each particular channel. When dual labeled samples are counted (e.g. ^3H and ^{14}C , or ^{35}S which has an energy spectrum similar to ^{14}C) counts that arise in the two channels are due in part to beta-particle decay of the two different isotopes whose energy spectra overlap. This portion of energy overlap in the two counting channels is called channel spillover (fig(A1).1). Quenching which is due to color and chemical interactions present in a sample, affects the light intensity of a sample by reducing the pulse heights formed when the photons are detected by the photomultiplier tubes. When quenching is present in a sample (fig(A1).1

Fig(A1).1. Typical arrangement for dual label counting. Channel.1 (^3H) measures counts arising from the low energy isotope and part of the high energy isotope (spillover, shaded area). Channel.2 measures counts arising almost entirely from the high energy isotope (^{14}C). The figure shows the difference between a unquenched (A) and a quenched (B) sample. Quenching reduces counting efficiency (relative area of the energy spectrum). The resulting shift in the energy spectra of the two isotopes increases the amount of ^{14}C events occurring in the low energy isotope (^3H) channel. (from E.C. Long, 1977)

B), it can drastically affect counting by reducing the counting efficiency of the two isotopes as well as by increasing the spillover of the higher energy isotope into the lower energy isotope's counting channel.

The LS7500 liquid scintillation system uses two quench monitoring techniques:

1. The H-number is an external standard technique in which a high energy gamma-source is placed near the sample vial. The resulting physical interaction produces recoil or Compton electrons. This interaction results in a typical pulse height distribution which is distorted by quenching (fig(A1).2). The true quench level of any sample can be quantitatively expressed as a shift in the Compton edge (H-number) of a sample relative to a unquenched sample.

Quench standards which contain the same isotope as the sample but a known amount of radioactivity are used to generate a quench curve which relates the counting efficiency of each standard to its corresponding H-number. A correction for the spillover between the two counting channels is generated simultaneously. Based on this calibration curve the microprocessor of the LS7500 counter automatically converts the sample's counts per

Fig(A1).2. Typical distribution of pulse heights formed by Compton interactions in a unquenched scintillation solution (A) and the distortion of Compton distribution in a quenched sample (B). (from D.L. Horrocks, 1977)

minute into decays per minute units.

2. Another technique for quench monitoring used in parallel with the H-number technique is the automatic quench correction (AQC). In this technique the programs counting channel limits are adjusted on the basis of the H-number of the sample. This compensates for variations in isotope counting due to variable quenching between samples which would otherwise result in a different extent of spillover between counting channels (fig(A1).3).

Fig(A1).3. Panel A depicts the effect of quenching (H-number) on the counting efficiency of tritium (^3H -CH.1) and ^{14}C (^{14}C -CH.2) and the spillover of ^{14}C into the tritium channel (^{14}C -CH.1). Note that as quenching increases (H-number increases) the spillover of ^{14}C into the tritium counting channel.1 also increases. Data in panel B are from the same quench standards as in A which were counted under AQC conditions. Note that ^{14}C spillover (^{14}C -CH.1) does not increase as quench increases. (from E.C. Long, 1977)

APPENDIX II

CALCULATION OF THE DILUTION FACTOR FOR ION ANALYSIS

The dilution factor, D, of the equation 4 (page 35), was calculated according to the relationship:

$$D = V_F / (V_{AD} / V_{AI})$$

where,

V_F is the total volume of solution at the end of dilution with double distilled deionized water (μ l).

V_{AD} is the aliquot of nitric acid digestate which was diluted (μ l).

V_{AI} is the total volume of nitric acid digestate, which includes the pellet water (V_T of equation 4) in the silicone oil experiments but not in the dried samples of the nylon mesh experiments.

Typical values of these paramaters in the cell suspension experiments were:

$$V_T: 40,800\mu\text{l}$$

$$V_{AD}: 1020\mu\text{l}$$

$$V_{AI}: 800\mu\text{l}$$

hence,

$$\begin{aligned} D &= 40,800 / (800 / 1020) = \\ &= 52,020\mu\text{l} \end{aligned}$$

Therefore, this results in a dilution of $\text{HNO}_3:\text{H}_2\text{O}$ (vol/vol) of about 1:50.

