HS

THE DEVELOPMENT AND DISTRIBUTION OF BINUCLEATION IN CARDIAC MYOCYTES OF DIFFERENT SPECIES

A Thesis presented to
The School of Graduate Studies
The University of Ottawa

by

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In partial fulfillment of requirements for
the degree of

Master of Science

in the

Department of Physiology
Faculty of Medicine

September, 1977

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CONTENTS

ACKNOWLEDGEMENT

I wish to thank, first of all, Dr. Borivoj Korecky, who supervised my research, for his advice and help, and for the opportunity to learn from him.

I was able to make many important improvements in the setting up of experiments and this thesis due to the useful suggestions of Dr. Karel Rakusan.

I would also like to thank Dr. Gordon A. Kinson and the other members of the Department of Physiology who were always willing to assist in any capacity.

Finally, I would like to thank my sister, Doreen Sweet, who so skillfully typed this manuscript.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

It has been observed that up to 80% of the muscle cells in the adult rat heart are binucleated. This observation was extended using the technique of nuclear counting in enzymatically dispersed myocytes from different regions and at different ages in the rat. About 80% of the myocytes thus obtained from the right ventricle and from different regions of the left ventricle of the adult rat were found to be binucleated. This level was reached by the fourteenth day of post-natal life. When changes in the rate of body and heart growth were induced in litters of different size, it was evident that the attainment of the adult proportion of binucleated myocytes was more closely related to body and heart weight than to age.

In the beef heart, the overall proportion of binucleated myocytes was found to be 48%, with a uniform distribution in different regions.

In the rabbit, dog and cat hearts, far lower percentages of binucleated cardiac muscle cells were observed in newborn and young animals than in adults of the given species.

INTRODUCTION

Two ways by which growth may occur are; (1) the number of cells increase, (2) the size of the existing cells increase. There are varying levels of growth by increasing cell size and cell numbers. Throughout life blood cell turnover occurs. Thus, cells must constantly be formed to replace those which are continually being lost. This process occurs throughout life, well after body growth is complete.

In other organs there is a lower rate of cell turnover. Cell numbers increase in the lungs up to around 10 years of age, but further enlargement of the lungs after this time will be due to cell enlargement and not to increases in cell numbers in the human.

Finally, there are organs in which the only mode of cellular change in postnatal life is through enlargement of the existing cells. In both the heart and skeletal muscle no proliferation of muscle cells take place, under normal conditions, after birth (Goss 1966).

The myogenic cells of developing mammalian heart muscle are derived from splanchnic mesoderm. In the fetus, during the first stages of cardiac development, there is very active proliferation of undifferentiated myogenic cells. The cardiac myoblast is a dividing cell simultaneously synthesizing cell specific proteins (Weinstein, Hay 1970, Oberpriller, Oberpriller 1971, Hay, Low 1972, Rumyantsev 1972). As the myofibrillar mass accumulates, the spindle-shaped cardiac myoblasts are gradually transformed into cylindrical, fully functioning myocytes, just prior to birth (Manasek, 1968). However, even at this advanced stage of development, mitoses are still frequent; this is well documented in studies of neonatal hearts (Rumyantsev 1963, Bishop 1971). The cells continue to divide, but only up to about 3 weeks postnatally in the rat.

It appears that sometime during development when the amount of DNA per cell sharply rises, cardiac myocyte nuclei in some species become polyploid while others tend towards polynucleation. Examples of both polynucleation and polyploidization can be seen in Figure 1.

Cellular growth and nuclear proliferation can be gauged by several techniques. Autoradiography is used to detect newly formed DNA. The presence of polypoidy in the nucleus can be detected by cytophotometry. Nuclear counts can be made on longitudinal and transverse sections in fixed stained myocardium, as well as on isolated individual heart muscle cells. In order to note whether mitotic activity is occurring, colchicine may be used, as it stops mitosis in the metaphase stage which is readily identifiable by microscopy. Through the use of morphological techniques, such as counting cells in transverse histological sections and biochemical techniques involving the combination of cytophotometric and biochemical DNA determinations, the number of muscle cells within the heart can be estimated.

(a) Autoradiography

Autoradiography has been used by several investigators to determine the prevalence of replication of nuclear material. In this technique, a labelled purine or pyrimidine base, such as H^3-thymidine, is introduced into the animal. For a limited period, newly formed DNA incorporates this label which can be detected by exposing a photographic emulsion

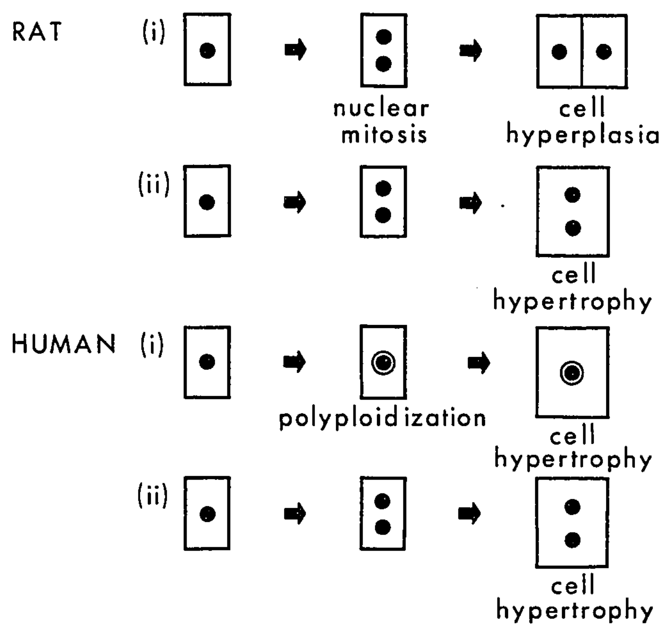

Figure 1. Schematic diagram illustrating possible pathways of myocyte growth and deposition of new nuclear material in human and rat hearts.

applied to the section. The proportion of nuclei thus labelled is an index of the rate of replication of nuclear material.

This technique has been used to observe DNA synthesis and nuclear proliferation in the rat heart during histogenesis, in normal postnatal and under artificially induced growth stimuli. Sasaki et al. (1970) found that about 8.3% of the heart muscle cell nuclei were incorporating label in 2 day old rats and that this value dropped to 0.15% at 3 weeks of age. Similarly, after the third week of life, the incorporation of thymidine was negligible (0.2 - 0.0%) (Rumyantsev, 1963) in the rat heart.

