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UNIVERSITY OF GHANA, LEGON

**DEVELOPMENT OF TRANSVERSE
TUBULAR SYSTEMS (T-TUBULES)
IN THE HAMSTER (*MESOCRICETUS
AURATUS*) MYOCARDIUM.**



**THIS THESIS IS SUBMITTED TO THE
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UNIVERSITY OF GHANA, LEGON

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DECLARATION

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DEDICATION

TO:

- 1) The Most High God and**
- 2) Ernestina & Kweku Jnr. Adjenti.**



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LIST OF FIGURES.

Figures	Page
1 Electron micrograph of left ventricular myocytes of pre-natal hamster (14½ days)	29
2 Electron micrograph of left ventricular myocytes of a 15½ day old pre-natal hamster	30
3 Electron micrograph of the left ventricular myocytes of a 16½ day old pre-natal hamster	31
4 Electron micrograph of right ventricular myocytes of a 14½ day old pre-natal hamster.	34
5 Electron micrograph of the right ventricular myocytes of a 15½ day old pre-natal hamster.	35
6 Electron micrograph of the right ventricular myocytes of a 16½ day old pre-natal hamster.	36
7 Electron micrograph of the left ventricular myocyte of a day old post natal hamster.	38
8 Electron micrograph of the left ventricular myocytes of a day old post-natal hamster (20 hours after birth).	39
9 Electron micrograph of the right ventricular myocytes of a day old post- natal hamster.	41
10 Electron micograph of a transverse section of the right ventricular myocytes of a day old postnatal hamster.	42

- 11 Electron micrograph of the left ventricular myocytes of a 7-day old post-natal hamster. 44
- 12 Electron micrograph of the left ventricular myocytes of a 7-day postnatal hamster. 45
- 13 Electron micrograph of the left ventricular myocyte of a 7-day old postnatal hamster showing regular indentations of the sarcolemma. 46
- 14 Electron micrograph of the right ventricular myocyte of a 7-day old postnatal hamster showing shallow indentations in the Z-line regions. 48
- 15 Electron micrograph of the right ventricular myocyte of a 7-day old postnatal hamster showing some cellular inclusions. 49
- 16 Electron micrograph of the right ventricular myocytes of a 7-day old hamster showing indentations on the sarcolemmal surface. 50
- 17 Electron micrograph of the left ventricular myocyte of a 14-day postnatal hamster showing T-tubules. 52
- 18 Electron micrograph of the left ventricular myocyte of a 14-day postnatal hamster. 52
- 19 Electron micrograph of a 14-day postnatal hamster showing well-developed myofibrils. 54
- 20 Electron micrograph of the left ventricular myocyte of 21 days postnatal hamster showing T-tubules and T-SR couplings. 56
- 21 Electron micrograph of the left ventricular myocyte of a 21-day

postnatal hamster showing definitive T-tubules.	56
22 Electron micrograph of the left ventricular myocyte of a 21-day postnatal hamster showing T-tubules and extent of development of the myofibrils.	57
23 Electron micrograph of the right ventricular myocytes of a 21-day postnatal hamster showing the extent of development of the myofibrils and T- tubules.	59
24 Electron micrograph of the right ventricular myocytes of a 21-day postnatal hamster.	59
25 Electron micrograph of the right ventricular myocytes of a 21-day postnatal hamster showing cellular inclusions.	60
26 Electron micrograph of the right ventricular myocytes of a 21-day postnatal hamster showing T-SR couplings.	60
27 Electron micrograph of the right ventricular myocytes of a 21-day postnatal hamster showing well-developed myofibrils and pinocytotic vesicles.	61

CONTENTS

<u>Content</u>	<u>Page</u>
Declaration	ii
Dedication	iii
Acknowledgement	iv
List of Figures	vi
Abstract	xi
Chapter One Introduction and Literature Review	1
1.1 General Introduction	1
1.2 Literature Review of the Transverse Tubular Systems (T-Systems)	4
Chapter Two Materials and Methods	17
2.1 The biology and taxonomy of the hamster	17
2.2 Breeding of hamsters	17
2.3 Grouping of hamsters	18
2.3.1 Pre-Natal Group	19
2.3.2 Post-Natal Group	21
2.4 Preparation of Tissues for light and electron microscopy	23
2.4.1 Light Microscopy	24
2.4.2 Electron Microscopy	25
Chapter Three Results	27

3.1	Pre-natal Ventricular Cells (Days 14½, 15½ and 16½)	27
3.1.1	Left Ventricle	27
3.1.2	Right Ventricle	32
3.2	Post-Natal Stage	37
3.2.1	Day 1 (Left Ventricle)	37
3.2.2	Day 1 (Right Ventricle)	40
3.2.3	Day 7 Post-Natal (Left Ventricle)	43
3.2.4	Day 7 Post-Natal (Right Ventricle)	47
3.2.5	Day 14 Post-Natal (Left Ventricle)	51
3.2.6	Day 14 Post-Natal (Right Ventricle)	53
3.2.7	Day 21 Post-Natal (Left Ventricle)	54
3.2.8	Day 21 Post-Natal (Right Ventricle)	57
Chapter Four	Discussion	62
	References	78
	Appendix I (Buffers and Fixatives)	93
	Appendix II (Preparation of Epon 812 Resins)	95
	Appendix III (Preparation of Stains)	97

ABSTRACT

With the aid of an electron microscope the development of transverse tubules (T-tubules or system) was studied in pre- and post-natal hamsters (*Mesocricetus auratus*) labelled with horse radish peroxidase (HRP). Thin sections (70-90 nm) and semi-thin sections (800-1000 nm) were cut using an ultramicrotome and examined under an electron microscope. No evidence of T-tubules was observed during the pre-natal and first week in neonatal stages of development when myocytes were characterised by large cytoplasmic spaces with few and partially developed but functioning myofibrils. Between seven and fourteen days of post-natal life, myofibrils developed rapidly and the A-and I-bands became more distinct. By the seventh day, shallow indentations of the sarcolemma at the Z-line regions appeared, representing the early beginnings of the T-system. Wide T-tubule invaginations of the sarcolemma with cross-sectional diameter of 200-205 nm began to appear in right and left ventricular myocytes by the twenty-first day of post-natal life in some Z-line regions of myofibrils. At this stage also, myofibrils had adopted adult forms with well organised cross-striations. Mitochondria were also well developed, occupying the spaces between rows of myofibrils.

This study supports the view that T-tubules are primarily adapted for homeostatic functions of the mammalian cardiac myocytes, improving efficiency of transport of metabolic substrates and products, and that they are not for excitation-contraction coupling as presumed by some workers. It also shows that the wide T-system observed in the studies of myocytes of an adult hamster is pre-determined, as this feature is noticeable as early as the third week of post-natal development, coinciding with maturation of the myofibrils for active adult life.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The ultrastructure of myocytes and other features of the mammalian heart have been extensively investigated by many workers and the various findings have been reviewed by several workers including Challice and Viragh (1974), Endo (1977), Fabiato (1983), Canale *et al.*, (1986), and Navaratnam (1987). In general, three types of myocardial cells have been described. These are; (i) the atrial cells that also contain the natriuretic hormone, referred to as atrial granules (Palade, 1961, Debold *et al.*, 1981), (ii) the ventricular cells which are the chief working myocytes and (iii) the specialised impulse-generating and conducting myocytes.

Each type of cardiac cell is bounded by a cell membrane referred to as the sarcolemma, and each cell contains a single nucleus with a cytoplasmic compartment containing organelles such as myofibrils, mitochondria, lipid bodies, glycogen granules and a network of endoplasmic reticulum referred to as the sarcoplasmic reticulum (SR). The SR is continuous with the

Golgi apparatus that is found at one pole of the nucleus. Especially in ventricular myocytes, the sarcolemma is invaginated at intervals to form transverse tubules referred to as T-tubules. From extensive investigations conducted so far, the longstanding controversy of whether cardiac cells are in syncytium or not, has been settled by the identification of well-defined intercellular junctions referred to as intercalated discs (van Breemen, 1953, Sjöstrand and Andersson, 1954; Challice and Viragh, 1974).

Morphometric investigations of the cardiac cell types and their cytoplasmic elements in different mammals have also been conducted at the ultrastructural level. The results of these studies clearly reveal that there are considerable differences in volume fractions of myofibrils, mitochondria, lipid bodies, SR, and T-tubules (Anversa *et al.*, 1971, Canale *et al.*, 1986; Navaratnam, 1987; Tagoe *et al.*, 1995; and Dennis, 2000). These differences have been related to functional capacities of cardiac cells in the various mammalian species. For example, work on the mammalian heart including those of the grey seal (*Halichoerus grypus*), a diving mammal (Ayettey, 1979), the insect-eating bat (*Pipistrellus pipistrellus*) which is an arboreal mammal (Ayettey *et al.*, 1991) and the hamster (*Mesocricetus auratus*) and

hedgehog (*Erinaceus europaeus*), mammals with the capacity to hibernate (Olsson, 1972; Ayetey, 1980; Ayetey and Navaratnam, 1981) reveal higher mitochondrial concentrations in ventricular myocytes, compared to similar cells in mammals that do not have special physiologic adaptations like the mouse (*Mus musculus*) (Bossen *et al.*, 1978) and the rat (*Rattus rattus*) (Ayetey, 1978; Schaeper *et al.*, 1985; Kim *et al.*, 1994). Also the width of primary T-tubules measuring from 350 to 450 nm have been reported in the ventricular cells of the insect-eating bat (Ayetey *et al.*, 1991), in the grey seal these tubules measure from 400 to 450 nm (Ayetey, 1979); in the hamster the primary tubules measure from 450 to 500 nm (Ayetey and Navaratnam, 1981) and from 500 to 550 nm in the hedgehog (Ayetey, 1980) as compared to primary T-tubular width in mammals which have no special physiologic adaptations. Some of these mammals having no special physiologic adaptations in which narrow primary T-tubular widths have been observed include; the ferret (*Mustela putorius furo*), measuring from 50 to 80 nm (Simpson and Rayns, 1968), rat measuring from 100 to 130 nm (Forssman and Girardier, 1970; Ayetey and Navaratnam, 1978) and man from 100 to 150 nm (Nayler, 1975). This adds to the view that the quantitative differences observed in the diameter of primary

T-tubules may be adaptive for higher functional capacities of myocardial cells in these mammals.

Of particular interest in the present investigation are the T-system and the role it is considered to play in cardiac cell function. It is generally accepted that these T-tubules are involved in the excitation-contraction process (Endo, 1977; Fabiato, 1983). However, its absence in some cardiac cells has raised doubts as to whether they are essential for this activity in myocytes. This also indicates that the T-tubules may play roles other than or in addition to excitation-contraction coupling. Some workers, for example, (Ayettey and Navaratnam, 1978) have already suggested that these tubules enhance the metabolic efficiency of cardiac cells, as they increase surface area of the sarcolemma for the transport of metabolic substrates and products across the cell membrane.

1.2 Literature review of the Transverse Tubular System (T- System)

The T-tubule system had attracted much interest among researchers for nearly forty-five years. The first investigator to identify invaginations of the sarcolemma with the aid of the

electron microscope was Lindner (1957) who worked on the ventricular myocardium of the dog (*Canis familiaris*). In the same year, Porter and Palade (1957) also described similar tubules in skeletal muscles of the rat. Andersson-Cedergren (1959) found these tubules in the rat and was the first to refer to them as T-tubules. She was also the first to suggest that this tubular system is related to the contractile process. Subsequently, several workers have identified T-tubules in ventricular myocytes in many mammalian species. These include Simpson and Oertalis (1961) in the sheep (*Ovis aries*), Nelson and Benson (1963) in the rabbit (*Lepus cuniculus*) and human, Rayns *et al.*, (1975) in the guinea pig (*Cavia porcellus*), Page (1967), Forssman and Girardier (1970) in the rat and Forbes and Sperelakis (1977) in the mouse (*Mus musculus*). These tubules occur in the I-and Z-band regions in cardiac and skeletal muscle cells. In skeletal muscle cells, the T-tubule couples with the SR on either side of its sarcolemma, giving rise to a triadic system (Huxley, 1964). In cardiac cells, however, the SR coupling is mainly found on one side of the sarcolemmal membrane, giving rise to a diadic system (Simpson and Rayns, 1968).

The SR that couples with T-tubules in cardiac cells is morphologically distinct, in that it has fine linearly-organised luminal granules and electron dense plaques that project like foot processes towards the T-tubule membrane (Manasek, 1968; Franzini-Armstrong, 1970; Challice and Viragh, 1973; Rayns *et al.*, 1975; Ishikiwa and Yamada, 1975; Ayetey, 1978; Ayetey and Navaratnam, 1978). These feet-like processes at the T-SR junctional gap had been described earlier in skeletal muscles. The feet-like processes do not, however, contact the T-tubule membrane, there being a narrow gap of about 2 nm between the plaque and the tubule. It is considered that this arrangement facilitates transfer of the electrical impulse from the T-tubule to the SR membrane, as the gap of 2 nm is small enough for such process (Fabiato, 1983; Meissner, 1994; Schneider, 1994).