APPENDIX III

CALCULATION OF THE EXPECTED CHANGE IN SODIUM AND POTASSIUM CONCENTRATION IN EXPERIMENTS WHICH USED INULIN TO MEASURE V_{ex} RELATIVE TO EXPERIMENTS USING SULFATE

The last column of table 1 (page 51) represents the expected change in the mean values of estimated intracellular sodium and potassium concentration in experiments using inulin to measure V_{ex} relative to experiments using sulfate. The calculation used equation 6 (page 66, Discussion) as follows:

$$I_{EST} = F_{RND}[I]_e + F_{ROD}[I]_i \quad (6)$$

the extracellular concentrations of sodium ($[Na^+]_e$) and potassium ($[K^+]_e$) in table 1, isolation saline, were:

$$[Na^+]_e = 90mM$$

$$[K^+]_e = 30mM$$

assuming that sulfate equilibrates the round cell volume, the mean values of intracellular sodium and potassium concentration when sulfate measured V_{ex} can be taken to represent the rod shaped myocyte ion content:

$$[Na^+]_i = 59mM$$

$$[K^+]_i = 111mM$$

assuming that inulin would not penetrate 30% of hypercontracted myocytes, the expected change in the mean value of intracellular ion concentration will be:

$$\begin{aligned} \text{Na}_{\text{EST}} &= 0.30(90) + 0.70(59) = \\ &= 68\text{mM} \end{aligned}$$

$$\begin{aligned} \text{K}_{\text{EST}} &= 0.30(30) + 0.70(111) = \\ &= 87\text{mM} \end{aligned}$$

The figure below shows that $[\text{Na}^+]_i$ did not depend on the percentage of hypercontracted cells when $\text{H}_2^{35}\text{SO}_4$ was the extracellular volume marker. In similar experiments when ^3H -inulin was the extracellular volume marker, $[\text{Na}^+]_i$ increased significantly ($p < 0.01$) with $\text{Na}_T/(\text{Na}_T + \text{K}_T)$. The latter is directly proportional to percent hypercontracted cells (fig 5, page 44).

Fig(A3)1. Intracellular sodium concentration as a function of percent hypercontracted cells (data shown in table 1, page 52, silicone oil method, ^{35}S -sulfate).

APPENDIX IV

CALCULATION OF ACTIVITY COEFFICIENT

By definition the activity of an ion is the product of concentration and activity coefficient. For sodium ion the intracellular activity can be written as:

$$a(i)_{Na^+} = A_{cf} \cdot [Na^+]_i$$

where,

$a(i)_{Na^+}$ is the intracellular activity of sodium ion (mM).

A_{cf} is the activity coefficient.

$[Na^+]_i$ is the intracellular concentration of sodium ion (mM).

Assuming that Wittenberg and Gupta, 1984, measured intracellular activity of sodium:

(for myocytes incubated in nominally calcium free saline)

$$a(i)_{Na^+} = 23 \pm 3 \text{ mM}$$

The intracellular concentration of sodium estimated in this study for myocytes in a corresponding saline was:

$$[Na^+]_i = 59 \pm 12 \text{ mM}$$

hence, the activity coefficient can be estimated as:

$$\begin{aligned} A_{cf} &= a(i)_{Na^+} / [Na^+]_i = \\ &= 23/59 = 0.39 \end{aligned}$$

As pointed out by Lee and Fozzard, this is an apparent

activity coefficient since it is much lower than what would be expected if all the sodium was in a homogeneous solution (i.e. 0.745, Lee and Fozzard, 1975). In the case of newly isolated myocytes, the low value of activity coefficient implies that a large portion of intracellular sodium may be bound or sequestered in organelles.

REFERENCES:

1. Alexen, P.H., and J.H.B. Bridge. 1985.
Electrochemical ion gradients and
and $\text{Na}^+/\text{Ca}^{++}$ exchange stoichiometry.
J. Gen. Phys. 85: 471-475.
2. Alto, L.A. and N.S. Dhalla. 1979.
Myocardial cation contents during induction of
calcium paradox.
Am. J. Physiol. 237: H713-H719
3. Altschuld, R. ,L. Gibb , A. Ansel, C. Hohl,
F.A. Kruger, and G.P. Brierley. 1980.
Calcium tolerance of isolated rat heart cells.
J. Mol. Cell. Cardiol. 12: 1383-1395
4. Barry, W.H., Y. Hasin, and T.W. Smith. 1985.
 Na^+ -Pump inhibition, enhanced calcium influx via
sodium-calcium exchange, and positive inotropic
response in cultured heart cells.
Circ. Res. 56: 231-241
5. Bendukidze, Z., G. Isenberg, U. Klockner. 1985.
 Ca^{++} -tolerant guinea-pig ventricular myocytes
as isolated by pronase in the presence of $250\mu\text{M}$
free calcium.
Basic Res. Cardiol. 80 (suppl.1): 13-18.