Neffgen and Korecky (1971) induced cardiac hypertrophy in rats, just after the weaning period and found significant DNA formation occuring in both muscle and non-muscle cells. Morkin and Ashford (1968) induced cardiac hypertrophy in adult rats and found that DNA formation was limited almost entirely to interstitial and connective tissue cells and did not occur to any significant extent in muscle cell nuclei. Thus, it appears that significant DNA-replication in rat myocytes is confined to the pre-natal and early post-natal periods.

Although the formation of new DNA may be observed, neither hypertrophy nor hyperplasia of cells can be gauged solely by autoradiographic technique as it only reveals formation of nuclear compounds.

(b) Cytophotometry

Newly formed DNA may be deposited in existing nuclei, rather than newly formed nuclei; this phenomenon is known as polyploidization. To determine the ploidy class of a heart muscle cell nucleus, both the concentration of DNA in the nucleus and the surface area of the nucleus in section should be known. Through the use of cytophotometry, the concentration of DNA is calculated within a given area and this value is multiplied by the nuclear volume (nuclear sectional surface area X number of sections X thickness of each section). These results were compared to the amount of DNA in fibroblast nuclei, which is 6 X 10^{-12}g, the quantity of DNA in an average diploid cell nucleus (Vendrely, Vendrely 1949). The overwhelming majority (> 95%) of myocytes in the normal rat heart are diploid (Grove et al. 1969). Similarly, high percentages were found in mice (Peterson and Baserga 1965), and in other species (Pfitzer 1971). These findings are detailed in Table 1.

In contrast to these species, in the human heart, only about half (47%) of the myocytes are reported to be diploid (Kompmann et al. 1966, Sandritter and Adler 1971). In hypertrophied adult hearts, Capers (1964) found little or no increase in the quantities of DNA in the muscle cell nuclei. This finding was however, strongly contradicted by the results of Sandritter and Scomazzoni (1964), Kompmann et al. (1966) and Sandritter and Adler (1971). They showed that in the human, as the mass of the heart increased, so did the percentage of polyploid muscle cell nuclei in the left ventricle (Table 1). These results are controversial as the latter investigators considered enlarged hearts to weigh in excess of 500 g, whereas Capers used 400 g as the point where hypertrophy begins. Therefore, an overlap existed where hearts between 400 - 500 g have been considered both normal and enlarged. As seen in Table 1, hearts weighing 250 - 500 g were classified as one group with respect to ploidy values. With this in mind, Capers' finding that normal and enlarged hearts have similar ploidy values in muscle cell nuclei may not be altogether wrong.

Table 1

PLOIDY FREQUENCIES OF

LEFT VENTRICULAR MYOCYTES (%)

<table>
<tr><th>SPECIES</th><th></th><th>2C</th><th>4C</th><th>8C</th><th>16C</th><th>32C</th><th></th></tr>
<tr><td>Rat</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>6½ weeks</td><td></td><td>96</td><td colspan="4">4</td><td rowspan="2">) I</td></tr>
<tr><td>17-18 weeks</td><td></td><td>98</td><td colspan="4">2</td></tr>
<tr><td>Rabbit
Cat
Dog
Cattle</td><td>Adult</td><td>93-98</td><td>2.7</td><td>-</td><td>-</td><td>-</td><td>) II</td></tr>
<tr><td>Man
Heart weight</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>150 g</td><td></td><td>45</td><td>47</td><td>8</td><td>-</td><td>-</td><td rowspan="3">) III</td></tr>
<tr><td>250-500 g</td><td></td><td>20</td><td>50</td><td>35</td><td>5</td><td>-</td></tr>
<tr><td>500-750 g</td><td></td><td>0-10</td><td>10-40</td><td>45-65</td><td>15-30</td><td>0-5</td></tr>
</table>

I Grove et al. 1969

II Pfitzer 1971

III Kompmann et al. 1966
Sandritter and Adler 1971

It is probable, but cannot be determined, that some or all of the hypertrophic hearts used by Capers weighed between 400 - 500 g, while the normal adult hearts were in the vicinity of 250 - 400 g. These hearts would fit into the 250 - 500 g ploidy group of Kompmann and Sandritter. According to Linzbach (1960), the upper limit of normal heart growth is 500 g.

(c) Counting the Number of Nuclei Per Cell

Nuclear counts have been performed in mammalian hearts, by basically 3 methods:

(i) serial sectioning and reconstruction of cells in fixed, stained myocardium

(ii) examination of intact fibers in fixed, stained myocardium

(iii) staining of enzymatically dispersed isolated myocytes

Method (i) involves making consecutive sections of myocardium and through observing these sections, the cells are visually reconstructed and their nuclei can be counted. This technique has been used by Korecky (unpublished data), although it was very laborious and few cells were found. Method (ii) consists of counting nuclei of intact muscle cells present in longitudinal sections of heart muscle. This technique has been used by Hort (1953) and Bardoli et al. (1967). In method (iii) heart muscle cells are isolated and stained. The individual intact cells are then observed under the light microscope and the nuclei are counted. This technique has been used by Schneider and Pfitzer (1972) and Korecky (unpublished data).

Korecky found (unpublished data) that approximately 80% of the muscle cells in adult rat left ventricles contained 2 nuclei. Values for adult animals of other species appear in Table 2. It appears necessary

Table 2

PERCENTAGE OF BINUCLEATED CELLS IN

HEARTS OF DIFFERENT SPECIED

(Korecky, unpublished)

SPECIES	SAMPLE.	No.	Perfusion	No.	Stirring	No.	Shaking
RAT							
adult S.D.	L.V.	4	78±1.8	4	81±1.3		
adult S.D.	R.V.	2	82±2.9				
old S.D.	L.V.	7	81±0.9				
adult L.	L.V.					9	79±1.6
GUINEA PIG	L.V.	4	81±1.6	3	70±2.2		
RABBIT	L.V.	4	78±0.8				
KITTEN	L.V.	6	76±1.5				
DOG	L.V.			4	45±2.3		
BEEF	L.V.					8	45±1.1
MAN	L.V.P.					4	10±2.2

Mean values in percentage SEM are given. Sprague Dawley, L. - Lewis inbred. No. - number of animals, LV - left ventricle, RV - right ventricle, LVP - left ventricular papillary muscle.

to confirm and extend these findings. It would be of interest to view heart muscle cells of animals of differing ages within a given species to see if levels of binucleated cells vary with age.

It is also unclear as to whether the distribution of binucleated cells is uniform throughout all regions of the heart of these adult animals. It has been shown that in the human heart, the frequency of binucleated myocytes appears to vary with age. Schneider and Pfitzer (1972) found only 8% of the muscle cells to be binucleated in newborns, while this value increased to 33% in a group ranging in age from 4½ months to 14 years (Figure 2). Hort (1953) found that in newborns, only 4.1% of the left ventricular muscle cells were binucleated, whereas 33.8% were in hearts of infants 1.5 - 6 months of age (Figure 3).