The morphology of T-tubules has been carefully researched, with the finding that T-tubule diameters, for example, can be altered by varying ionic composition of the extracellular fluid (Legato *et al.*, 1968). In his study of the ultrastructure of the cardiac cell, Page (1978) noted that the T-system is lined by a basement membrane that is continuous with the surface sarcolemma. As already noted, the lumen of the T-tubules also

vary in calibre in different species (Sommer and Johnson, 1970; Ayetey, 1979; 1980; Ayetey and Navaratnam, 1981; Ayetey *et al.*, 1991). From such ultrastructural study of the human ventricular myocytes, Katz (1992) referred to the lumen of the T-tubule as “carrying” the extracellular space towards the centre of the myocardial cell, thereby increasing its surface area substantially. Cytochemically, the T-tubular membranes, like the rest of the surface sarcolemma, contain membrane-bound enzymes such as adenylate and guanylate cyclases and basic adenosine triphosphatase (Schultze, 1984; Malouf and Meissner, 1984). Similarly, the membrane of T-tubules possess two proteins, amphiphysin II and caveolin III (De Camilli *et al.*, 2002) which are also implicated in sarcolemmal degeneration.

The architecture of the T-system has been documented quite well from the works of a number of workers including Forssman and Girardier (1970) and Ayetey and Navaratnam (1978). In these studies, the extracellular space was labelled with electron-dense material such as horseradish peroxidase (HRP) stained with diaminobenzidine (DAB) to define the T-tubule invaginations. From the work of these investigators, four main components of the tubules have now been described: (i) primary,

(ii) secondary and (iii) tertiary transverse tubules and (iv) longitudinally oriented tubules.

The primary transverse tubules are the direct invaginations of the plasma membrane. The only morphological difference established so far between this part of the membrane and the surface sarcolemma is the lack of pinocytotic vesicles, small and globular invaginations of the sarcolemma that remain in the vicinity of the sarcolemma in the subsarcolemmal space (Fawcett and McNutt, 1969; Ishikawa and Yamada, 1975; Forbes and Sperelakis, 1976). Peroxidase labelling renders these vesicles as separate structures from the T-tubules (Ayetey, 1978; Ayetey and Navaratnam, 1978). The primary T-tubules, in general, are located in I-band regions close to the Z-band. They are associated with diadic couplings as described previously.

Secondary T-tubules are transversely oriented branches of the primary T-tubules and are found in the I- and Z- band regions. They are at right angles to the primary ones, but are narrower than the parent tubules from which they arise, being approximately half the diameter of the primary ones; they also do not contain any identifiable basement membrane such as found in

primary tubules (Ayetey and Navaratnam, 1978). The secondary T-tubules, like the primary ones, are associated with SR to form diadic couplings. Investigations so far have not revealed any branches off the secondary T-tubules (Ayetey and Navaratnam, 1978).

Longitudinal T-tubules are branches of the primary T-tubules but, unlike the secondary T-tubules which run transversely across myofibrils, they (the longitudinal tubules) run in a direction parallel to the long axis of the myofibrils (Forssman and Girardier, 1970; Ayetey and Navaratnam; 1978, Ayetey, 1978). The longitudinal T-tubules are narrower than their parent primary T-tubules measuring about half the diameter of the primary ones. They are, therefore, of the same calibre as the secondary transverse tubules. These longitudinal T-tubules lack any identifiable basement membrane. In the absence of extracellular markers such as HRP, these tubules can easily be confused with longitudinally running SR tubules, especially if they are narrow. Longitudinal T-tubules enter into coupling with profiles of SR to form diads (Ayetey and Navaratnam, 1978). Adjacent longitudinal T-tubules are linked at the A-band levels by narrow tubules referred to as the tertiary T-tubules. These

tertiary tubules have been well demonstrated in rat ventricular cells (Ayettey and Navaratnam, 1978). They are narrower than the longitudinal and secondary transverse tubules, measuring about half the diameter of the secondary transverse and longitudinal tubules. They are located in A-band regions close to the M-line at the centre of the A band. They form diadic couplings with SR profiles, but less so than in other parts of the T-system (Ayettey and Navaratnam, 1978). The tertiary T-tubules, like secondary and longitudinal T-tubules lack luminal basement membrane coat (Navaratnam, 1987).

The literature on cardiac ultrastructure clearly reveals that the T-tubules are not present in all cardiac cells. Even in most mammalian hearts investigated so far, T-tubules are either rare or absent in atrial cells (Forssman and Girardier, 1970, Hibbs and Ferrans, 1969). In the impulse generating and conducting cells that are not designed primarily for contractile activity, T-tubules are rare or absent. Most workers, including Sommer and Johnson (1968) had been unable to identify these tubules in specialised myocardial cells. Page (1967), however, reported the presence of T-tubules in Purkinje impulse-conducting cells in the cat heart. After labelling the extracellular space with HRP,

rudimentary forms of the T-system have also been described in cells in different parts of specialised impulse-generating and conducting system including the nodes (Ayettey and Navaratnam, 1978). Surprisingly, an extensive T-system has even been reported in conducting Purkinje cells of the fruit-eating bat (*Eidolon helvum*) even without peroxidase labelling (Ayettey *et al.*, 1993).

Investigations in non-mammalian vertebrates have so far failed to identify with certainty, T-tubules in ventricular, atrial or the specialised impulse generating and conducting cells. No T-tubules have been found in fishes (Yamamoto, 1966), amphibians, (Staley and Benson, 1968; Sommer and Johnson, 1969), reptiles (Leak, 1967; Hirakow, 1970) and birds (Akester and Akester, 1971; Hikida, 1972). Even after HRP-labelling, no T-tubules were found in the myocardium of frog (*Bufo regularis*) myocardium (Page and Niedergerke, 1972) and the domestic fowl (*Gallus domesticus*) (Ayettey, 2002 personal communication). Only Hirakow (1970) had reported some form of T-tubules in developing avian and turtle heart cells.

T-tubules have been found in myocardial cells of invertebrates like crustaceans with myocytes being even smaller in

size than in non-mammalian vertebrates (Myklebust *et al.*, 1977; Myklebust, 1977). T-tubules have also been reported in skeletal muscle cells of all vertebrates (Ross *et al.*, 1989).

Until reports of Myklebust and co-workers (Myklebust *et al.*, 1977) confirmed the existence of T-tubules in crustacean myocardial cells, the absence of T-tubules in non-mammalian vertebrates had been attributed to small diameters of cardiac myocytes in these animals (Hirakow, 1970). Girardier (1965) had considered that there was a critical size of myocyte below which no T-tubules were found. This however, could not explain the absence of T-tubules in atrial cells, some of which are of the same diameter as ventricular myocytes that possess such tubules in the same animal species (Eisenberg and Eisenberg, 1968). From comparative studies of the different chambers of the mammalian heart, Fawcett and McNutt (1969) noted that the presence or absence of T-tubules could not be predicted from cell diameter alone. They however, observed that T-tubules were rare or absent in atrial cells with average diameter of 5-6 μm (Fawcett and McNutt, 1969). For example, T-tubules are virtually absent in ferret (*Mustela putorius furo*) atrial muscle cells with average diameter of 1-2 μm (Forbes and Sperelakis, 1977). Ayetey (1978) who noted the presence of T-tubules in the specialised nodal cells

of the rat argued that there could be the possibility of occurrence of T-tubules in many lower vertebrates but that they might be too narrow to be identified without extracellular labelling with HRP, lanthanum hydroxide or ferritin. To date, however, there has not been any conclusive report of existence of T-tubules in the non-mammalian vertebrates.

As noted earlier, quantitative differences exist in the size of T-tubules in ventricular cells of mammals. Bats, seals, hamsters and hedgehogs, have been reported to possess wide calibre primary T-tubules. Diameters range from 350-450 nm in the insect-eating bat (*Pipistrellus pipistrellus*) (Ayettey *et al.*, 1991), 400-450 nm in the grey seal (*Halichorus grypus*) (Ayettey, 1979), 450-500 nm in the hamster (*Mesocricetus auratus*) (Ayettey and Navaratnam, 1981) and 500-550 nm in the hedgehog (*Erinaceus europaeus*) (Ayettey, 1980). It is noteworthy that all these animals exhibit some special characteristics such as diving in the seals, high exercise tolerance in the bats and hibernation in the hedgehogs and hamsters (Spector, 1956; Ayettey and Navaratnam, 1981). The golden hamster, for example, can alter its heart rate from a normal level of about 450 beats per minute to 4-15 beats per minute during hibernation (Spector, 1956) without any deleterious effect to the myocardium such as ventricular arrhythmia. The grey

seal, *Halichorus grypus* also reduces its heart rate from about 120 beats per minute to 4 beats per minute during diving without ventricular arrhythmia (Harrison and Ridgway, 1976). It had been suggested therefore that the wide T-tubules in these mammals may have a protective effect in the myocyte in extreme bradycardia (Ayettey, *et al.*, 1991; Anaglate *et al.*, 1994; Dennis *et al.*, 1999; Tagoe *et al.*, 1999).

The significance of the occurrence of T-tubules and the variations in their sizes has been considered. As these T-tubules are not present in all cardiac cells, it cannot be said that they are essential for excitation-contraction coupling process as suggested by earlier workers (Endo, 1977). Since these tubules, by their invaginations, increase surface area (Page, 1978) the possible role of these tubules in improving metabolic efficiency had been discussed (Ayettey *et al.*, 1993; Dennis *et al.*, 1999).

Little information is available on the development of T-tubules in either cardiac or skeletal muscle, although the development of the vertebrate heart has been extensively studied over many years (Balinsky, 1970; Chacko, 1972; Challice and Viragh, 1974). Huxley (1964) and Simpson (1965) using fate map techniques in the study of the origin of organelles in

different mammals, showed that the T-system has the same origin as the SR, arising from the endodermal germ layer. This study was reviewed and confirmed in a subsequent study on rat embryos by Chacko (1972), who noted the presence of SR tubules in the 7-day pre-natal rat myocytes but gave no indication of when the T-tubules first appeared.

Investigations in mammals like the mouse (*Mus musculus*) (Challice and Viragh, 1973a; Ishikawa and Yamada, 1975; Forbes *et al.*, 1984); hamster (*Mesocricetus auratus*) (Colgan *et al.*, 1978); rat (*Rattus rattus*) (Schiebler and Wolff, 1966; Hirakow and Gotoh, 1975; Nakata, 1977; Hirakow *et al.*, 1980); cat (*Felis domestica*) (Sheridan *et al.*, 1979; Maylie, 1982; Gotoh, 1983); rabbit (*Lepus cuniculus*) (Hoerter *et al.*, 1981; Page and Buecker, 1981); dog (*Canis familiaris*) (Bishop, 1973; Legato, 1975; 1979) and opossum (*Didelphis virginiana*) (Hirakow and Krause, 1980) indicate that the development of T-tubules is a post-natal event. In the human embryo (Leak and Burke, 1965), rhesus monkey (*Macaca mulatta*) (Allen and Carstens, 1967), sheep (*Ovis aries*) (Brook *et al.*, 1984, Sheldon *et al.*, 1976), cow (*Bos indicus*) (Forsgren and Thornell, 1981), and guinea-pig (*Cavia porcellus*) (Forbes and Sperelakis, 1976,

Hirakow and Gotoh, 1980), however, the T-tubules had been observed at pre-natal stages.

The present study is to investigate the development of the T-tubules in the hamster, a mammal with wide T-tubules in the adult ventricular cells. As this animal survives under extreme conditions of severe bradycardia, it is important to establish when the tubules first appear and whether they occur at the same time in myocardial cells of both right and left ventricles. This study was further intended support the argument that T-tubules are not essential for excitation-contraction coupling but that they are part of adaptive features for efficient homeostatic mechanism.

CHAPTER TWO

MATERIALS AND METHODS

2.1 The biology and taxonomy of the hamster.

The golden hamster is a popular pet and a research animal. Hamsters are known for their short gestation period, pugnacious disposition, cheek pouches, large immature litters and the ability to escape confinement. Hamsters do well on pelleted chows and usually remain healthy and active throughout their short life span (Hoffman *et al.*, 1968).

Hamsters are mammals belonging to the order: Rodentia, family: Cricetidae, genus: *Mesocricetus* and species: *M. auratus* (Menzies, 1961).

2.2 Breeding of hamsters

The hamsters were bred at the Laboratory Animals Unit of the Noguchi Memorial Institute for Medical Research, Legon. The animals were kept in metallic cages of about 2 m³ and fed with vitamin-rich pelleted fish-chow and autoclaved water. The temperature in the breeding room was maintained at 18-20°C.

Hamsters have a 4-day estrous cycle. As a sexually mature female approached the estrous period, slight stringy,

translucent mucus was extruded from the vagina. Such females were then weighed and caged with mature males. The morning following estrous a tenaceous opaque vaginal discharge appeared. A receptive, non-belligerent behaviour of these females to the male hamsters indicated an impending estrous period. The caged female hamsters were weighed every morning since the cessation of the vaginal discharge upon mating and a rapid weight gain determined the day of pregnancy. Pseudopregnancy was usually avoided by the observation of a cessation of vaginal discharge of estrous within 5-9 days automatically upon fertilisation coupled with a rapid weight gain.

The gestation period of the hamsters lasted for 14-18 days, with an average of 16 days. Average litter size was six. The average life span of hamsters is 18-24 months, but older individuals have been reported (Menzies, 1961).

2.3 Grouping of hamsters

Six pregnant hamsters and twenty young from the breeding stock were used and divided into two batches; pre- and post-uterine (pre- and post- natal) hamsters. The post-natals were also referred to as neonatals. Each group was further sub-divided into

two: (i) treated (TT)-those treated with horse radish peroxidase (HRP) and (ii) control (CT)-those free from HRP treatment.