6. Berry, M.N., D.S. Friend, J. Scheuer. 1970.
Morphology and metabolism of intact muscle cells
isolated from adult rat heart.
Circ. Res. 26: 679-687
7. Bkaily, G., N. Sperelakis, and J. Doane. 1984.
A new method for preparation of isolated single adult
myocytes.
Am. J. Physiol. 247: H1018-H1026
8. Bloom, S. 1970.
Spontaneous rhythmic contraction of spread
heart muscle cells.
Science 167: 1727-1729.
9. Borgers, M. and H. van Belle. 1985.
Intracellular Ca^{++} shifts during Ca^{++} paradox.
Basic Res. Cardiol. 80 (suppl.2): 25-30.
10. Borgers, M., F. Thone, and L. ver Donk. 1985.
Sarcolemma bound calcium. Its importance in cell
viability.
Basic Res. Cardiol. 80 (suppl.1): 31-36.
11. Burrows, M.T. 1910.
The cultivation of tissue of the chick embryo
outside the body.
J. Am. Med. Assoc. 55 : 2057-2058.
12. Burrows, M.T. 1912.

Rhythmical contraction of isolated heart muscle
outside the organism.

Munch Med. Wochenschr. 59: 1473-1475.

13. Bustamante, J.O., T. Watanabe, and T.F. McDonald.
1981.
Single cells from adult mammalian heart: isolation
procedure and preliminary electrophysiological study.
Can. J. Physiol. Pharmacol. 59: 907-910

14. Cheung, J.Y., I.G. Thompson, J.V. Bonventre. 1982.
Effects of extracellular calcium removal and anoxia
on isolated rat myocytes.
Am. J. Physiol. 243: C184-C190

15. Claycomb, W.C., and H.D. Bradshaw Jr., 1983.
Aquisition of multiple nuclei and the activity of
DNA polymarase alpha and reinitiation of DNA
replication in terminally differentiated adult
cardiac muscle cells in culture.
Dev. Biol. 99: 331-337

16. Claycomb, W.C., and R.L. Moses. 1985.
Culture of atrial and ventricular cardiac muscle
cells from the adult squirrel monkey Suimiri sciureus
Exp. Cell Res. 161: 95-100.

17. Claycomb, W.C., and M.C. Palazzo. 1980.
Culture of the terminally differentiated adult
cardiac muscle cell:
A light and scanning EM study.
Dev. Biol. 80: 466-482

18. Cobbold, P.H. , and P.K. Bourne. 1984.
Aequorin measurements of free calcium in single heart cells.
Nature 312: 444-446

19. Cobbold, P.H., P.K. Bourne, and K.S.R. Cuthbertson. 1985.
Evidence from aequorin for injury of metabolically inhibited myocytes independently of free Ca^{2+} .
Basic Res. Cardiol. 80 (suppl.2): 155-158

20. Cohen, C.J., B.P. Bean, R.W. Tsien. 1984.
Maximum upstroke velocity as an index of available sodium conductance: comparison of V_{max} and voltage clamp measurements of sodium current in rabbit Purkinje fibers.
Circ.Res. 54: 636-651

21. Colquhoun, R., E. Neher, H. Reuter, C.F. Stevens, 1981.
Inward current channels activated by intracellular calcium in cultured cardiac cells.
Nature (Lond.) 294: 752-754.

22. Cooper IV, G., W.E. Mercer, J.K. Hooper, P.R. Gordon, R.L. Kent, I.K. Lauva, T.A. Marino. 1986.
Load regulation of the properties of adult feline cardiocytes: the role of substrate adhesion.
Circ. Res. 58: 692-705

23. Corabeuf, E., P. Gautier, and P. Gouiraudou. 1981.

Potential and tension changes induced by sodium removal in dog Purkinje fibers: role of an electrogenic sodium-calcium exchange.
J. Physiol. 311: 605-622.