(d) Observation of Mitotic Activity

The principle involved is that treatment with colchicine, which stops mitotic activity of the cells, makes it possible to observe the arrested nuclei in metaphase which are clearly identifiable microscopically. The frequency of such nuclei can be used to indicate whether mitotic activity still occurred in the heart muscle at the time of sacrifice.

Shafiq et al. (1968) attempted to note the presence of mitotic activity in heart muscle cell nuclei of 3 week old rats. However, they found mitotic activity only occuring in connective tissue cells and 'free' cells. Some of these 'free' cells were closely associated with myocardial fibers. However, these cells were always physically separated from the myocardial fibers by their plasma membranes, as seen under the electron microscope. Thus, Shafiq concluded that mitotic activity occurred

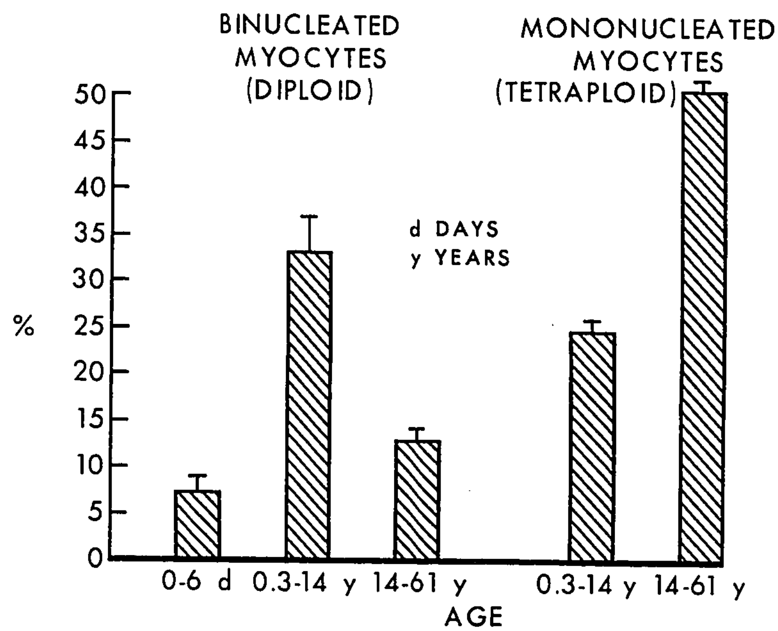

Figure 2. Percentages of binucleated left ventricular myocytes and tetraploid nuclei in the human heart at different ages (Schneider and Pfitzer, 1972).

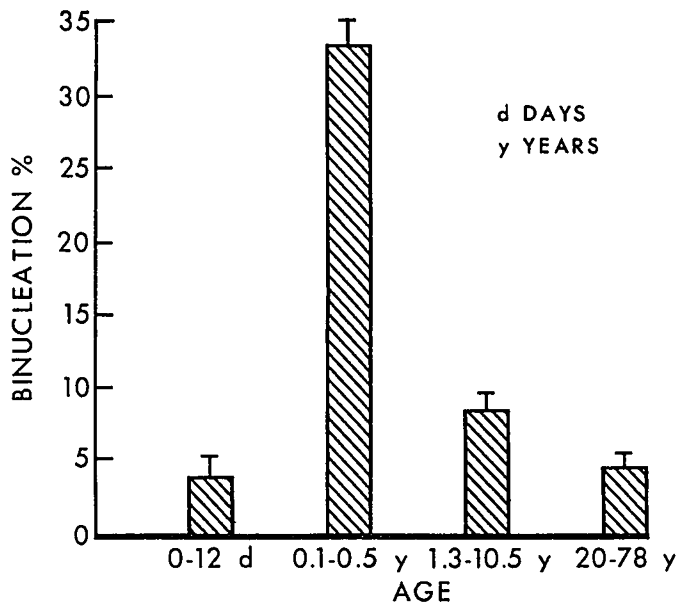

Figure 3. Percentages of binucleated left ventricular myocytes at different ages in the human heart (Hort, 1953).

in undifferentiated myoblasts and other 'free' cells, but not in fully formed muscle fibers in the rat heart.

Rumyantsev (1963) suggested that by about 2 weeks of age, mitotic activity in rat heart muscle cells had ceased. Sasaki et al. (1970) found very little mitotic activity extending to about 4 weeks postnatally (Figure 4).

From these findings, it appears that sometime between 2 and 4 weeks of age, mitotic activity ends in rat cardiac myocytes, confirming the findings of auto-radiographic observations (p. 3-5).

Even though mitosis can be halted in metaphase with the use of colchicine, this technique fails to answer the question whether, following chromosomal duplication, complete cellular endoreplication occurs. Therefore, we still would not know whether a mononucleated cell would give rise to two mononucleated cells or one binucleated cell.

(e) Counting the Number of Cells in the Heart

Cell numbers, at different ages, in the heart have been counted in order to see by what manner growth of the heart takes place.

(1) Morphological Methods

The heart muscle fibers per unit area were counted in transverse histological sections by Rakusan et al. (1963). Arai et al. (1968) did not calculate the total number of muscle fibers as they formed a very complex network; instead, they obtained the total length of heart muscle fibers by dividing the muscle volume of the left ventricle by the mean cross sectional area of the muscle fibers. Hort (1953) estimated the number of heart muscle cells through the use of nuclear dilution techniques within sections of heart muscle. This technique involved the

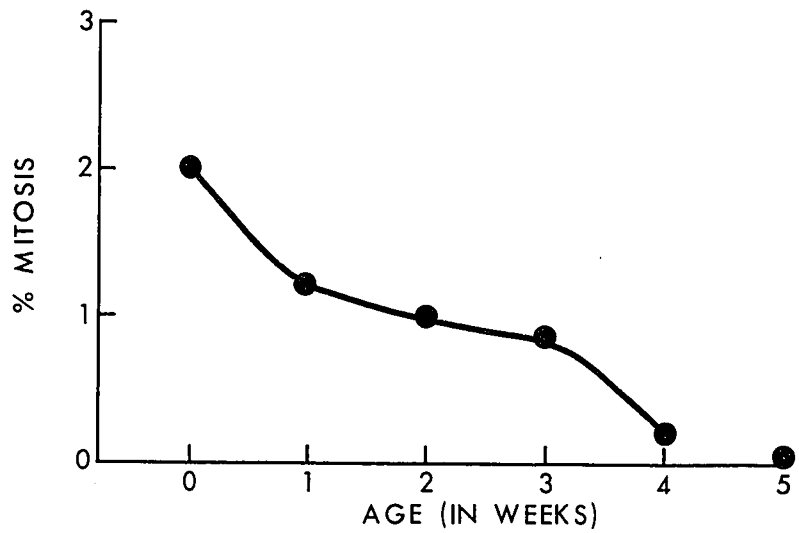

Figure 4. Mitotic activity of heart myocyte nuclei in the rat. (Sasaki et al. 1970).

counting of nuclei of intact muscle cells present in longitudinal sections of cardiac muscle. The number of cells per unit area were calculated by multiplying the number of nuclei observed by the predetermined cell number/nuclei number ratio. The unanimous finding is that there is no further increase in cell numbers after the 3rd-4th week of life (Rakusan and Poupa, 1963, Poupa et al. 1970, Zak, 1973).