2.3.1 Pre-natal Group

Two pregnant hamsters each (one treated and one control) were sacrificed 14½, 15½, and 16½ days after conception. Twenty-four to forty-eight hours prior to sacrifice, one pregnant hamster was injected with 0.1 ml HRP dissolved in 5 ml isotonic saline through the femoral vein. Foetuses from this material were designated as treated (TT), while foetuses from the remaining untreated pregnant mothers were the controls (CT).

Following full anaesthetisation of the pregnant hamsters with ether, the uteri were quickly removed within 1-2 mins. The removal of the uteri was further made possible by immobilising the hamsters with dissecting pins through their fore and hind limbs. The uteri were then placed in a petri dish containing isotonic saline. The foetuses were quickly freed from the placental tissues with a pair of fine forceps.

While still breathing, half of the foetal hearts of materials from the treated batch were further injected with 0.1 ml solution of the HRP dissolved in 5 ml isotonic saline. This was to make up for

any probable loss of HRP through the maternal circulation. By using a hypodermic syringe fitted with a fine needle of aperture size 16 mm, the apex of the foetal heart was carefully located through the transparent skin of the thorax. The heart apices were pierced gently through the thorax and 0.1 ml of HRP dissolved in 5 ml isotonic saline was injected into the ventricular chamber. Contraction of the foetal heart enabled circulation of the HRP into all tissues including the heart. The foetuses were then placed in a sterile hood for 30-60 mins.

After the HRP treatment, the foetuses were washed in a freshly prepared isotonic saline solution in a petri dish. With the foetuses still breathing, their thoracic cages were cut open with a pair of fine scissors under the dissecting microscope. The hearts were carefully dissected out and were fixed in Karnovsky's fixative in 0.1M sodium cacodylate buffer for 20-30 mins. The foetal hearts were subsequently washed free of excess fixative in 0.1M sodium cacodylate buffer for 3 mins. The treated hearts were injected with 0.1 ml of 3,3- diaminobenzidine trihydrochloride (DAB) dissolved in 10 ml of 0.1M sodium cacodylate buffer mixed with 1% Hydrogen peroxide (H_2O_2) solution for 30-60 mins. DAB is an incubating medium that enhances adequate penetration of HRP into the heart tissues.

In handling DAB, which is poisonous, protective clothing and hand gloves were worn and the treatment was carried out in a fume cupboard in order to avoid contact with the skin. The hearts were then rinsed thoroughly with 0.1M sodium cacodylate buffer solution, cut into pieces of about 1mm³ with a single edged blade and finally post fixed in 1% osmium tetra oxide (OsO₄) buffered in 0.1M sodium cacodylate solution for 3 hours.

Foetuses of anaesthetised pregnant hamsters used as control were given the same treatment as described above, except that they were not treated with HRP and DAB.

2.3.2 Post-natal Group

Tissues designated as treated in this case were processed differently. Three litter age, all from the same mother were picked at random. They were injected through the cephalic vein with 0.1 ml of HRP dissolved in 5 ml isotonic saline, twelve to forty-eight hours prior to being sacrificed at age 1, 7, 14 and 21 days old. These neonates were subsequently anaesthetised with ether, their thoracic cages cut open and the hearts perfused for 10 mins with 2% glutaraldehyde in 0.1M sodium cacodylate buffer. This was to

harden the otherwise tender tissues of these neonatals and also to stabilise the ionic components of the tissues by stopping further enzymatic activity. The neonatal hearts were later perfused with Karnovsky's fixative in 0.1M sodium cacodylate buffer for another 10 mins. This process further fixes the tissues and consequently minimises autolysis. The hearts were subsequently injected through the apex with 10 ml of DAB dissolved in 0.1M sodium cacodylate and 1 % H₂O₂ and then allowed to incubate for 30-60 mins. The hearts were then removed, washed in a solution of 0.1M sodium cacodylate buffer solution for 3 mins after which left and right ventricles were identified and dissected under a dissecting microscope in a petri dish filled with Karnovsky's fixative.

The dissected heart parts were again washed in 0.1M sodium cacodylate buffer solution in order to rinse off any excess Karnovsky's fixative from the tissues, cut into smaller pieces of about 1mm³, and then post-fixed in 1% OsO₄ in 0.1M sodium cacodylate buffer for 3 hours. As stated earlier, hearts of control hamsters were not treated with HRP and DAB but for the microscopic work the tissue pieces were processed in same way as was done for treated animals.

2.4 Preparation of Tissues for Light and Electron Microscopy

The 1 mm³ of heart tissues from Control and Treated materials in both pre- and post-natals were dehydrated through increasing series of alcohol, i.e 50%, 70%, 95%, and 100%. The tissues were dehydrated in two changes of 50% alcohol for 5 mins each, in three changes of 70% alcohol for 5 mins each, in three changes of 95% alcohol for 5 mins each, and in three changes of 100 % alcohol for 10 mins each. The heart tissues were subsequently embedded in four stages of the epon mixture (see appendix II) in order to ensure that tissues were properly embedded in the epon mixture so as to avoid break up of tissues during sectioning. Thus, the tissues were embedded in: (i) 3 parts of 100% alcohol and one part of the epon mixture for 30-60 mins; (ii) 1: 1 part of 100 % alcohol and epon mixture for 30-60 mins; (iii) 1: 3 parts of 100 % alcohol and epon mixture.

The dehydrated tissues were then passed through propylene oxide for 10 mins at 4°C to clear any alcohol that may be present.

Finally, the tissues were embedded in freshly prepared mixture of epon and then cured in an oven at 30-60°C for 48-72 hours.

2.4.1 Light Microscopy

Five blocks from each of the foetal and neonatal tissues were selected at random and used for the study. Semi-thin (800-1000 nm) sections were cut using *LEICA ULTRA CUT R* ultramicrotome, and a Diatome knife. These sections were examined with the light microscope after staining with toluidine blue. The sections were picked on drops of distilled water on clean slides, fixed properly on the slides by heating for 1-2 mins on a *GALLENKAMP HOTPLATE 300* at 60-70°C, flooded with toluidine blue and then heated again for another 1-2 mins. The stain was washed off with distilled water, the slide dried and mounted in Distrene, Plasticizer and Xylene (DPX) and then cover slipped. The slides were examined under an *OLYMPUS* light microscope fitted with a camera for taking pictures. The light microscope at this stage was used in order to check whether the relevant regions of the tissue were being sectioned for the electron microscopic (EM) work. Measurement of sarcomere length was done using a *REICHERT* monocular light microscope fitted with

an eye piece graticule. Pictures were subsequently taken on the relevant sites and printed for examination.

2.4.2 Electron Microscopy

Greyish looking ribbon-like, thin sections (70-90 nm) were picked on 200 mesh copper grids, dried on a filter paper for about 5 mins and stained with 2% uranyl acetate in 50 % alcohol for 10-15 mins. The uranyl acetate was rinsed gently with a jet of distilled water for 1 min. The staining was carried out in an enclosed container to reduce contact since uranium is highly radioactive. The slides were subsequently counter stained with 2% lead citrate for 6-10 mins, washed carefully with distilled water for 1 min, and dried on a filter paper for 5 mins. Sodium hydroxide pellets were placed near the lead citrate on beeswax in order to absorb any carbon dioxide (CO₂) from the system to prevent any possible reaction with the lead. Stained sections were viewed in a *JEOL JEM-1010* electron microscope at 80 KV. Pictures of specimens were taken at magnifications ranging between 2,500 and 20,000 and prints were made at final magnifications of 5,000-

40,000. The final prints were used in studies of organelles, especially T-tubules.

CHAPTER THREE

RESULTS

Two types of micrographs are obtained; (i) sections treated with horseradish peroxidase (HRP) and (ii) those free from HRP treatment or controls. The HRP is only intended to infiltrate the extracellular space and consequently the T-tubules but it has failed to penetrate these regions. Therefore, all the micrographs show similar features.

3.1 Pre-Natal Ventricular Cells (days 14½, 15½, and 16½).

3.1.1 Left Ventricle

(Figures 1, 2 and 3) show that the myocytes have large cytoplasmic space. These myocytes are irregularly arranged and there is a limiting membrane, the sarcolemma that surrounds the individual myocytes. The sarcolemma forms clear intercalated discs (ID) at the cell junctions between these myocytes. There are no sarcolemmal specialisations indicative of T-tubules, but are

present in the subsarcolemmal regions what appear as internal membrane systems perceive to be SR tubules, (Fig. 1).

The most prominent organelles encountered at this stage include the myofibrils, nuclei and mitochondria. The myofibrils appear to be poorly developed with scattered myofilaments. In each myocardial cell is a single, centrally-located nucleus which contains fine chromatin material as well as a prominent nucleolus. Mitochondria abound in the cytoplasmic spaces. They are irregularly arranged along the strands of myofibrils. The most frequent forms seen are cylindrical, round and dumb-bell.

Large amounts of lipid bodies are also encountered. These are usually present in the form of dark, round bodies without visible limiting membrane figs.

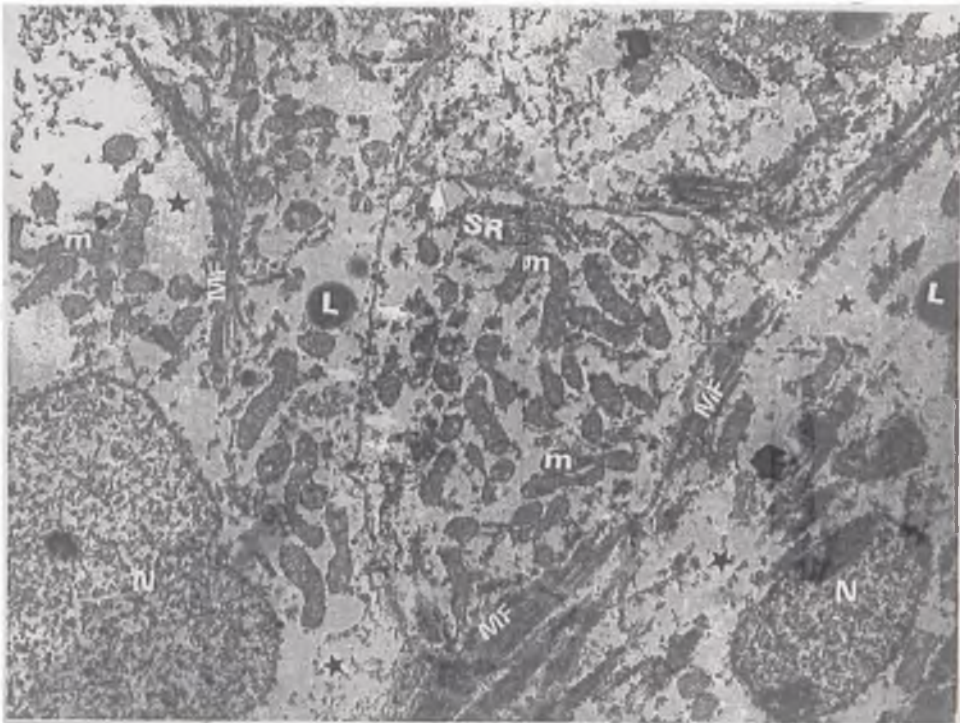


Fig 1: Electron micrograph of left ventricular myocytes of pre-natal hamster (14½ days of pregnancy) showing large cytoplasmic (intracellular) spaces (stars), poorly-formed myofibrils (MF), mitochondria (m), intercalated disc (arrow), lipid bodies (L), subsarcolemmal SR tubules and nuclei (N). Note the absence of T-tubules. X8, 000.