24. Coronado, R., R. Latorre, 1982.
Detection of K^+ and Cl^- channels from calf cardiac sarcolemma in planar lipid bilayer membranes.
Nature (Lond.) 298: 849-852.
25. Crevey, B.J., G.A. Langer, and J.S. Frank. 1978.
Role of Ca^{2+} in maintenance of rabbit myocardial cell membrane structural and functional integrity.
J. Mol. Cell. Cardiol. 10: 1081-1100.
26. Dani, A.M., A. Cittadini, and G. Inesi. 1979.
 Ca^{++} -Transport and contractile activity in dissociated mammalian heart cells.
Am. J. Physiol. 237: C147-C155
27. Desilets, M. and M. Horackova. 1982.
 Na^+ -Dependence of $^{45}Ca^{2+}$ uptake in adult rat isolated cardiac cells.
Biochim. Biophys. Acta 721: 144-157
28. Dhalla, N.S., P.K. Singal, V. Panagia, J.A.C. Harrow, U.B. Anand-Strivastava, and R.E. Beamish. 1984.
Progress and problems in understanding the involvement of calcium in heart function.
Can. J. Physiol. Pharmacol. 62: 867-873

29. Dow, J.W., N.G.L. Harding, T. Powell. 1981.
Isolated cardiac myocytes: I. Preparation of adult myocytes and their homology with intact tissue and II. Functional aspects of mature cells.
Cardiovasc. Res. 15: 483-514 and 549-579
30. Dulbecco, R. 1952.
Production of plaques in monolayer tissue culture by single particles of an animal virus.
Proc. Nat. Acad. Sci. (USA) 38: 747-752
31. Fabiato, A., F. Fabiato. 1972.
Excitation-contraction coupling of isolated cardiac fibers with disrupted or closed sarcolemmas.
Circ. Res. 31: 293-307.
32. Fabiato, A. 1985.
Spontaneous versus triggered contractions of "calcium tolerant" cardiac cells from adult rat ventricle.
Basic Res. Cardiol. 80: (suppl.2): 83-88.
33. Farmer, B.B., M. Mancina, E.S. Williams, A.M. Watanabe. 1983.
Minireview: Isolation of calcium tolerant cells from adult rat hearts: review of the literature and description of a method.
Life Sci. 33: 1-18

34. Forbes, M.S. and N. Sperelakis. 1985.
Intercalated discs of mammalian heart: a review of
structure and function.
Tissue & Cell 17: 605-648

35. Fozzard, H.A., C.T. January, and J.C. Makielski.
1985.
New studies of the excitatory sodium currents in
heart muscle.
Circ. Res. 56: 475-485

36. Frangakis, C.J., J.J. Bahl, H. McDaniel, and
R. Bressler. 1980.
Tolerance to physiological calcium by isolated
myocytes from the adult heart; an improved cellular
preparation.
Life Sci. 27: 815-825

37. Frank, J.S., G.A. Langer, L.M. Nudd, and
K. Seraydarian. 1977.
The myocardial cell surface, its histochemistry, and
the effect of sialic acid and calcium removal on its
structure and cellular ionic exchange.
Circ. Res. 41: 702-714

38. Fry, D.M., D. Scales, and G. Inesi. 1979.
The ultrastructure of membrane alterations of
enzymatically dissociated cardiac myocytes.
J. Mol. Cell. Cardiol. 11: 1151-1163.

39. Gangola, P., and A.E. Shamoo. 1986.
Synthesis and characterization of a peptide segment

of $(Ca^{2+}+Mg^{2+})$ -ATP-ase. A candidate for calcium transport site.

J. Biol. Chem. 261: 8601-8603

40. Ganote, C.E. .1985.
Cell to cell interactions contributing to the "oxygen paradox".
Basic. Res. Cardiol. 80 (suppl.2): 141-146

41. Goshima, K, S. Wakabayashi, A. Masuda. 1980.
Ionic mechanism of morphological changes of cultured myocardial cells on successive incubation in media without and with Ca^{2+} .
J. Mol. Cell. Cardiol. 12: 1135-1157

42. Grinwald, P.M., and W.G. Nayer. 1981.
Calcium entry in the calcium paradox.
J. Mol. Cell. Cardiol. 13: 867-880.

43. Harding, D., and C. Fry. 1985.
Effect of temperature on calcium exchange in digitonin-treated rat ventricular myocytes.
Biochim. Biophys. Acta 847: 136-139

44. Haworth, R.A., D.R. Hunter, and H.A. Berkoff. 1980.
The isolation of Ca^{2+} -resistant myocytes from the adult rat.
J. Mol. Cell. Cardiol. 12: 715-723

45. Hearse, D.J., S.M. Humphrey, and G.R. Bullock. 1978.
The oxygen paradox and the calcium paradox: two facets of the same problem?