(2) Biochemical Methods

Initially, it was believed that by dividing the amount of DNA in one heart cell nucleus into that contained in the whole heart, the total number of heart cells could be calculated. However, not all heart cells contain one nucleus, specifically muscle cells may well be binucleated. Even if the ratio of cells to nuclei within the heart were known, the number of cells could not be obtained by dividing the amount of DNA in a diploid nucleus into that contained in the heart and correcting for the cell to nuclear ratio. The reason for this is that not all nuclei within the heart contain the same amount of DNA.

Taking these factors into account, Adler and Sandritter (1971), through the combined use of cytophotometric DNA determinations and biochemical DNA determinations in the whole heart calculated the number of muscle cells in the heart. The genom was used as the basic unit; it contains 6×10^{-12}g of DNA, the quantity of DNA in a diploid nucleus (Vendrely, Vendrely 1949). The total number of genoms in the heart was obtained by dividing the total DNA in the heart by the DNA in one genom. From several sections of heart muscle, a mean value for 100 muscle cell nuclei and corresponding number of connective tissue cell nuclei was obtained with respect to their total DNA content and number of genoms.

The number of these genoms divided by the mean number of 100 muscle cell nuclei and corresponding connective tissue nuclei will equal the number of genoms in the heart divided by the total number of nuclei in the heart. Once the total number of nuclei is calculated, the total number of muscle cell nuclei can be obtained by multiplying the total number of nuclei by the fraction:

$$\frac{\text{100 muscle cell nuclei}}{\text{100 muscle cell nuclei + corresponding number of connective tissue nuclei}}$$

Finally, the total number of muscle cells in the heart is calculated by multiplying the total number of muscle cell nuclei in the heart by the fraction of muscle cells to muscle cell nuclei, this mean value obtained from the sections containing 100 muscle cell nuclei. In this study, the number of heart muscle cells were found to be in the range of 2×10^9 in normal hearts, and in maximally hypertrophied human hearts, there is an increase in the number of heart muscle cells of up to 100%.

Cardiac Growth (i) Rat:

Cellular proliferation in early life appears to be influenced by the growth rate of the rat (Rakusan et al., 1978). At 3 weeks of age, rats from smaller litters had attained greater heart and body weights than those from larger litters. These larger sized animals also had greater numbers of left ventricular muscle cells than the smaller animals.

Neffgen and Korecky (1971) induced cardiac enlargement by nutritional anemia in young postweaning rats. By autoradiography, it was evident that in these rats, there was a considerable production of new DNA in the heart. Since previous studies of early onset cardiomegaly (Poupa et al. 1964) showed that cell size in the muscle tissue of enlarged hearts of anemic animals was essentially the same as in normal myocardium. Neffgen and Korecky concluded that if cardiac enlargement occurred in the early postweaning life of the rat, both muscle and non-muscle cells undergo hyperplasia. However, if the growth stimulus is applied in the adult rat, hypertrophy of the muscle cells along with non-muscle cell hyperplasia results.

Cardiac Growth (ii) Human:

In observing the human heart, Linzbach (1947) using as parameters either fiber diameters or nuclear densities, concluded that all normal human hearts have the same number of myocardial fibers. This number remains constant during physiologic growth and any increase in weight during this period can be accounted for by enlargement of the individual fibers. Similarly, Adler (1975) concluded that from birth, no increase in the number of heart muscle cells takes place.

Heart growth was also observed under conditions of chronic cardiac hypertrophy. Linzbach (1960) did not see cell hypertrophy as the only mode of growth in chronically hypertrophic hearts. He arrived at the figure of 500 g as the "critical heart weight" for human hearts. Growth up to this weight is solely accomplished by muscle cell hypertrophy, however, beyond this value muscle cell hyperplasia also took place. Similarly, Adler (1975) found the number of muscle cells in hypertrophied adult human hearts (over 500 g) to double when compared to normal sized adult hearts.

The previous results indicate that cellular hyperplasia of cardiac myocytes takes place in the human heart under conditions of chronically increased load, resulting in cardiac enlargement. It is not clear however, whether or not the mode of cell replication is identical to that which exists in the normally growing fetal heart.

(f) Conclusions

In conclusion, it appears that in the adult rat, up to 80% of the cardiac muscle cells contain 2 nuclei. Almost all muscle cell nuclei are diploid. DNA production and cell proliferation occur up to the first few weeks of postnatal life. Hyperplasia of muscle cells takes place only if an artificial growth stimulus occurs not later than the end of the weaning period.

In contrast, within the human heart, up to 33% of the muscle cells have been shown to be binucleated in children under 14 years of age. However, in the adult, this value falls to 10% or less. As the heart enlarges pathologically, greater percentages of higher ploidy class muscle cell nuclei are formed. While normally muscle cell proliferation stops around birth, this potential remains dormant and under conditions of chronically augmented load, resulting in excessive cardiac enlargement (> 500 g), muscle cell proliferation may resume.

(g) Summary of Literature

Through the use of autoradiography, it was established that in the rat, DNA formation appears to occur up to the third week of postnatal life in the heart muscle cells. When the hearts were induced to hypertrophy, DNA formation was apparent in both muscle and non-muscle cells of young postweaning rats, but only non-muscle cell DNA production occurred in adult rats.

Almost all muscle cell nuclei within the hearts of both young and adult rats appear to be diploid. Even after extensive hypertrophy of the hearts, there was no real evidence for an increase in the percentage of polyploid nuclei. In contrast, several investigators concluded that as the human heart increases in size (specifically greater than 500 g), the percentage of polyploid nuclei increases.