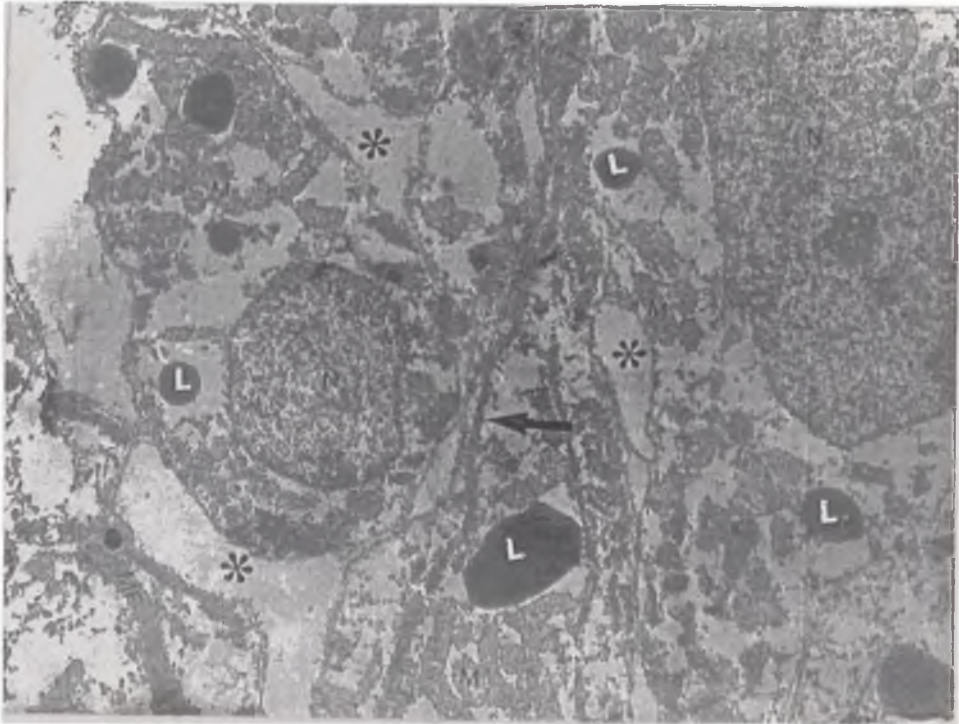


Fig.2: Electron micrograph of left ventricular myocytes of a 15½ day old pre-natal hamster showing large cytoplasmic spaces (), lipid bodies (L), intercalated disc (arrow), myofibrils (MF), mitochondria (M) and nuclei (N). No T-tubules seen X 8,000.*

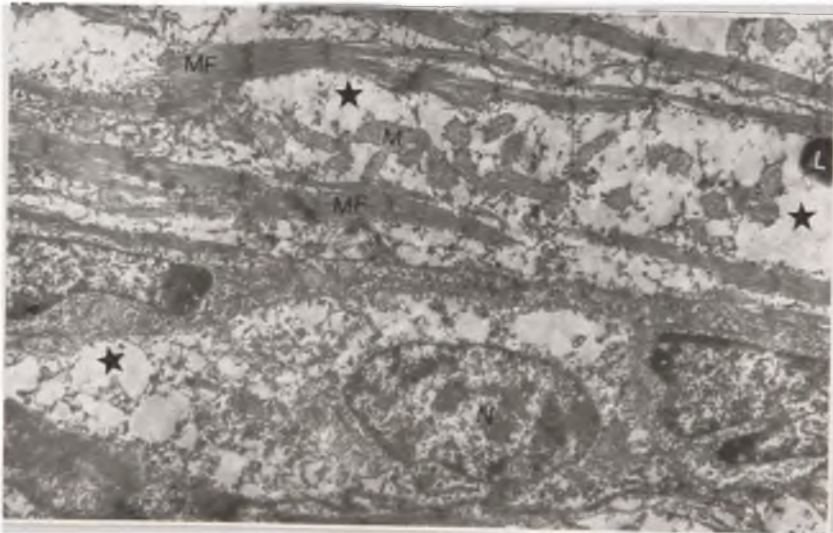


Fig.3: Electron micrograph of the left ventricular myocytes of a pre-natal hamster (16½ days of gestation) showing myofibrils (MF) separated by large cytoplasmic spaces (stars), numerous mitochondria (M), lipid body (L) and nucleus (N). Note the absence of T-tubules. X 8,000

3.1.2 Right Ventricle

The myocytes appeared similar to those observed in the left ventricle. The cells adjoin each other in different planes with clearly defined intercalated discs without specialisations occurring at the cell junctions (Figs. 4, 5 and 6). Occasionally, one or two desmosomes are seen along these cell junctions (Fig. 4). No T-tubules are observed, however, there is present SR in the non-specialised regions of the intercalated discs (Fig. 4).

Myofibrils and mitochondria, as seen in the left ventricular myocytes are the most frequently encountered cellular organelles from this portion of the heart (Fig. 5). The myofibrils are poorly-formed with the myofilaments being irregularly arranged (Fig. 5). Large cytoplasmic spaces separate adjacent myofibrils (figs.5 and 6). Frequently, long columns of mitochondria are also seen in the cytoplasmic spaces. Occasionally, these mitochondria are found packed closely to the long axis of the poorly-formed strands of myofibrils (Figs. 4, 5 and 6). Most common mitochondrial shapes seen are cylindrical, round and dumb-bell (Figs 4, 5 and 6).

Each myocyte is usually associated with a nucleus, which appear to be centrally placed. Frequently, full outlines of these

nuclei are difficult to observe as portions usually appear outside the field of view (Figs. 4, 5 and 6). Few lipid bodies are also encountered in the cytoplasmic spaces close to mitochondria (Fig. 4).

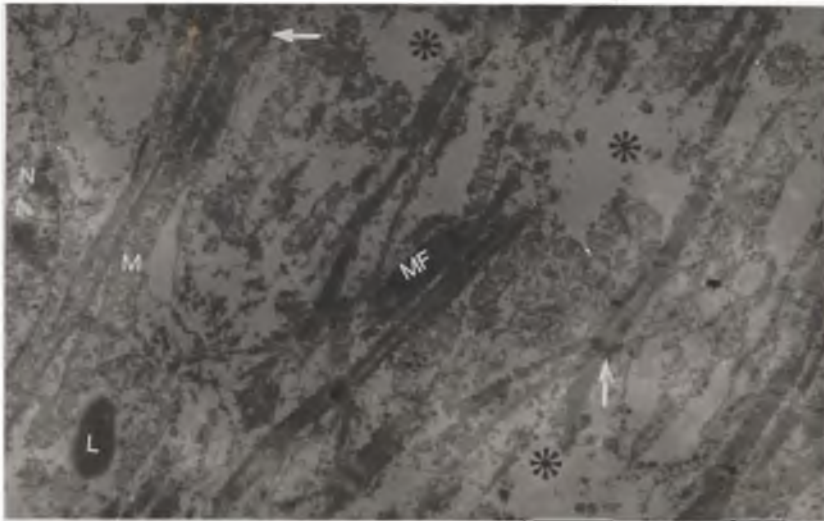


Fig.4: Electron micrograph of right ventricular myocytes of 14 1/2 day old pre-natal hamster showing myofibrils (MF), mitochondria (M), lipid body (L) and nucleus (N). Upper arrow indicates an Intercalated Disc and bottom arrow shows a desmosome. T-tubules are, however absent. X8, 000

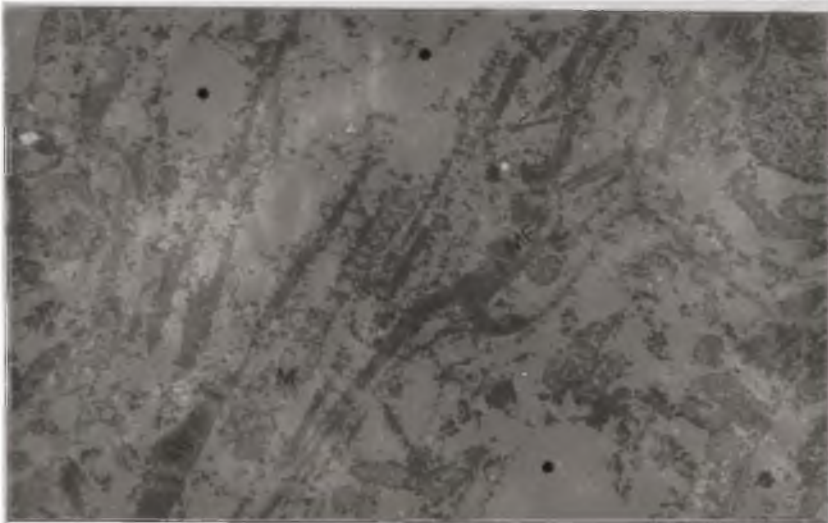


Fig.5: Electron micrograph of the right ventricular myocyte of a 15½ day old pre-natal hamster showing poorly-formed myofibrils (MF), mitochondria (M) and large cytoplasmic spaces (). No T-tubule is seen. X 8,000.*

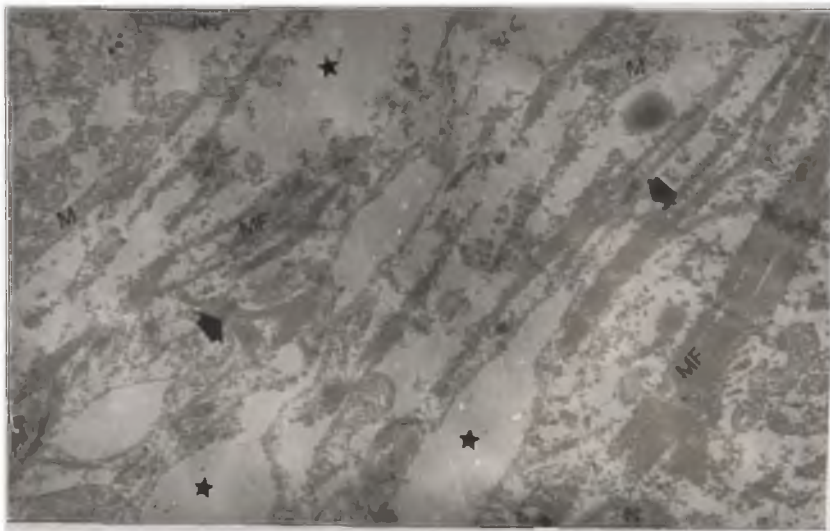


Fig.6: Electron micrograph of the right ventricular myocyte of a 16½ day old pre-natal hamster showing myofibrils (MF), mitochondria (M), nuclei (N) and large cytoplasmic spaces (). Arrows indicate SR tubules at intercellular junctions. Note the absence of T-tubules. X 8,000.*

3.2 Post-natal Stage

3.2.1 Day 1 Left Ventricle

In Figs. 7 and 8, myocytes at this stage bear close resemblance to those observed in the pre-natal stage. The myofibrils appear as the most dominant cellular organelle. Large cytoplasmic spaces are seen to separate adjacent bands of myofibrils. Generally, myofibrils at this stage appear to be better organised than those encountered in the pre-natal stages as evidence by relatively, closely packed myofilaments and well-defined Z-lines in these micrographs (Figs. 7 and 8).

The sarcolemma enveloping each myocyte lacks T-tubules. Scalloping and invagination of the sarcolemma are seen at this stage. No definitive T-tubules, however, are observed on the sarcolemmal surface. As observed in the pre-natal stages, mitochondria occur close to myofibrils with large numbers of glycogen granules and lipid bodies adjoining columns of mitochondria and the long axis of myofibrils (Fig. 7). Each myocyte is seen to be associated with a prominent elongated nucleus, (Fig 8).

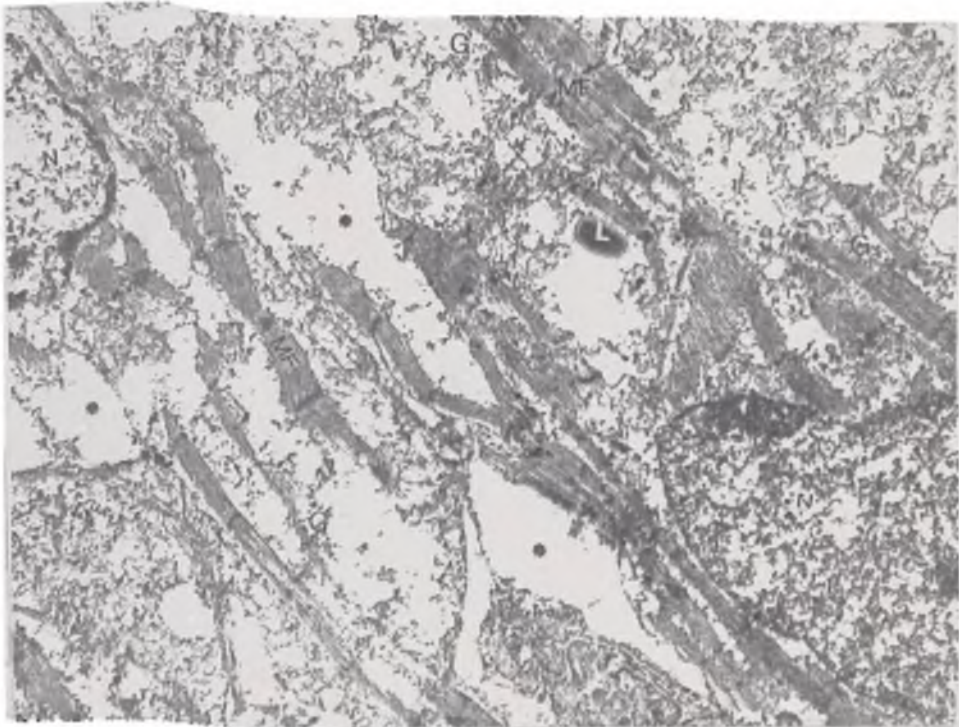


Fig. 7: Electron micrograph of the left ventricular myocyte of a day old post-natal hamster (20 hours after birth) showing relatively well organised myofibrils (MF), nuclei (N), cytoplasmic spaces (), glycogen granules (G) and lipid body (L) Note the absence of T-tubules. X 8,000.*

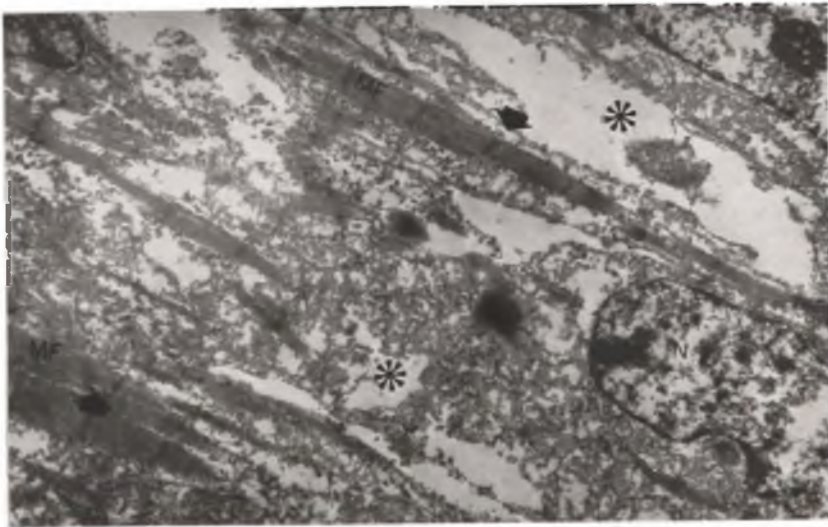


Fig. 8: Electron micrograph of the left ventricular myocyte of a day-old post-natal hamster (20 hours after birth) showing relatively well-formed myofibrils (MF) with clearly demarcated Z-lines (arrows), nucleus (N) and large cytoplasmic spaces (). Note the absence of T-tubules. X 8,000.*

3.2.2 Day 1 Right Ventricle

Figures 9 and 10 show development of organelles in the right ventricle of day old hamsters. Organelles seen in the right ventricular myocytes appear similar to those observed in the left ventricular cells. At lower magnifications, (Fig. 9) myofibrils and mitochondria are seen as the most prominent cellular organelles. Large cytoplasmic spaces are frequently encountered. In cross-section, these intercellular spaces show blood vessels and connective tissue (Fig. 10).