- J. Mol. Cell. Cardiol. 10: 641-668
46. Hohl, C.M., R.A. Altschuld, G.P. Brierley. 1983.
Effects of calcium on the permeability of
isolated adult heart cells to sodium.
Arch. Biochim. Biophys. 221: 197-205.
47. Horackova, M. 1984 .
Transmembrane calcium transport and the activation of
cardiac contraction.
Can. J. Physiol. Pharmacol. 62: 874-883
48. Horrocks, D.L., 1977
The H-number concept.
Beckman Instruments Inc., Fullerton, Ca.
49. Houser, S.R., A. Bahinski, and L.H. Silver. 1985.
Passive membrane properties of isolated feline
ventricular myocytes.
Am. J. Physiol. 248: H622-H630.
50. Isenberg, G. 1977.
Cardiac Purkinje fibers. $[Ca^{++}]_i$ controls
steady-state potassium conductance.
Pflügers Arch. 371: 71-76
51. Isenberg, G., and U. Klockner. 1982.
Calcium tolerant ventricular myocytes prepared by
preincubation in a "KB medium".
Pflügers Arch. 395: 6-18
52. Isenberg, G., and U. Klockner. 1982.

Isolated bovine ventricular myocytes:
characterization of the action potential.
Pflügers Arch. 395: 19-29

53. Jacobson, S.L. 1977.
Culture of spontaneously contracting myocardial cells
from adult rats.
Cell Struct. Funct. 2: 1-9
54. Jacobson, S.L., M. Banfalvi, and T.A. Schwarzfeld.
1985.
Long term primary cultures of adult human and rat
cardiomyocytes.
Basic. Res. Cardiol. 80 (suppl.1): 79-82
55. Jacobson, S.L., C.B. Kennedy, and G.A.R. Mealing.
1983.
Evidence for functional sodium and calcium ion
channels in the membrane of cultured
cardiomyocytes of the adult rat.
Can. J. Physiol. Pharmacol. 61: 1312-1316.
56. Jacobson, S.L., and H.M. Piper. 1986.
Cell cultures of adult cardiomyocytes as models of
the myocardium.
J. Mol. Cell. Cardiol. 18: 661-678.
57. Johnson, E.A., and M. Lieberman. 1971.
Heart, excitation and contraction.
Annu. Rev. Physiol. 33: 479-510.
58. Juhasz, A., 1987.

Some electrophysiological and pharmacological properties of cultured heart cells from the adult rat ventricle.

M.Sc. Thesis, Carleton University.

59. Kameyama, M., M. Kakei, R. Sato, T. Shibasaki, H. Matzuda, R.H. Irisawa. 1984.
Intracellular Na^+ activates K^+ channel in mammalian cardiac cells.
Nature 308: 354-356.
60. Kim, D., J.D. Marsh, W.H. Barry, and T.W. Smith. 1984.
Effects of growth in low potassium medium or ouabain on membrane Na-K-ATP-ase, cation transport, and contractility in cultured chick heart cells.
Circ. Res. 55: 39-48.
61. Kim, D., and T.W. Smith. 1986.
Effect of growth in low- Na^+ medium on transport sites in cultured heart cells.
Am. J. Physiol. 250: C32-C39
62. Kono, T. 1969.
Roles of collagenases and other proteolytic enzymes in the dispersal of animal tissues.
Biochim. Biophys. Acta 178: 397-400.
63. Kunze, D.L., A.E. Lacerda, D.L. Wilson, and A.M. Brown. 1985.
Cardiac Na^+ -currents and the inactivating, reopening and waiting properties of single cardiac

Na⁺-channels.

J. Gen. Physiol. 86:691-719

64. Lambert, M.R., J.D. Johnson, K.G. Lamka, G.P. Brierley and R.A. Altschuld. 1986.
Intracellular free Ca²⁺ and the hypercontracture of adult rat heart myocytes.
Arch. Biochem. Biophys. 245: 425-435.

65. Lamers, J.M., J.T. Stinis, and T.J.C. Ruigrok. 1984.
Biochemical properties of membranes isolated from Ca⁺⁺-depleted rabbit hearts.
Circ. Res. 54: 217-226.