The adult rat left ventricle contains binucleated myocytes comprising about 80% of the myocyte population. However, in humans, at birth, less than 10% of the muscle cells contain 2 nuclei. The highest percentage of binucleated muscle cells, around 33%, occurs in early life up to about 14 years. Throughout adult life, only about 10% of the myocytes appear to contain 2 nuclei. The percentage of binucleated muscle cells did not increase in either the human or rat hearts after undergoing hypertrophy.

Mitotic activity has been detected in heart muscle cells of the early postnatal rat. There has been some discussion as to whether or not this mitotic activity is occurring in fully developed muscle cells or only in their precursors.

Muscle cell proliferation appears to stop around the weaning period in the rat heart. Investigators see muscle cell hyperplasia occurring only if cardiac enlargement is induced early in the life of the rat; if hypertrophy is induced in the adult, only muscle cell hypertrophy takes place. In the human heart, the number of muscle cells appear to reach a plateau at birth or just after. Some investigators believe that in excessive cardiac enlargement, there is a re-appearance of muscle cell proliferation.

STATEMENT OF THE PROBLEMS

(1) It has been found that 80% of the muscle cells in the adult rat heart contain 2 nuclei. It is not known whether this frequency of binucleated myocytes is uniform throughout all regions of the heart.

Thus, lower and higher percentages of binucleated cells may exist in different regions of the rat heart, but the overall average for the heart is 80%. Therefore, different regions of the heart will be inspected in order to note whether there is variation from the 80% value of binucleation throughout the heart.

(2) The percentage of binucleated muscle cells in the human heart has been investigated at different ages. It appears that at birth, there are very low frequencies of binucleated cells, but in early life their numbers rise to over 30% of the muscle cell population. By adulthood, these values decline sharply. It is not known whether in other species values obtained in the adult represent a constant state or whether variations in the number of binucleated muscle cells exist throughout life. Therefore, hearts of animals of various ages of given species will be examined to determine the percentages of binucleated muscle cells with respect to age.

(3) The percentage of binucleated myocytes are apparently quite high in the adult rat heart. It is not known whether factors such as rate of heart or body growth may influence the occurence of binucleation. Therefore, litters of varying numbers will be set up from birth in order to note whether heart and body growth or age is more closely related to development of binucleated muscle cells. In all above species, the frequency of binucleated cells will be determined in samples of myocytes obtained by enzymatic disruption.

(4) Since the method used to obtain isolated myocardial cells yields only a fraction of all myocytes comprising the myocardium, this sample may be biased in favour of binucleated cells which, for some reason, may be better able to survive the procedure. Therefore, it is essential

to validate this technique. In order to do this, serial sections of rat left ventricle will be made so that cells can be visually reconstructed as they actually occur in the heart, both binucleated and any other, and the results from the enzymatically dispersed ventricles may be compared to those from the serial sections.

EXPERIMENTAL PROTOCOL

(1) The proportion of binucleated cells was determined in isolated myocytes obtained by enzymatic dispersion from the right ventricle and from different regions of the left ventricle in both the adult rat and beef hearts.

(2) Left ventricles of fetal and young rats were surveyed, in an attempt to record the development of the proportion of binucleated muscle cells.

(3) Assuming the development of binucleation has been recorded from birth, litter sizes were controlled in order to modify the rate of growth of the heart and of the body in order to establish whether age or heart size had a greater influence on the development of binucleated cells.

SUMMARY OF METHODOLOGY

(a) Regional Binucleation in the Heart

A total of 6 adult rat hearts and 3 adult beef hearts were used. In 3 of the rat hearts, the number of binucleated cells were recorded in the right ventricle and in the apex and base of the left ventricle. In the other 3 rat hearts, distributions of binucleated myocytes were obtained from the combined subendocardial tissue of the apex and base and combined subepicardial tissue of the apex and base of the left ventricle. In the 3 beef hearts, separate determinations were made in the right ventricle, apex, base, subendocardial apex, subendocardial base, subepicardial apex and subepicardial base of the left ventricle. The cell isolation technique (shaking) was employed. This technique is described on pages 25-27.

(b) Appearance of Binucleated Cells with Respect to Age

The studies dealing with the appearance of binucleated cells with respect to age, involved rats ranging in age from the nineteenth day of gestation (2 days prior to birth) to 24 days of age. The rabbits were between the fifty-second day of gestation (9 days before birth) and 3 days of age. The dogs were of 2 ages, the fifty-first day of gestation (10 days before birth) and 7 days of age. Newborn cats (6) were also observed.

The left ventricles were enzymatically disrupted, the cells thus isolated were fixed, stained and binucleated myocytes were counted. Aside from rats over 21 days of age, all hearts of a given age group were pooled. The pooling procedure was necessary in order to provide a great enough yield of cells to be counted on the slides. The cell isolation technique (shaking) was employed.

(c) Serial Sections of Newborn Rat Left Ventricle

In the serial section technique, cells of the rat left ventricle were reconstructed from 5 μ sections. The percentage of binucleated cells obtained using the cell isolation method was compared to hematoxylin-HCl stained serial sections from newborn rat hearts. The sections were first examined under low microscope power to locate an area where the cells were sectioned approximately perpendicular to their long axis. Using the viewing screen mounted on the bright field standard WL Zeiss research microscope magnified 630X, cell contours were outlined with a marker on a transparent plastic sheet (cells and their nuclei were drawn and numbered for easier identification in subsequent sections).

The number of nuclei per cell was then counted. A cell was considered to begin or end when portions of the intercalated disc became visible. In some instances, the intercalated disc was not visible. Therefore, an estimated average cell length of 33 μ was obtained for a newborn rat and only cells ranging from 25 μ to 45 μ were considered as single cells.

Three rats were thus used. Since the newborn rat hearts were so small, only a limited number of myocytes (33) could be identified which fulfilled the above requirements.

(d) Fast, Normal and Slow Growing Rats

Less than 8 hours after birth, the young rats from 8 litters were removed and the males and females separated. Subsequently, the newborn males and females were randomly returned to each mother forming 3 litters of fast growing animals (4 per litter), 2 litters of normally growing animals (8 per litter) and 3 litters of slow growing animals (16 per litter). Each litter contained equal numbers of males and females. By using this process the chances of potential genetic differences in growth occurring in the experimental litters were reduced. This method was used by Rakusan (1975). In his experiments, a total of 22 litters comprising fast, normally and slow growing animals were used. The results of his experiments clearly indicated that heart growth was proportional to body growth rather than to age.