Few desmosomes are found at the cell junctions where the sarcolemma adjoining adjacent cells come into close apposition (Fig. 9). No T-tubules are seen in these myocytes.

The nucleus contains fine chromatin material that appear denser than the surrounding organelles (Fig. 10). Large numbers of glycogen granules are also seen in the myocytes.



Fig. 9: Electron micrograph of the right ventricular myocytes of a day old post-natal hamster showing long strands of myofibrils (MF), large cytoplasmic spaces (), lipid body (L), mitochondria (M) and a desmosome (arrow). No T-tubules are seen. X4,000.*

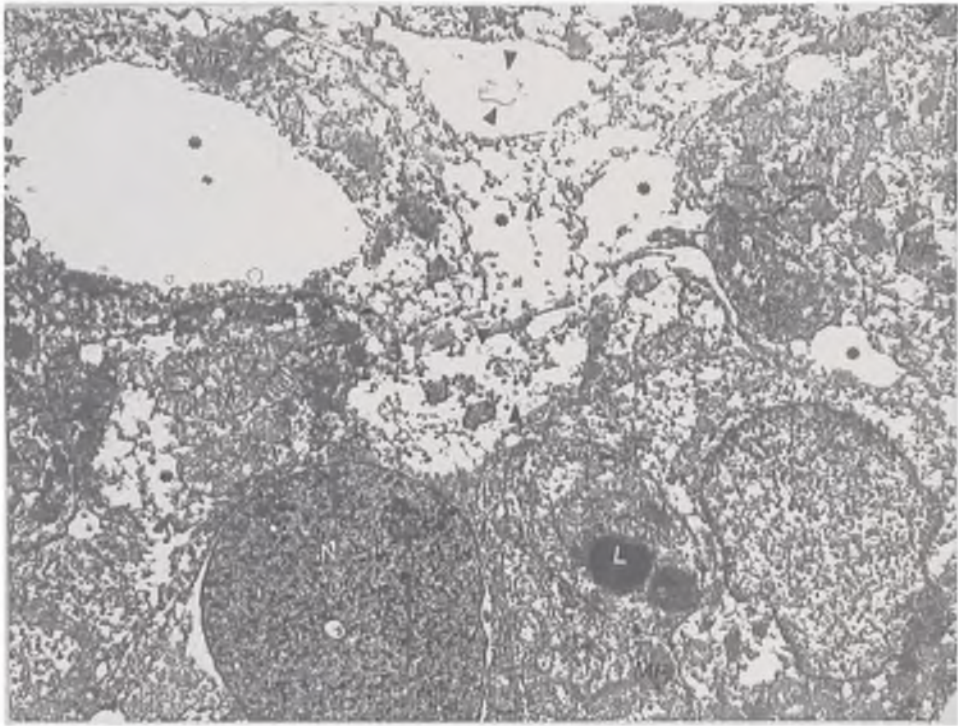


Fig. 10: Electron micrograph of a transverse section of the right ventricular myocytes of a day old postnatal hamster showing cytoplasmic spaces occupied by blood vessels and connective tissue (arrowheads), mitochondria (M), myofibrils (MF), lipid body (L) and nuclei (N). X8, 000.

3.2.3 Day 7 Left Ventricle.

In Figs. 11, 12 and 13 the myofibrils appear to be relatively more organised by way of the closeness of their strands than previously encountered. The large cytoplasmic spaces that characterised myocyte in the previous stages also appear reduced. The nucleus is centrally located (Fig. 11).

There are shallow indentations of the sarcolemma in the Z-line regions of the myofibrils. These indentations measure from 150 to 200 nm across their widest diameter. Close to the indentations are profiles of SR (Fig. 12). Two adjacent, dark lines running perpendicular to the long axis of the myofibrils limit a single sarcomere (Fig. 11).

Mitochondria are seen close to the long axis of myofibrils. Occasionally, lipid bodies are seen in the cytoplasm of these myocytes, adjacent to the mitochondria (Figs. 11 and 13). The myocytes appear to be joined end-to-end with well-defined IDs in the intercellular junctions (Figs. 13).

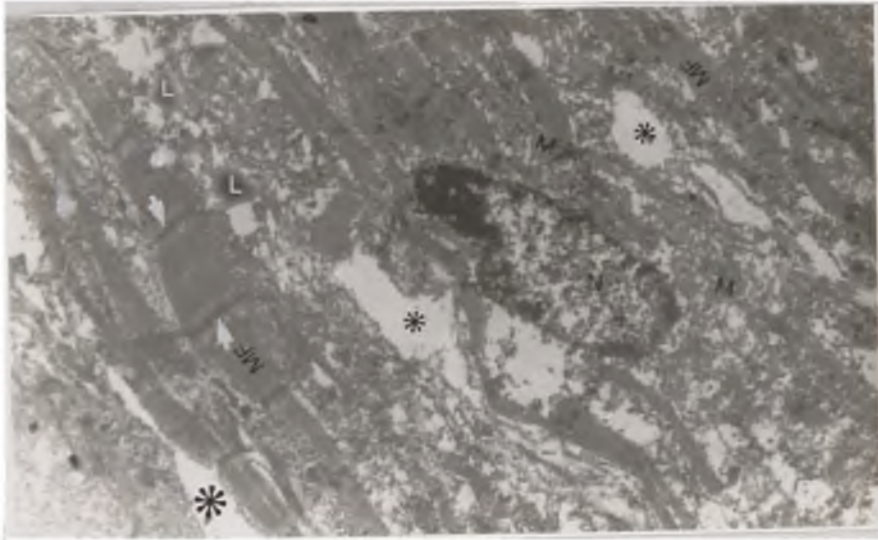


Fig. 11: Electron micrograph of the left ventricular myocyte of a 7-day hamster (post-natal) showing well-developed myofibrils (MF) with reduced cytoplasmic spaces (), centrally placed nucleus (N), Z-lines delimiting sarcomeres on myofibrils (white arrows), mitochondria (M) and lipid bodies (L). Note the absence of T-tubules X 8,000.*

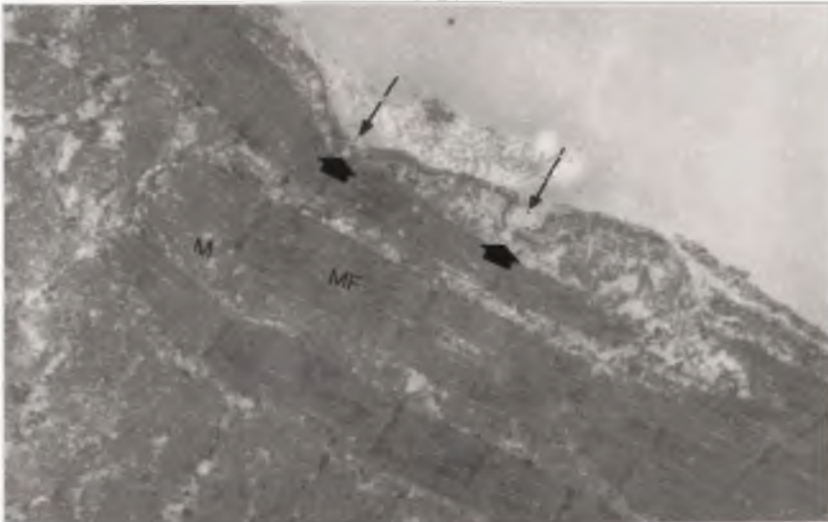


Fig. 12: Electron micrograph of the Left ventricular myocyte of a 7-day old post-natal hamster. Note the regular indentation of the sarcolemma in the Z-line region (small arrows), T-SR tubular couplings (big arrows) and the reduced cytoplasmic space. Mitochondria (M) are seen adjacent to myofibrils (MF). X 20,000.



Fig. 13: Electron micrograph of the left ventricular myocytes of a 7-day old post-natal hamster showing regular indentations of the sarcolemma indicating the beginning of T-tubules (arrows) and lipid bodies (L). White arrow shows thickened sarcolemma with its overlying basement membrane to form an ID. Definitive T-tubules are, however, absent. X 5,000.

3.2.4 Day 7 Right Ventricle

As also observed in the left ventricular cells, reduced intracellular spaces characterised these myocytes. At high magnification, (Fig. 14), only myofibrils and mitochondria are visible.

The sarcolemma of the myocytes is vaguely seen, but regular indentations along its surface at the Z-line regions of the myofibrils are shown (Figs.14 and 16). Definitive T-tubules are, however, absent.

Numerous mitochondria are found close to the long axis of the myofibrils (Fig. 14). Frequently, desmosomes are seen in the intercellular regions of these myocytes (Fig. 15) with the sarcolemma occasionally characterised with pinocytotic vesicles (Fig. 16).

Few lipid bodies are present in the cytoplasm of these myocytes, close to mitochondria (Fig. 15). Large numbers of glycogen granules are also frequently seen in the cytoplasm of the myocytes.

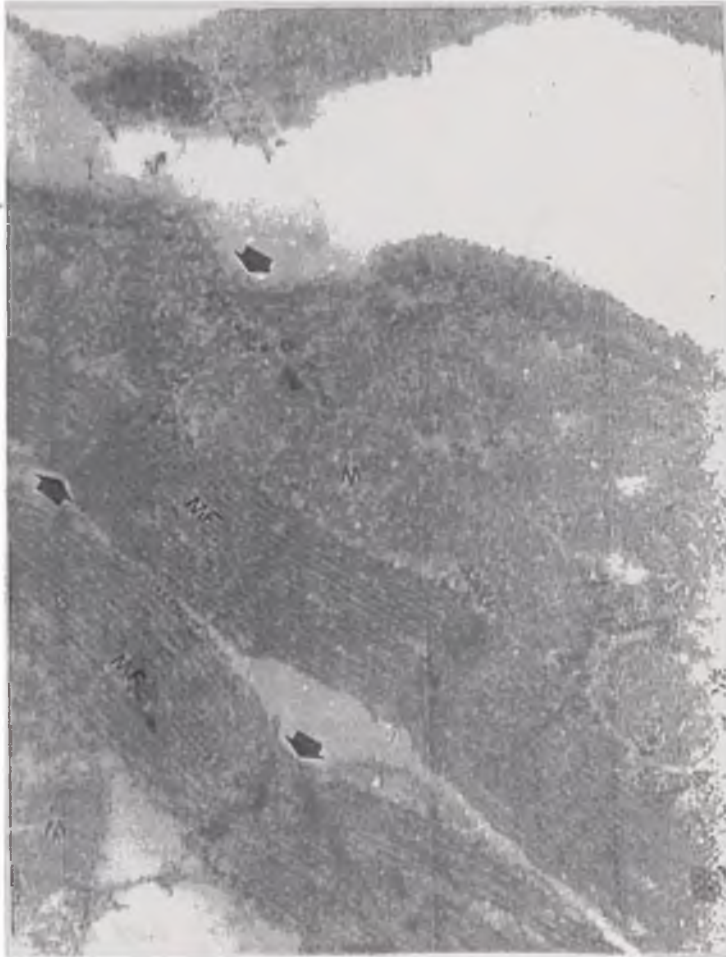


Fig. 14: Electron micrograph of right ventricular myocyte of a 7-day post-natal hamster showing the depth of sarcolemmal indentations (arrows) in the Z-line region of the myofibrils (MF) and mitochondria (M) adjacent to the myofibrils. Definitive T-tubules are absent. X 40,000.

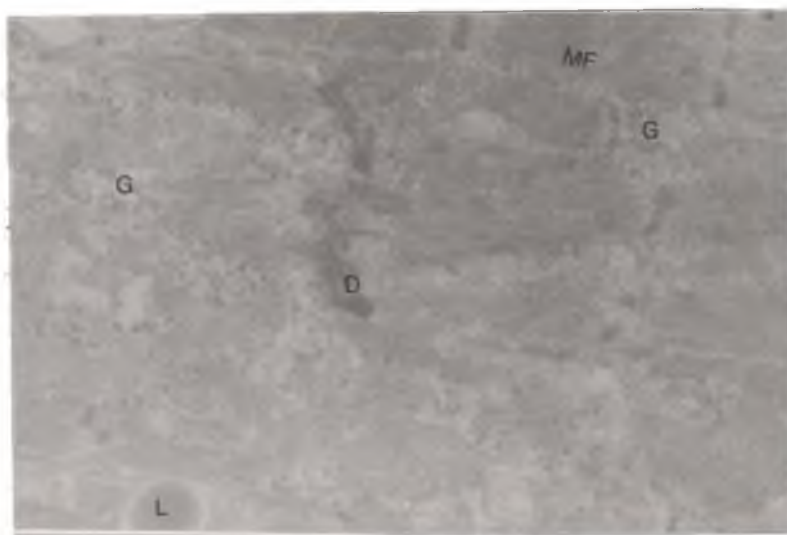


Fig. 15: Electron micrograph of right ventricular myocyte of a 7-day post-natal hamster showing myofibrils (MF), lipid bodies (L), glycogen granules (G) and desmosome (D). X 20,000



Fig. 16: Electron micrograph of the right ventricular myocyte of a 7-day post-natal hamster showing indentations on the sarcolemmal surface (big arrows), pinocytotic vesicle (small arrows), glycogen granules (G) and cut section of a nucleus, N (extreme left). X10, 000.