66. Lee, C.O., P. Abete, M. Pecker, J.K. Sonn, and M. Vassalle. 1985.
Strophanthidin inotropy: role of intracellular sodium ion activity and sodium-calcium exchange.
J. Mol. Cell. Cardiol. 17: 1043-1053

67. Lee, C.O., and H.A. Fozzard. 1975.
Activities of potassium and sodium ions in rabbit heart muscle.
J. Gen. Phys. 65: 695-708

68. Lee, C.O. 1985.
200 Years of digitalis: the emerging central role of the sodium ion in the control of cardiac force.
Am. J. Physiol. 249: C367-C378

69. Lelievre, L.G., D. Mansier, D. Charlemagne, and

- B. Swynghedauw. 1984.
Effects of calcium on the heterogeneity of
the Na^+, K^+ -ATP-ase forms in rat heart.
Basic Res. Cardiol. 79 (suppl): 128-133.
70. Libby, P. 1984.
Long-term culture of contractile mammalian heart
cells in a defined serum-free medium that limits
non-muscle cell proliferation.
J. Mol. Cell. Cardiol. 16: 803-811
71. Lieberman, M., G.R. Horres, N. Shigeto, L. Ebihara,
J.F. Aiton, and E.A. Johnson. 1981.
Cardiac muscle with controlled geometry: application
to electrophysiological and ion transport studies.
In: Excitable cells in tissue culture. Editors:
P.G. Nelson and M. Lieberman. Plenum Publishing Co.
72. Long, E.C., 1977
AQC on the Beckman LS-8000 series.
Beckman Instruments Inc., Irvine, Ca.
73. Lundgren, E., T. Borg, S. Mardh. 1984.
Isolation, characterization and adhesion of
calcium-tolerant myocytes from the adult
rat heart.
J. Mol. Cell. Cardiol. 16: 355-362
74. McDonald, T.F., and R.L. DeHaan. 1973.
Ion levels and membrane potential in chick heart

tissue and cultured cells.

J. Gen. Physiol. 61: 89-109

75. Matsuda, H. 1983.
Effects of intracellular calcium injections
on steady-state membrane currents in
isolated single ventricular cells.
Pflügers Arch. 397: 81-83.
76. Mazet, F., B.A. Wittenberg, and D.C. Spray. 1984.
Fate of intercellular junctions in isolated adult
rat cardiac cells.
Circ. Res. 56: 195-204
77. Meier Jr., C.F., G.M. Briggs, W.C. Claycomb. 1986.
Electrophysiological properties of cultured adult
rat ventricular cardiac muscle cells.
Am. J. Physiol. 250: H731-H735
78. Moscona, A. 1952.
Cell suspensions from organ rudiments
of the early chick embryo.
Exp. Cell Res. 3: 535-541.
79. Moscona, A., and H. Moscona. 1952.
The dissociation and aggregation of cells
from organ rudiments of early chick embryo.
J. Anat. 86: 287-301.
80. Moses, R.L., and W.C. Claycomb. 1982.
Ultrastructure of terminally differentiated
adult rat cardiac muscle cells in culture.

Am. J. Anat. 164: 113-131

81. Muir, A.R. 1966.
The effects of calcium ion depletion
on the ultrastructure of the rat heart.
J. Anat. 100: 437-438.
82. Muir, A.R., 1967.
The effects of divalent cations on the
ultrastructure of the perfused heart.
J. Anat. 101: 239-246.
83. Mullins, L.J. 1979.
The generation of electric currents in cardiac
fibers by $\text{Na}^+/\text{Ca}^{++}$ exchange.
Am. J. Physiol. 236: C103-C110
84. Mullins, L.J. 1981.
Ion transport in heart. Raven Press, NY.
85. Nag, A.C., and M. Cheng. 1981.
Adult mammalian cardiac muscle cells in culture.
Tissue & Cell 13: 515-523
86. Nag, A.C., M. Cheng, D.A. Fischman and R. Zak. 1983.
Long-term cell culture of adult mammalian
cardiac myocytes: electron microscopic and
immunofluorescent analyses of myofibrillar structure.
J. Mol. Cell. Cardiol. 15: 301-317.
87. Page, E. 1962.
Cat heart muscle in vitro .II The steady state

resting potential in quiescent papillary muscles.

J. Gen. Physiol. 46: 189-200

88. Page, E., 1962.

Cat heart muscle in vitro . III The extracellular space.

J. Gen. Physiol. 46: 201-214

89. Page E., 1965.

Ion movement in heart muscle: tissue compartments and the experimental definition of driving forces.

Ann. NY. Acad. Sci. 127: 34-48

90. Page, E., and R.S. Bernstein. 1964.

Cat heart muscle in vitro V Diffusion through a sheet of right ventricle.

J. Gen. Physiol. 47: 1129-1140

91. Page, E., and E. Gross-Page. 1968.

Distribution of ions and water between tissue compartments in the perfused left ventricle of the rat heart.

Circ. Res. 22: 435-446

92. Page, E., L.P. McCallister, and B. Power. 1971.

Stereological measurement of cardiac ultrastructures implicated in excitation-contraction coupling.