In the present set of experiments, at 14 days of age, 4 animals were sacrificed from the fast growing litters. The left ventricles were individually disrupted; the nuclei in the myocytes were counted and the percentage of binucleated myocytes was recorded. Similarly, 4 left ventricles were processed and ratios of binucleated cells were obtained from the normally growing litters. A total of 8 left ventricles (4 pair of pooled hearts) were obtained from the slow growing litters and the percentage of binucleated myocytes recorded. At 21 days of age, 4 animals were sacrificed from the slow growing litters, and the proportion of binucleated myocytes in their left ventricles was recorded. The heart weights and body weights of the animals used in these experiments were recorded. The cell isolation technique (shaking) was used.

MATERIALS AND METHODS

One method of counting the number of binucleated myocytes in the heart is to isolate the fully intact myocytes, because it obviates the difficulties inherent in serial sections. In the cell isolation technique the heart is enzymatically disrupted and the isolated cells are then fixed and stained. In this way, individual whole cells and their nuclei may be viewed under the light microscope. The cell isolation technique employed in this study involved initial mechanical digestion of the myocardium with subsequent enzymatic disruption by shaking in an enzyme solution containing 0.05% hyaluronidase and 0.025% collagenase. Retrograde perfusion of the hearts with enzyme containing solution was deemed to be inpractical due to the anticipated wide range of heart weights from different species (young rats to adult beef). A uniform technique for all heart sizes was therefore developed.

A detailed description of this technique is as follows: the animal was sacrificed and the heart removed and placed in calcium-free Ringer's solution at room temperature. When a beef heart was examined, only a small piece (5-10 g) of the left ventricle was placed in this solution.

The Ringer's solution contained:

143.1 mM NaCl
3.6 mM KCl
1.2 mM $MgSO_4$
5.5 mM $C_6H_{12}O_6$
3.0 mM Hepes

The solution was buffered to a pH of 7.4 using 0.5 N NaOH and 0.5 N HCl. The hearts were left overnight in this solution since a greater number of whole myocytes were obtained than if the hearts were processed immediately after being removed from the animal.

The next day, the left ventricle was separated and minced in 2 or 3 ml. of the Ringer's solution and poured into an Erlenmeyer flask. It was digested in 5 ml. of Ringer's solution containing 0.05% hyaluronidase and 0.025% collagenase. The flask was shaken by hand at room temperature for 10 minutes. From the cell suspension which was obtained, a fraction was poured off into a test-tube. Another 5 ml. of enzyme solution were added to the remaining quantities of partly digested left ventricle in the flask and shaken for a further 10 minutes. Another fraction was poured off into a second test-tube.

From each fraction, 2 or 3 drops were placed on a glass counting chamber and observed under a phase contrast standard Zeiss WL research microscope (with Neofluar achromatic optic) at 630X magnification. These observations were made in order to ascertain whether a sufficient concentration of separate, whole myocytes was available for fixing and staining. The general purpose formaldehyde/glutaraldehyde fixative was used since this allowed for the greatest yield of cells. The fixative was prepared by adding 1 g. of paraformaldehyde and 4 drops of .5 N NaOH to 30 ml. of distilled water. The solution was heated to 70^{o}C to dissolve the paraformaldehyde. Distilled water was added, making the overall volume 100 ml. After cooling, 1.43 ml. of this solution was discarded and replaced by the same volume of 50% glutaraldehyde. Finally, 3.852 g. of sodium cacodylate were added and the solution buffered to a pH of 7.4 using .5 N NaOH and .5 N HCl.

One or more ventricles were pooled from the given species of rat, dog, cat and rabbit hearts. The ventricles were enzymatically disrupted to yield a cell suspension. A fraction of each suspension was used to make 2 slides. On each slide, 400 myocytes were counted and their number of nuclei recorded. The myocytes were observed in bright field at a magnification of 630X. The cells were placed on a SCWP02500 Millipore filter which secured the cells on its surface allowing for washing and staining of the cells without causing major changes in the shape and size of the cells, as the stained cells were similar in dimensions to cells observed before fixing and staining took place. This was necessary because the cells and their nuclei had to be visible in their fully intact form so they could be evaluated. This was done in the following manner: the filter was placed in a 95% ethanol solution. The filter was then placed, with its hydrophobic face upwards, on the wire mesh of a filter holder and inserted into the mouth of a suction flask. First, 15 ml. of distilled water were passed through the filter. Then 3 or 4 drops of cell suspension, followed by 15 ml. of distilled water were filtered to secure the cells on the filter. Finally, 15 ml. of 95% ethanol were filtered. The filter was then secured on a microscope slide and the cells stained using a Hematoxylin-HCl technique outlined in Table 3.

The filter was allowed to dry for at least 15 minutes. Then, a coverslip was placed over it.

(b) Evaluation of Data

Student's t-test was applied to test for statistical significance between (i) the different regions of the heart with respect to percentages of binucleation in the experiments dealing with regional distribution of binucleation; (ii) the percentages of binucleation, heart weight and body weight of fast, normal and slow growing rat hearts.

Table 3

STAINING PROCEDURE

80% ethanol 10 times

70% ethanol 10 times

50% ethanol 10 times

Distilled water 10 times

Harris hematoxylin 90 seconds

Running tap water (37°C) 120 seconds

1% HCl 60 seconds

Running tap water (37°C) 360 seconds

50% ethanol 60 seconds

70% ethanol 60 seconds

80% ethanol 60 seconds

95% ethanol 120 seconds

2 baths of propanol 120 seconds each

1:1 solution of propanol:xylol 120 seconds

3 baths of xylol 160 seconds each

RESULTS

The results of this study are summarized in Tables 4-10 and Figures 5-11.

(a) Regional Distribution of Binucleated Myocytes in the Heart

(i) Adult rat heart

In the right ventricle and in all regions observed in the left ventricle, approximately 80% (range: 79.4 - 81.0%) of the myocytes in the adult rat heart were binucleated (Table 4). A photograph of isolated myocytes appears in Figure 5.

(ii) Adult beef heart

In the right ventricle and different regions of the left ventricle 46.5 - 49% of the myocytes observed were binucleated (Table 4). A photograph of isolated myocytes appears in Figure 6.

(b) Appearance of Binucleated Myocytes with Respect to Age

(i) Rat

At 2 days before birth, only 5% of the left ventricular myocytes were binucleated. As summarized in Table 5, there appeared to be a progressive increase in the percentage of binucleated myocytes during the first 2 postnatal weeks; thereafter, this percentage remained stable at approximately 80%.

(ii) Rabbit

As seen in Table 6, there was an increase in the percentage of binucleated myocytes in the left ventricle of the rabbit. At 9 days before birth, 19.3% were binucleated, whereas by 3 days of age 27% of the myocytes were binucleated. In the adult, about 78% of the myocytes were binucleated.