3.2.5 Day 14 Left Ventricle

Myocytes appear to have advanced in development with the various organelles bearing close resemblance to those of mature myocytes (Figs. 17 and 18).

Mitochondria, which abound in the cytoplasm of these myocytes, occur in different shapes and sizes. The shapes usually encountered are oval, cylindrical and dumb-bell (Figs. 17 and 18).

Even though the sarcolemma is poorly seen, (Fig. 17) there appear to be some profiles of T-tubules in the Z-line regions of the myofibrils. Occasionally, pinocytotic vesicles are seen along the surface of the sarcolemma (Fig. 18). Frequently, close appositions of profiles of T-tubules and SR occur in regions other than the Z-line, especially in the subsarcolemmal region (Fig. 18). Few lipid bodies occur in the cytoplasm of these myocytes.

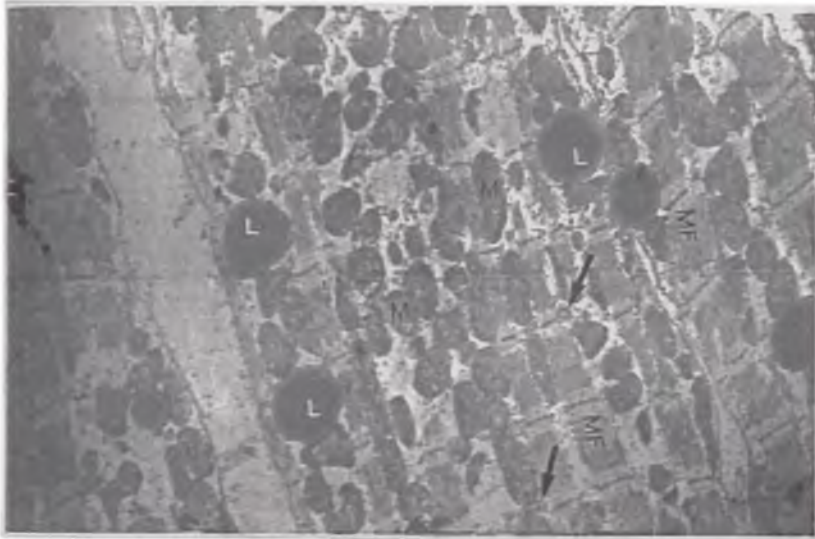


Fig. 17: Left ventricular myocytes of a 14-day old post-natal hamster showing T-tubules (arrows), well developed myofibrils (MF), mitochondria (M) and lipid bodies (L). X 12,000.

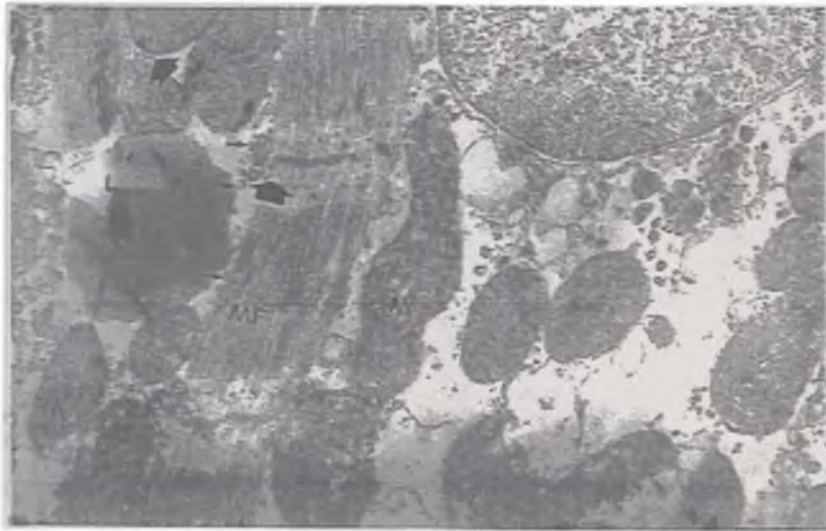


Fig. 18: Electron micrograph of the left ventricular myocyte of a 14-day old post-natal hamster. Note the presence of pinocytotic vesicles (small arrows), T-SR coupling (big arrow), myofibrils (MF), lipid body (L) and mitochondria (M). X 30,000.

3.2.6 Day 14 Right Ventricle

Fig. 19 shows micrograph from the right ventricle of 14 days post natal. In this figure myocytes are well developed. The myofibrils show well- defined sarcomeres that are limited on either side by dark Z-lines traversing the long axis of the myofibrils. These myofibrils are seen to occupy the entire cytoplasm of the myocytes and appear bulkier than those encountered in the earlier stages. The wide intracellular spaces encountered at the younger stages of development of the myocytes appear to be filled with closely packed myofibrils.

The sarcolemma show distinct invagination in the Z-line, giving the T-tubules a better defined appearance than observed at day 7. On the average these tubules measure 200 nm across their widest diameter.

Mitochondria lie adjacent to the myofibrils and numerous lipid bodies also frequently intercept or abut columns of these mitochondria. Few glycogen granules are also encountered in the myocytes at this stage.

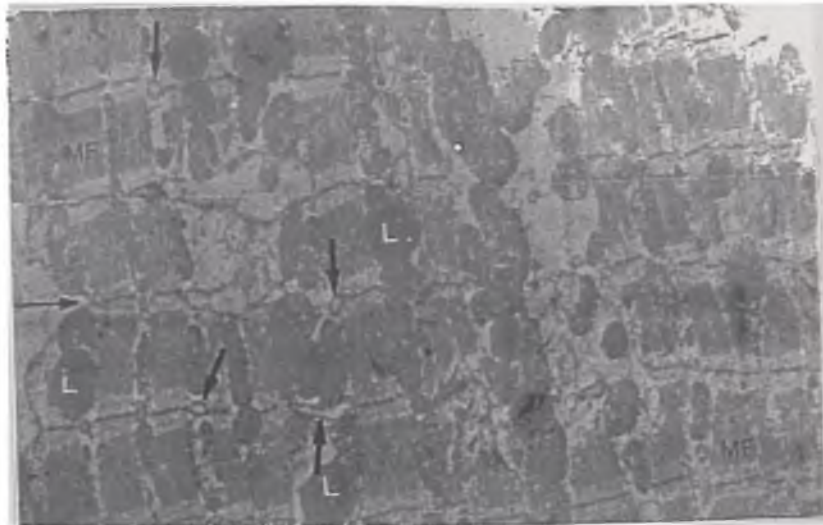


Fig. 19: Right ventricular myocytes of a 14-day post-natal hamster. Note the well-developed myofibrils (MF) with clearly shown Z-lines, lipid bodies (L) and T-tubules (arrows) X12, 000.

3.2.7 Day 21 Left Ventricle

Figures 20, 21 and 22 show left ventricular development, 21 days post natal. The myocytes resemble mature cardiocytes in appearance. The surface of the sarcolemma is characterised by the presence of numerous pinocytotic vesicles (Fig. 20).

T-tubules are seen to transcend deep profiles of the myofibrils. Frequently, close appositions between the T-tubules and SR are seen in the Z-line regions of myofibrils. The T-tubules are wider than those encountered at day 14, measuring on the average 205 nm across their widest diameter.

The cytoplasmic spaces appear to be reduced. Organelles such as myofibrils, mitochondria and lipid bodies occupy these intracellular spaces (Figs 21 and 22). The mitochondria are long and frequently, lipid bodies are seen to intercept columns of the mitochondria (Fig. 22). At higher magnifications (Figs. 20 and 21), the mitochondria appear to have two membranes; internal and external. The internal membrane is thrown into folds that project into the matrix of the mitochondria.

Glycogen granules occurred close to mitochondria and adjacent to myofibrils. The nucleus is seen to have dense chromatin material enclosed in a nuclear membrane (Fig.21). The myofibrils are well developed with clearly shown A-and-I bands.

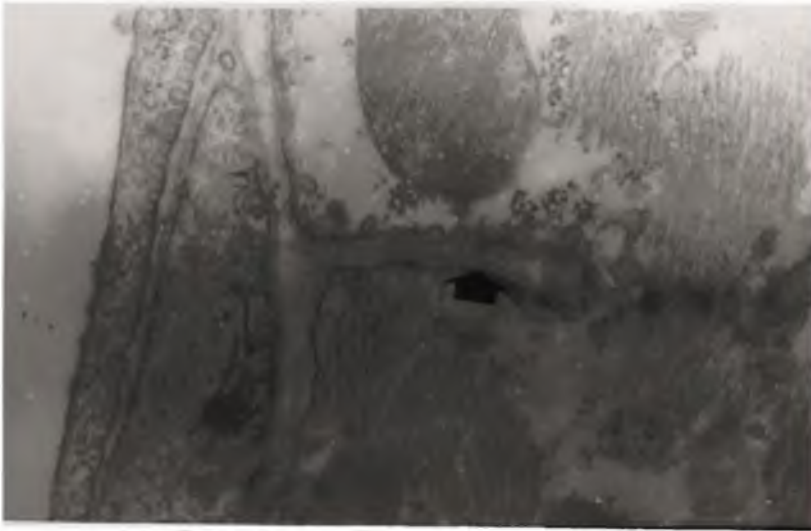


Fig. 20: Left ventricular myocyte of a 21 day old post-natal hamster showing T-tubules (T), T-SR coupling (big arrow), numerous pinocytotic vesicles along the sarcolemma (small arrows) and glycogen granules (G). X 60,000.

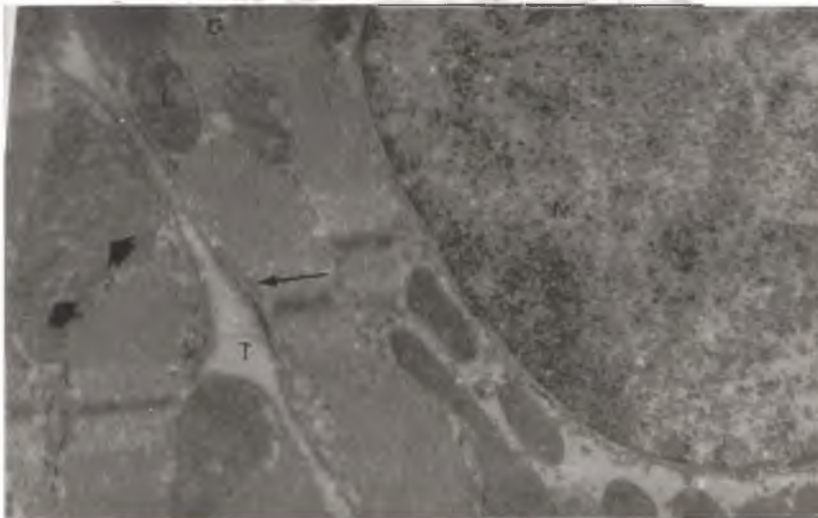


Fig. 21: Electron micrograph of the left ventricular myocyte of a 21-day old post-natal hamster. Note the presence of definitive T-tubule (T), T-SR coupling (long arrow), mitochondria with closely packed cristae (big arrows), lipid droplet (L), nucleus (N) and glycogen granules (G). X 20,000



Fig. 22: Left ventricular myocyte of a 21- day old post-natal hamster. Note the closeness of the myofibrils (MF) with clearly showing A-and I- bands, large lipid bodies (L), mitochondria (M) and T-SR couplings (arrows). X 30,000.

3.2.8 Day 21 Right Ventricle

The sarcolemma enclosing the myocytes is seen to invaginate deep in the I-band regions of the myofibrils (Fig. 23). The myofibrils are well developed as indicated by clearly defined A-and I-bands. Each sarcomere is limited on either side by Z-lines that cross the long of the myofibrils.

Mitochondria are seen to lie close to myofibrils (Figs. 23, 24, 25, 26 and 27) and lipid bodies are also seen close to these mitochondria (Figs. 24 and 27). The myocytes appear to abut each other closely. Clearly defined intercalated discs (IDs) mark the

regions of their apposition. Occasionally, dense plaques are found along the non-specialised regions of the intercalated discs (Fig. 24). Frequently, portions of the ID regions are made up of desmosome (Fig. 25). SR is found at non-specialised regions of the IDs (Fig. 26). Pinocytotic vesicles occur along the sarcolemma in the region of the intercellular space (Fig. 27).

Profiles of T-tubules are frequently seen in the Z-line regions (Figs. 23 and 26) with SR lying close to these tubules. The T-tubules appear wider than those observed in myocytes present in the heart of 7 and 14-day post-natal hamsters. They measure on the average, 205 nm across their widest diameter (Fig. 27). Numerous glycogen granules also are frequently seen.