PNAS USA 68: 1465-1466

93. Page, E., and Y. Shibata. 1981.

Permeable junctions between cardiac cells.

Ann. Rev. Physiol. 43: 431-441

94. Page, E., and A.K. Solomon. 1960.
Cat heart muscle in vitro. I Cell volumes
and intracellular concentrations in papillary
muscle.
J. Gen. Physiol. 44: 327-324

95. Pager, J. 1971.
Etude morphometrique du system tubulaire
transverse du myocarde ventriculaire de rat.
J. Cell. Biol. 50: 233-237

96. Pappano, A.J., and N.L. Sperelakis. 1969.
Low K^+ conductance and low resting potential
of isolated single cultured heart cells.
Am. J. Physiol. 217: 7076-1082.

97. Patlak, J.B., and M. Ortiz. 1985.
Slow currents through single sodium channels
of the adult rat heart.
J. Gen. Physiol. 86: 89-104

98. Pelzer, D., and H.M. Piper. 1984.
Low resting potential in single isolated heart cells
due to membrane damage by the recording
microelectrode.
Pflügers Arch. 400: 197-199.

99. Piper, H.M., I. Probst, P. Schwartz, F.J. Hutter,
and P.G. Spieckermann. 1982.
Culturing calcium stable adult cardiac myocytes.

- J. Mol. Cell. Cardiol. 14: 397-414.
100. Piper, H.M., R. Spahr, J.F. Huter, and P.J. Spieckermann. 1985.
The calcium and the oxygen paradox: non-existent on the cellular level.
Basic Res. Cardiol. 80: 159-163.
101. Piper, H.M., R. Spahr, I. Probst, and P.G. Spieckermann. 1985.
Substrates for attachment of adult cardiac myocytes in culture.
Basic Res. Cardiol. 80 (suppl.2): 175-180.
102. Powell, T., 1985a.
Isolated adult heart cells. The development of a new experimental model.
Basic Res. Cardiol. 80 (suppl.2): 9-12.
103. Powell, T. 1985b.
The isolation and characterization of calcium-tolerant myocytes.
Basic Res. Cardiol. 80 (suppl.2): 15-16.
104. Powell, T. 1985c.
Electrophysiological properties of isolated ventricular myocytes.
Basic Res. Cardiol. 80 (suppl.1): 87-92.
105. Powell, T., and R.P. Gould. 1972.
Intact isolated muscle cells from the adult rat heart.

J. Physiol. (Lond.): 16P-19P.

106. Powell, T., D.A. Terrar, V.W. Twist. 1980.
Electrical properties of individual cells isolated
from adult ventricular myocardium.
J. Physiol. (Lond) 302: 131-153
107. Preuner, J., 1985.
Functional alterations of the sarcolemma in
 Ca^{++} -free perfused hearts.
Basic Res. Cardiol. 80 (suppl.2): 19-24.
108. Regan, L.G., and R.B. Rogard. 1982.
Binding of labeled saxitoxin to the
TTX-resistant cardiac Na^{+} -channels
in rat.
Circ. Res. 66 (suppl.2): 96-102.
109. Reuter, H., 1967,
The dependence of slow inward current
in Purkinje fibers on the extracellular
calcium concentration.
J. Physiol. (Lond) 192: 479-492
110. Reuter, H., and N. Setz. 1968.
The dependence of calcium efflux from
cardiac muscle on temperature and external
ion composition.
J. Physiol. (Lond.) 195: 451-470
111. Ringer, S. 1883.
A further contribution regarding the influence

of the different constituents of blood on the contraction of the heart.

J. Physiol. (Lond.) 4: 29-42.

112. Ruano-Arroyo, G., G. Gerstenblith, and E.G. Lakatta. 1984.

'Ca-Paradox' in the heart is modulated by cell sodium during the calcium-free period.

J. Mol. Cell. Cardiol. 16: 783-793

113. Schanne, O.F., Bkaily, G. 1981.

Explanted cardiac cells: a model to study drug actions ?

Can. J. Physiol. Pharmacol. 59: 443-467.

114. Schilling, W.P., D.W. Schuil,

E.E. Bagwell, and G.E. Lindenhauer. 1984.

Sodium and potassium permeability of membrane vesicles in a sarcolemma-enriched preparation from canine ventricle.

J. Membr. Biol. 77: 101-114

115. Schwartz, P., H.M. Piper, R. Spahr,

J.F. Hutter, and P.G. Spiekermann. 1985a.

Development of new intracellular contacts between cardiac myocytes in culture.