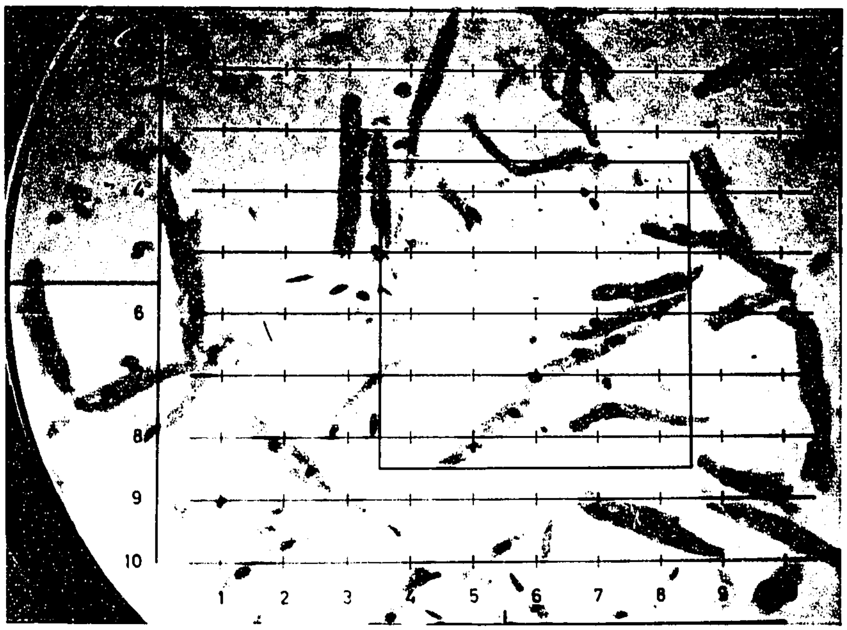

Figure 5. Photograph showing muscle cells of the adult rat left ventricle. The cells were obtained using the cell isolation (shaking) technique, magnification 630X.

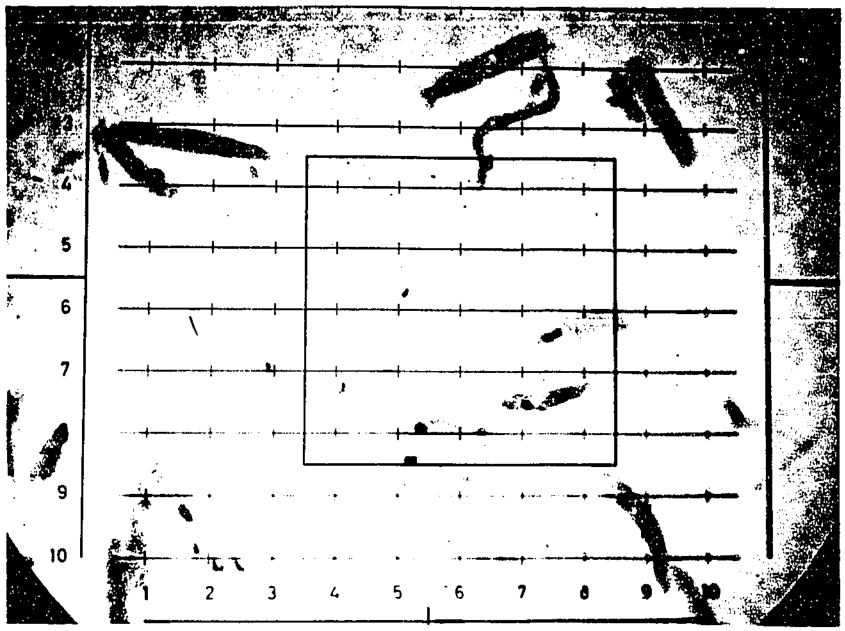

Figure 6. Photograph showing muscle cells of the adult beef left ventricle. The cells were obtained using the cell isolation (shaking) technique, magnification 630X.

Table 4

REGIONAL DISTRIBUTION OF BINUCLEATED MYOCYTES IN BOTH THE RAT AND BEEF HEARTS

	REGION	(MEAN) % BINUCLEATION	S.E.
ADULT BEEF HEART			
	right ventricle	48.6	0.50
	apex	49.5	1.34
	base	50.3	1.93
	subendocardium (apex)	47.9	1.30
	subepicardium (base)	46.8	0.88
	subendocardium (base)	48.9	1.14
	subepicardium (apex)	46.5	1.12
t	left ventricle	48.0	1.38
ADULT RAT HEART	right ventricle	80.5	.98
	apex	79.9	.55
	base	80.4	.56
	subendocardium (apex + base)	81.0	1.10
	subepicardium (apex + base)	79.4	.96
t	left ventricle	80.2	.79

In each region, eight different groups of 100 cells were counted and the number of nuclei per cell recorded, and then mean percentages of binucleation were calculated. A total of 3 beef hearts were used, 3 rat hearts were used for each region inspected (6 in total), no pooling was required.

t Represents the mean value of the four subregions inspected.

S.E. Standard error of the mean.

Table 5

NUCLEAR COUNTS IN LEFT VENTRICULAR CARDIAC MYOCYTES

ISOLATED BY ENZYMATIC DISRUPTION OF THE RAT HEARTS

AGE (Days from birth)	WEIGHT g	NUMBER OF ANIMALS	MONONUCLEATED % (Range)	BINUCLEATED % (Range)	POLYNUCLEATED %
-2	3.5	7	95.1 (93-98)	4.5 (2-7)	0.4
0	6.9	6	84.4 (72-89)	13.6 (10-25)	2.0
2	7.7	6	70.9 (61-78)	25.9 (20-34)	3.1
4	11.5	4	63.6 (54-72)	34.3 (27-44)	2.1
7	16	2	34.4 (29-43)	61.9 (55-67)	3.6
14	38	2	13.9 (4-23)	81.5 (71-95)	4.6
21	51	2	15.6 (12-21)	82.3 (76-86)	2.1
23-24	64	5	19.5 (9-28)	80.5 (72-90)	

Mean values (Range, greatest and least number of binucleated cells per 100 cells counted). The hearts of each age group were minced, pooled and four slides were prepared. Four hundred cells were evaluated in each slide. Only in the 23-24 day old group was each heart separately disrupted. Hematoxylin - HCl staining and 630X magnification were utilized.