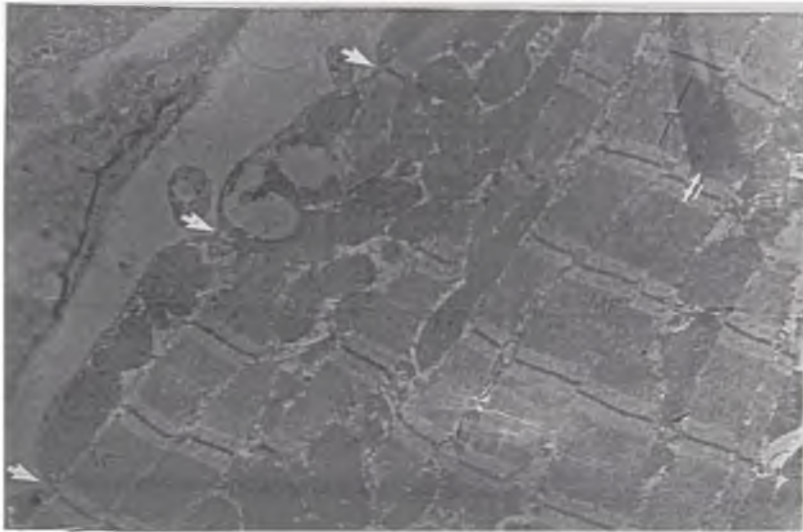


Fig. 23: Electron micrograph of the right ventricular myocyte of a 21-day old post-natal hamster showing well-developed myofibrils (MF) and invaginations of the sarcolemma at the Z-line regions (white arrows), well demarcated A- and I-bands and mitochondria (M). Note the presence of T-tubules (small black arrows). X 16,000.

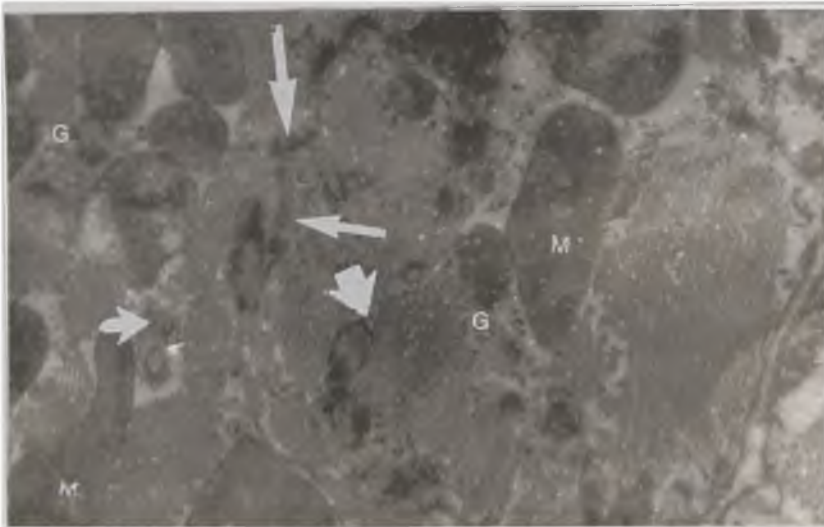


Fig. 24: Electron micrograph of a 21-day old post-natal hamster. Right ventricular myocyte showing SR-tubules (short arrows), mitochondria (M) and glycogen granules (G). Long arrows show dense plaques along intercalated disc. X 20,000.

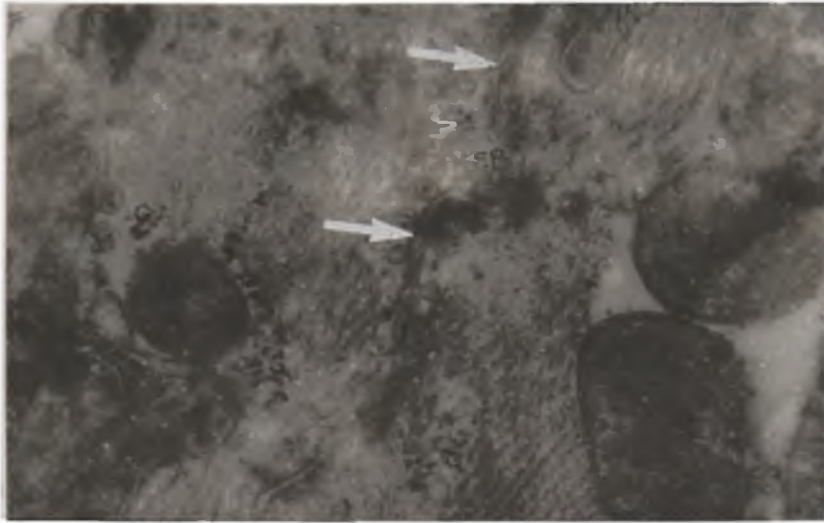


Fig. 25: Electron micrograph of the right ventricular myocyte of a 21-day old post-natal hamster showing intercalated disc (arrows), desmosome (D), SR, mitochondria (M), glycogen granules (G) and lipid droplet (L). X 50,000.



Fig. 26: Electron micrograph of the right ventricular myocyte of a 21-day old post-natal hamster showing T-SR coupling in the I-band region (arrow) of the myofibrils (MF) and glycogen granules (G). X 50,000.

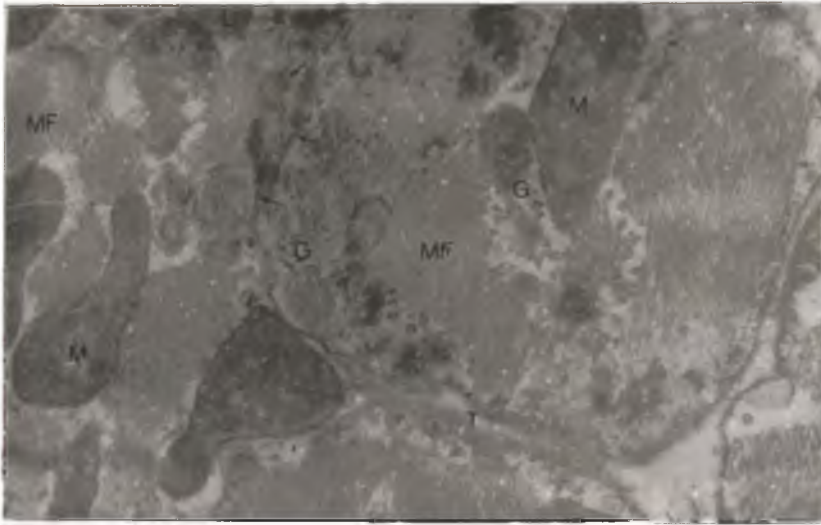


Fig. 27: Electron micrograph of the right ventricular myocyte of a 21-day old post-natal hamster. Note pinocytotic vesicles along the sarcolemmal surface in the region of the intercellular space (T), well-developed myofibrils (MF), mitochondria (M), intercalated disc (small arrows), lipid body (L) and Glycogen granules (G).

CHAPTER FOUR

DISCUSSION

The original plan of the project was to investigate both qualitative and quantitative changes in development of important features of the ventricular myocytes in the hamster including the T-tubular system. On account of technical problems encountered at the Noguchi Memorial Institute for Medical Research, Legon it was not possible to pursue the quantitative, morphometric study. The plan of the study was, therefore, reviewed to focus on qualitative aspects of the development of the T-tubule system in relation to its presumed role in excitation-contraction coupling. The results obtained in this study are reviewed and discussed below. The hamster was used as a model for the study because in the adult ventricular cells, the T-tubules are quite enormous in size. It was expected therefore, that early expressions of the development of the T-tubules would be readily noticed even in pre-natal life. It was also considered that these tubules might appear at the same time as the development of the myofibrils, if indeed the tubules are essential for excitation-contraction coupling.

Key observations in the above study are summarised below and discussed in relation to the process of excitation-contraction coupling and metabolic efficiency of the myocyte.

The first major observation is that the T-tubules begin to appear in the right and left ventricular cells at day 7 of post-natal life in the hamster. At this stage they are observed as indentations only in some but not all regions of the sarcolemma apposed to I-band portions of the myofibrils.

The second major observation is that the T-tubules progressively develop into their definitive form by day 21. As the sarcolemma invaginates it carries with it the SR couplings. The definitive T-tubules, therefore, have SR diadic couplings derived from the original surface SR couplings.

The third significant finding is that related to the calibre of the T-tubules. Even at early stages of post-natal development, the tubules are quite enormous in size. The large T-tubules observed in the adult are, therefore, determined very early. At day 21 of post-natal life, when definitive T-tubules began to appear, they measure on the average 205 nm compared to about 450 nm in the adult (Ayettey and Navaratnam, 1978) and 100-150 nm in other mammals such as the rat, dog and man (Fawcett and McNutt, 1969; Ayettey, 1978; Navaratnam, 1987). Up to day 21 of post-natal life no branchings of the primary tubules were observed. It is

possible therefore, that the secondary and tertiary transverse tubules and the longitudinally oriented tubules that branch from ~~the~~ primary tubules appear much later.

Lastly, it was noticed that the development of the T-tubules is commensurate with that of myofibrils. From day 7 to day 21 of post-natal life when the T-tubules assume more of the adult nature, the myofibrils also increase in volume qualitatively, occupying much of the cytoplasmic space. This also matches rapid increase in volume of mitochondria as observed qualitatively.

That the T-tubules begin to appear post-natally in the hamster is of interest since this supports the findings of a number of workers. Schiebler and Wolff (1966); Hirakow and Gotoh (1975); Nakata (1977) and Hirakow *et al.*, (1980) found that T-tubules appear between days 10-14 after birth in the rat whereas Schiebler and Wolff, (1966) first noticed their appearance between days 14-20 post-natal also in the rat. Other post-natal observations include those of Colgan *et al.*, (1978) in the hamster; Sheridan *et al.*, (1979); Maylie (1982) and Gotoh (1983) in the cat. The rest are Hoerter *et al.*, (1981); Page and Buecker (1981) in the rabbit; Bishop (1973); Legato (1975 and 1979) in the dog; Hirakow and Krause (1980) in the opossum, Ishikawa and Yamada (1975), and

Forbes *et al.*, (1984) in the mouse. Some workers, (Leak and Burke, 1965; Allen and Carstens, 1967; Forsgren and Thornell, 1981 and Hirakow and Gotoh, 1980) have, however, reported the presence of the T-tubules in some mammals such as man, rhesus monkey, sheep, cow and guinea pig during their pre-natal stages respectively. However, even in these species where T-tubules have been reported to occur in pre-natal stages of development, they (the T-tubules) appear after, and not before myofibrillar development.

The present work agrees with that of Colgan *et al.*, (1978) who found that the T-tubules first begin to appear after birth. They also noted the first expressions of the T-tubules between days 14-18 postnatal and the beginning of the adult form of the tubules at the end of the third week.

The above observations are of interest in the discussion of the role of the T-tubules in the excitation-contraction coupling process in myocardial cells. By definition, excitation-contraction coupling encompasses the sequence of steps that begins when an action potential depolarises the plasma membrane and ends with the binding of calcium to troponin C, the calcium receptor of the cardiac contractile protein (Lyman and Chatfield, 1955).

Depolarisation of the sarcolemma (including the T-system membranes) is thought to result in an influx of extracellular calcium which in turn induces the release of intracellular stores of calcium from the junctional SR, both of which are essential to initiate the excitation-contraction coupling process in cardiac myocytes (Staley and Benson, 1968).

It is difficult to discuss the role of T-systems with regards to excitation-contraction coupling without involving SR because of their close apposition (Simpson, 1965). The T-system as noted earlier, is usually continuous with the surface sarcolemma and, like the latter, couples with SR tubules which they carry deep into the myocytes by virtue of their invagination (Page, 1967; Forssman and Girardier, 1970).

Another argument in support of the implication of T-tubules in the excitation-contraction coupling process in mammalian cardiac cells is based on the rapid rate of spread of the excitatory impulse from the surface membrane to the contractile material in both atrial and ventricular cells (Staley and Benson, 1968; Forssman and Girardier, 1970). The assumption is that without T-tubules, the process will be much slower, depending on simple diffusion. Others such as Page (1978) and Forssman and

Girardier (1970) have suggested that the T-tubules increase the total surface area of the cell membrane and that this would rather impede swift propagation of the electrical impulse.

Based on the above arguments, T-tubules would also be expected to be developing at the time of the development of the myofibrils if they are essential in excitation-contraction coupling process. This should be so, especially as myocardial cells exhibit mechanical activity very early in the developing embryo. In the present study, the hearts that were investigated had begun contracting vigorously by the time they were removed at all the stages of development, beginning from 14½ days of pre-natal life. The T-tubules were not noticed until day 14 of post-natal life, although the myofibrils had started developing by day 14 of pre-natal life.

It could be argued from the above that, even without the T-tubules, the myocardial cell possesses a mechanism by which excitation-contraction coupling could be established. The absence of T-tubules during pre-natal life in the hamster, as had also been observed in species such as the mouse, rat, cat and dog, rules out possible participation of this system in the contractile process at this stage. These findings, therefore, challenge the general

assertion that the primary role of T-tubule is in excitation-contraction coupling (see Endo, 1977; Fabiato, 1983 and 1985 for review). An explanation must therefore be found for the excitation-contraction coupling mechanism in foetal myocytes in which no T-tubules had been observed. In such foetal myocytes, it could be argued that the process could be accounted for by simple diffusion of calcium ions as, at this stage, myofibrils are in their early stages of development and are few. As the volume of myofibrils increases, there may be need for invagination of the sarcolemma, to facilitate spread of the electrical impulse. Indeed, in this work the beginning of the T-tubules coincide with rapid increase in volume of myofibrils. However, as noted below, such explanation is tenuous, as T-tubules are not always present in adult myocytes where myofibrils are well developed.