Basic Res. Cardiol. 80 (suppl.1): 75-78.

116. Schwartz, P., H.M. Piper, R. Spahr,

and P.G. Spiekermann. 1985b.

Adaptation phenomena of adult myocytes in culture.

Basic Res. Cardiol. 80 (suppl2): 181-185

117. Schwarzfeld, T.A., and S.L. Jacobson. 1981.
Isolation and development in cell culture
of myocardial cells of the adult rat.
J. Mol. Cell. Cardiol. 13: 563-575.
118. Severs, N.J., A.M. Slade, T. Powell, and
V.W. Twist. 1985.
Ultrastructure of the sarcolemma and
intercalated discs in isolated rat myocytes.
Basic Res. Cardiol. 80 (suppl.2): 35-40.
119. Severs, N.J., A.M. Slade, T. Powell, V.W. Twist,
and R.L. Warren. 1982.
Correlation of ultrastructure and function
in calcium-tolerant myocytes isolated from
adult rat heart.
J. Ultrastruct. Res. 81: 222-239
120. Sheu, S.S., N.A. Fozzard. 1985.
 $\text{Na}^+/\text{Ca}^{++}$ Exchange in the intact cardiac cells.
J. Gen. Phys. 85: 476-478.
121. Silver, L.M. and S.R. Houser. 1985a.
Transmembrane potassium fluxes in isolated
feline ventricular myocytes.
Am. J. Physiol. 248: H614-H621
122. Silver, L.H. and S.R. Houser. 1985b.
Decreased sodium-potassium pump activity in
isolated hypertrophied feline ventricular myocytes.

Life Sci. 37: 607-615.

123. Smith, T.W., D. Kim, and W.H. Barry. 1984.
Studies of inotropism mechanisms of cardiac
glycoside in cultured heart cells.
Bas. Res. Cardiol. 79: 140-146

124. Snowdowne, K.W., R.J. Ertel, and A.B. Borle. 1985.
Measurement of cytosolic calcium with aequorin
in dispersed rat ventricular cells.
J. Mol. Cell. Cardiol. 17: 233-241

125. Sperelakis, N. 1979.
Origin of the cardiac resting potential.
In: Handbook of Physiology. The Cardiovascular
System. R.M. Berne, editor. Vol.1. The Heart.
pp 187-267. Am. Physiol. Soc., Bethesda, Maryland.

126. Sperelakis, N., and J.A. Schneider. 1976.
A metabolic control for calcium ion influx
that may protect the ventricular myocardial cell.
Am. J. Cardiol. 37: 1079-1085

127. Tuana, B.S., and N.S. Dhalla. 1982.
Purification and characterisation of
a Ca^{2+} -dependent ATP-ase from rat heart sarcolemma.
J. Biol. Chem. 257: 14440-14445

128. Vahouny, G.V., R. Wey, R.R. Starkweather, and
C. Davis. 1970.
Preparation of beating heart cells from adult rats.
Science 167: 1616-1618

129. Wakabayashi, S., and K. Goshima. 1981.
Kinetic studies on sodium-dependent calcium uptake by myocardial cells and neuroblastoma cells in culture.
Biochim. Biophys. Acta 642: 158-172
130. Wilson, D.L., A.E. Lacerda, D.L. Kunze, and A.M. Brown. 1985.
Single channels and whole cell sodium currents in heart cells.
Basic Res. Cardiol. 80 (suppl.2): 61-64.
131. Wittenberg, B.A., and R. Gupta. 1985.
VMR Studies of intracellular sodium ions in mammalian cardiac myocytes.
J. Biol. Chem. 260: 2031-2034
132. Wollenberger, A. 1985.
Isolated heart cells as a model of the myocardium.
Basic Res. Cardiol. 80 (suppl.2): 9-14.
133. Zimmerman, A.N.E., W. Daems, W.C. Hulsmann, J. Snijder, E. Wisse, and D. Durrer. 1967.
Morphological changes of heart muscle caused by successive perfusion with calcium-free and calcium containing solutions, (calcium paradox).
Cardiovasc. Res. 1: 201-209
134. Zimmerman, A.N.E., and W.C. Hulsmann. 1966.
Paradoxical influence of calcium ions on the permeability of cell membranes of the

isolated rat heart.

Nature 211: 646-647

135. Yates, J.C. and N.S. Dhalla. 1975.

Structural and functional changes associated
with failure and recovery of hearts after perfusion
with Ca^{++} -free medium.

J. Mol. Cell. Cardiol. 7: 91-103

END

18-02-90

FIN