Some attempts had been made to explain the absence of T-tubules in non-mammalian vertebrates the birds, reptiles, amphibians and fish. There is the suggestion that the smaller diameter of cardiocytes in these vertebrates compared to those of mammals, renders T-tubules unnecessary. Hirakow (1970) suggested that simple diffusion of calcium ions would be sufficient for the process. Indeed, Fawcett and McNutt (1969) had earlier proposed a similar mechanism for atrial cells of mammals which

are thinner than the ventricular ones and in which T-tubules are few or absent. This assumption, however, cannot be entirely valid as the cardiac cycle is rapid in some avians such as the humming bird (*Mellisuga helenae*) and the sparrow in which no T-tubules are found (Jewett *et al.*, 1971) and the myofibrils are well developed, the heart rate approximates 600 beats per minute (Anaglate *et al.*, 1994).

It seems that the excitement of discovering T-tubules in some cardiac and skeletal muscle cells in general, has influenced many researchers to attempt to explain excitation-contraction by that fact alone. Obviously, this does not fit the anatomical observations in all species. Attempts must be made, therefore, to find a better anatomical basis for the excitation-contraction coupling process that will not depend on T-tubules. In all likelihood, this structure is the SR for the following reasons;

(i) the SR tubules are widespread and are present where T-tubules are also found, taking part in either diadic coupling in cardiac muscle or triadic coupling in skeletal muscle (Huxley, 1964; Simpson and Rayns, 1968). (ii) SR couplings are not limited to T-tubules. There are several sites on the surface (uninvaginated) sarcolemma where SR forms couplings similar in morphology to those observed in relation to T-tubules. At such sites, typical

features such as SR luminal granules and feet-like processes extending from the SR membrane towards the sarcolemma are present (Ayettey and Navaratnam, 1978).

In this study, several SR couplings with the surface sarcolemma were noted even before T-tubules began to appear. Interestingly, the sites of T-tubule formation coincide with regions of the sarcolemma that had SR couplings. This observation is very important, as it suggests quite clearly that even before the T-tubules are formed, the structural basis for excitation-contraction coupling is laid in the SR-surface sarcolemmal couplings as observed in the finch (*Coereba flaveola*) (Sommer and Waugh, 1976) and in the humming bird (Jewett *et al.*, 1971).

Of primary importance in excitation-contraction coupling, therefore, is the surface SR coupling and not the T-tubules that develop from them. This assumption supports the observation made by Hirakow and Gotoh (1975) who, after investigating development of T-tubules in the rat, suggested that the excitation-contraction coupling process might be a basic function of the SR-sarcolemmal coupling referred to as the sarcolemmal complex. Indeed Hirakow and Gotoh (1975) concluded that, during uterine life, the sarcolemma alone (with the SR coupling) is sufficient to

carry out this process. They further suggested that in the rat, as could be applied to other mammals, the sarcolemmal complex becomes more specialised and decentralised in the form of T-tubules to facilitate more efficient excitation-contraction coupling.

Biochemical studies further support the view that the surface sarcolemmal SR complexes are of prime importance in the initiation of the contractile process. Schultze (1984), and Malouf and Meissner (1984) working separately reported that the T-tubules and the surface sarcolemmal-SR complexes are similar in biochemical nature with regard to membrane-bound enzyme systems such as ATPase, adenylate and guanylate cyclases. Recently, De Camilli *et al.*, (2002) isolated in the T-tubules two enzymes, amphiphysin II and caveolin III that are believed to be involved in the formation of the sarcolemma.

An interesting observation in a research on the heart muscle of the sparrow (*Passer domesticus*) must also be noted. Anaglate *et al.*, (1994) could not find T-tubules in ventricular cells of this species but reported specialised forms of SR at sites normally occupied by T-tubules in mammalian myocytes. In this study, the SR contains luminal granules but had no feet processes. They also occupied some, but not all I-band regions. Similar couplings were

found in relation to the surface sarcolemma. The interpretation of these observations was that the impulse along the surface sarcolemma was propagated through the SR membrane network from the SR-sarcolemmal coupling to the I-band region for the release of calcium ions (Anaglate *et al.*, 1994). This also means that SR luminal granules are present even where the T-tubules are not found. It is possible that in all species where T-tubules are not found, similar SR tubules with luminal granules exist but that the luminal granules are lost during the preparations of materials for microscopy.

The above observations and discussions reveal also the need for further research into the development of T-tubules. The process of sarcolemmal invaginations to produce T-tubules should be investigated more closely. Reasons must be given why the indentations occur at the I-Z regions and not elsewhere. If this is pre-determined genetically, it must be explained why the process of indentation of the sarcolemma occurs at some but not all I-Z regions. Something must account for the differential growth in the sarcolemma that allows development of the T-system. A whole field of structural, biochemical and physiological studies in cardiac development remains to be investigated in relation to T-tubules. Even before these investigations are carried out, there is the need

for a careful study of T-tubular development in ventricular cells of other mammalian vertebrates. It is likely that the process might be similar to that in the hamster with the T-system appearing after birth. This might be related to the increased volume of myofibrils required for increased metabolic activities and not primarily for excitation-contraction.

As noted in this study, the contractile elements (myofibrils) which are the chief energy consumers of cardiac cells increase in volume and adopt a more adult form at the same time as the T-tubules become significantly more definitive. The development of the T-tubules is, therefore, most likely a mechanism to meet the nutritional requirements of the ventricular myocytes. By their invaginated nature, the T-tubules increase surface area and also reduce the distance for diffusion of both metabolic substrates and products. This would greatly facilitate metabolic activities in the cardiocyte. The enormous size of the T-tubules at a very early stage of post-natal development indicates the preparedness of the myocardium for survival under extreme conditions. This suggests that the animal would be capable of hibernation by the third or fourth week of post-natal life. Whether this is so or not needs to be tested by subjecting three to four week old hamsters to hibernating conditions. It must, however, be kept in mind that the T-system

observed by the third week is not as elaborate as that in the adult, as it did not have secondary, tertiary and longitudinal branches.

Ultrastructural studies on the ventricular myocardium of the hamster during hibernation indicate a significant increase in diameter of the T-tubules (Dennis *et al.*, 1999). This supports the view that T-tubules are adaptive features for metabolic efficiency, and so they protect the myocardium from fatal arrhythmia in extreme conditions. As noted by Johansson (1991), arrhythmia, in ventricular cells could be due to inadequate supply of nutrients to the contractile elements or to accumulation of metabolic products, or both. Wide T-tubules would, therefore, protect the cell by ensuring rapid removal of metabolic products from the interior of the cell.

As reviewed already, wide primary T-tubules have been seen in the ventricular myocytes of some other mammals that have adaptive physiology. In the arboreal fruit-eating bat (*Eidolon helvum*) with high exercise tolerance, the T-tubules range between 350 and 450 nm in diameter (Ayetey *et al.*, 1993). In the grey seal (*Halichoerus grypus*) a diving mammal, the T-tubules measure 450 nm (Ayetey, 1979; Ayetey *et al.*, 1996). In the adult hamster (*Mesocricetus auratus*), the tubules are 450-500 nm in diameter

(Ayettey and Navaratnam, 1981). In the hedgehog (*Erinaceus europaeus*) which is capable of hibernating they are also wide, measuring 500-550 nm (Ayettey, 1980). In comparison, the T-tubules measure 50-80 nm in the ferret (*Mustela putorius furo*) (Simpson and Rayns, 1968), 100-130 nm in the rat (*Rattus rattus*) (Forssman and Girardier, 1970; Ayettey and Navaratnam, 1978), 150 nm in man (Nayler, 1975) and about 200 nm in the cat (*Felis domestica*) (Fawcett and McNutt, 1969). Although investigators have not covered all animals with specific physiologic adaptations, the trend so far is that there is significant difference between the size of the T-tubules of the myocardial cells and that of ordinary mammals. Ideally, the more important investigation will be the determination of functional volume of the T-tubule in relation to the cytoplasmic volume in all species. This will give more relevant information on the extent to which the T-tubule increases the surface area and contributes to metabolic efficiency. Some work has already been done in this regard (see Ayettey *et al.*, 1993; Anaglate *et al.*, 1994; and Dakubo *et al.*, 1995) but more information is required for a better understanding of the adaptive features in cardiac cells.

Other evidence that T-tubules are more of a feature for metabolic efficiency in myocytes in which they occur than for

initiation of the contractile process, is obtained from the work of McAllister and Page (1973). These workers stimulated the growth of cardiac cells in the rat by the administration of thyroxine. As a result, there was increase in contractile elements as well as increase in the total T-tubular surface area. It could be deduced from this work that T-tubule growth is related to growth of the energy consuming elements as well as increase in energy-generating elements to maintain maximum degree of efficiency of the myocyte. No doubt the present work also showed initial T-tubule size of 205 nm that would increase to about 500 nm in adult life with increase in myofibrillar elements.

As noted at the beginning of the discussion, the original objective in this study was to establish not only qualitative but quantitative basis of development of the myocyte in the hamster. All being well, this will be pursued as part of a programme for a Ph.D as this information is essential for the appreciation of the developmental features that equip the myocyte to be so unique in function.

Other aspect of the development of the cardiac myocyte that should be investigated is the vasculature. Adequate blood supply is critical to the efficiency in function of any tissue and

hence every cell. The degree of development of the vasculature and especially the capillary bed must be a major determining factor in how efficient the cell can function under extreme conditions.

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APPENDIX I

BUFFERS AND FIXATIVES

1) Sodium Cacodylate Buffer:

Solution A: 4.28 g of $(\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O})$ is dissolved in distilled water and made up to the 100 ml.

Solution B: 1 ml of concentrated hydrochloric acid (HCl) is diluted with 60.3 ml distilled water to make a 0.2M solution. To prepare 0.2M buffer at pH 7.4, 2.7ml solution B is added to 50 ml solution A and diluted further to 200 ml.

2) Karnovsky's Fixative (2% paraformaldehyde + 2.5% Glutaraldehyde in 0.1M sodium cacodylate buffer).

Solution A: 10% paraformaldehyde solution:

2.0g of powdered paraformaldehyde is dissolved in 20 ml of double distilled water heated to 60-65 °C in a fume cupboard to form 10% paraformaldehyde solution. Few drops of 1.0N sodium hydroxide is added until the solution becomes clear. It is then cooled before use.

Solution B: 10 ml 25% glutaraldehyde solution is measured into a conical flask.

To prepare Karnovsky's fixative 50 ml of 0.2M sodium cacodylate buffer is mixed with solutions A and B and topped to the 100 ml with distilled water.

3) 1% Osmium tetroxide (Os O₄) in 0.1M sodium cacodylate buffer.

An ampoule containing 1.0g solid Os O₄ is broken and dropped into a clean brown glass-stoppered bottle. 10 ml 0.2M sodium cacodylate buffer is added in a fume cupboard, shaken carefully and left overnight at 4 °C in a refrigerator to store. pH is adjusted to 7.4.

APPENDIX II

PREPARATION OF EPON 812 RESINS

MIXING PROCEDURE:

<u>Mixture A</u>	<u>Small Amount</u>	<u>Large Amount</u>
Epon 812	5 ml	62 ml
DDSA (Dodecyl succinic anhydride)	8 ml	100 ml

Mixture B:

Epon 812	8 ml	100 ml
NMA (Nadic methyl anhydride)	7 ml	90 ml

Final Embedding Mixture:

Mixture A	13 ml	162 ml
Mixture B	15 ml	190 ml
DMP-30 (2, 4, 6-tri dimethyl- aminomethyl phenol) 2% v/v	16 drops	5.3 to 7.1 ml

Disposable plastic syringes (10 or 20 ml) are used to measure the resin into a small beaker. Immediately before use, the two mixtures A and B are blended and the accelerator DMP-30 is added the proportion of 1.5-2.0%. Special teflon stirring rods are used to mix all the ingredients thoroughly so that the mixture becomes homogenous. The amount of accelerator added is critical in obtaining satisfactory polymerisation. An excessive amount of accelerator makes

the block dark in colour and too brittle for satisfactory sectioning. The final mixture is capped with aluminium foil and stored in the freezer at 4 °C.

APPENDIX III

PREPARATION OF STAINS

1.0% Toluidine blue stain

1.0g of toluidine blue is dissolved in 100 ml of distilled water containing 1.0g of sodium tetraborate. The solution is filtered with grade 1 filter paper and stored at room temperature.

Uranyl acetate

To make a 0.5% saturated solution of uranyl acetate, 0.1g of the chemical is added to 20 ml of 50% alcohol in a small beaker and stirred constantly for 30 mins. The beaker is covered with a thick hollowcardboard cylinder to protect the solution from light. The saturated solution is then centrifuged and the supernatant stored in cupboard at room temperature. A wrapping foil protects the solution from light.

Lead citrate

1.33g of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and 1.76g of sodium citrate are added to 30ml distilled water. The mixture is allowed to stand for 30 minutes with intermittent vigorous shaking till a milky solution is formed. 8.0ml of 0.1M sodium hydroxide (NaOH) is added, and the solution is made up to 50ml with distilled water.