Table 6

NUCLEAR COUNTS IN LEFT VENTRICULAR CARDIAC MYOCYTES

ISOLATED BY ENZYMATIC DISRUPTION OF THE RABBIT HEARTS

AGE (Days from birth)	WEIGHT g	NUMBER OF ANIMALS	MONONUCLEATED % (Range)	BINUCLEATED % (Range)	POLYNUCLEATED %
-9	6	10	78.6 (72-84)	19.3 (14-24)	2.1
-2	40	4	80.1 (74-84)	18.4 (14-22)	1.5
0	48	2	69.5 (63-73)	28.4 (20-37)	2.1
3	95	2	70.5 (65-74)	27.3 (21-32)	2.1

Mean values (Range, greatest and least number of binucleated cells per 100 cells counted). The hearts of each age group were minced, pooled and four slides were prepared. Four hundred cells were evaluated in each slide. Haematoxylin -HCl staining and 630X magnification were utilized.

(iii) Dog

In the dog, 10 days before birth only 5% of the myocytes in the left ventricle were binucleated. For dogs 6-60 months of age, 46% of the myocytes were found to be binucleated (Table 7).

(iv) Cat

Only 12% of the myocytes were binucleated in newborn animals, whereas approximately 76% of the left ventricular myocytes were binucleated in cats 1 to 2 months of age (Table 8).

(c) Serial Sections

In order to verify the percentages of binucleated myocytes obtained using cell isolation techniques, an alternative technique, serial sectioning of newborn rat left ventricle was used. This technique enabled us to retain all of the left ventricular myocytes for observation, which we could not do using the cell isolation technique. Using serial sections, we found that an average of 12% of the myocytes contained 2 nuclei. In the first rat, out of the 15 reconstructed cells, 2 were binucleated. In the second rat, only 5 cells could be viewed and none contained 2 nuclei; while 2 of the 13 cells observed were binucleated in the third rat heart (Table 9). This compared with 13.6% binucleated cells obtained from enzymatically disrupted hearts of similar age (Table 5).

(d) Fast and Slow Growing Animals

The last set of experiments involved the use of experimental litters of fast, normal and slow growing rats. All three groups were sacrificed at 14 days of age (results detailed in Table 10). The fast

Table 7

NUCLEAR COUNTS IN LEFT VENTRICULAR CARDIAC MYOCYTES
ISOLATED BY ENZYMATIC DISRUPTION OF THE DOG HEARTS

AGE	WEIGHT g	NUMBER OF ANIMALS	BINUCLEATED % (Range)
-10 days		5	5.0 (3-7)
7 days		5	14.6 (10-18)
6-60 months	11-30 kg.	4	46.0 (40-55)

Mean values (Range, greatest and least number of binucleated cells per 100 cells counted). The hearts of each age group were minced, pooled and four slides were prepared. The hearts of the dogs 6-60 months of age were individually disrupted. In each slide 400 cells were evaluated. Hematoxylin-HCl staining and 630X magnification were utilized.

Table 8

NUCLEAR COUNTS IN LEFT VENTRICULAR CARDIAC MYOCYTES

ISOLATED BY ENZYMATIC DISRUPTION OF THE CAT HEARTS

AGE (Days from birth)	WEIGHT g	NUMBER OF ANIMALS	BINUCLEATED %	(Range)
0	115	6	12.3	(7-18)
30-60	305	6	76.0	(70-84)

Mean values (Range, greatest and least number of binucleated cells per 100 cells counted). The hearts of the newborn cats were minced, pooled and four slides were prepared. The hearts of the older animals were treated individually. In each slide 400 cells were evaluated. Hematoxylin -HCl staining and 630X magnification were utilized.

Table 9

SERIAL SECTIONS OF NEWBORN RAT
LEFT VENTRICLES

RAT NUMBER	TOTAL CELL NUMBER	BINUCLEATED CELL NUMBER	PERCENTAGE OF BINUCLEATED CELLS
1	15	2	13.3
2	5	0	0
3	13	2	15.3

The cells were visually reconstructed in fixed stained sections of undisrupted myocardium, 630X magnification was used.

Table 10

TABLE OF RESULTS FOR THE EXPERIMENTS INVOLVING FAST, NORMAL AND SLOW GROWING LITTERS

	AGE (days)	GROUP	No. OF ANIMALS USED	BODY WEIGHT g	HEART WEIGHT mg	HEART WEIGHT / BODY WEIGHT	BINUCL-EATION %	POLY-NUCLEATION %
A	14	Fast (4 litter)	4	35.5	264	0.74	81.1	0.9
			±	0.65	8.13	0.02	0.24	0.38
B	14	Normal (8 litter)	4	29.8	222	0.75	80.7	1.3
			±	1.25	8.60	0.04	1.08	0.29
C	14	Slow (16 litter)	8	19.4	160	0.83	59.6	1.1
			±	0.84	5.37	0.02	1.04	0.31
D	21	Slow (16 litter)	4	28.3	214	0.76	78.9	1.4
			±	1.89	9.52	0.02	1.75	0.74
Significance								
		A:Bp		.005	.005	N.S.	N.S.	N.S.
		A:Cp		.001	.001	.005	.001	N.S.
		A:Dp		.01	.005	N.S.	N.S.	N.S.
		B:Cp		.001	.02	.05	.001	N.S.
		B:Dp		N.S.	N.S.	N.S.	N.S.	N.S.
		C;DP		.001	.001	.001	.001	N.S.

Average values: ± standard error of the mean.   N.S. nonsignificant.

growing rats' mean heart weight was 264 mg., mean body weight of 35.5 g. and 81.1% of the left ventricular myocytes were binucleated. The normally growing rats had a mean heart weight of 222 mg., body weight of 29.8 g. and 80.7% binucleation. In the slow growing rats, the mean heart weight was 160 mg., mean body weight was 19.4 g. and the proportion of binucleated cells was 59.6%. Another group of 4 slow growing rats were sacrificed at 21 days of age. Their mean heart weight was 214 mg., mean body weight 28.3 g. and 79% of the left ventricular myocytes were binucleated.

Results of the Student's t-test showed that mean body and heart weights of the slow growing group at 14 days of age and those of either the normal or fast growing groups, or the slow growing 21 day olds were statistically significantly different. The slow growing 14 day old group of rats was also significantly different from the other groups for percentages of binucleated myocytes. These results are summarized in Table 10.

In figures 7-9, percentages of binucleated myocytes were plotted against corresponding heart weights, body weights and ages for fast, normal and slow growing rats.

As both body weight and heart weight increased, the trend towards greater numbers of binucleated myocytes also resulted. Approximately 60% of the left ventricular myocytes had 2 nuclei in rats weighing around 19 g. with a mean heart weight 160 mg. However, the rats which had body weights of 25 g. or greater and heart weights near to or greater than 200 mg. had binucleated myocytes comprising 80% of the myocyte population of the left ventricle.

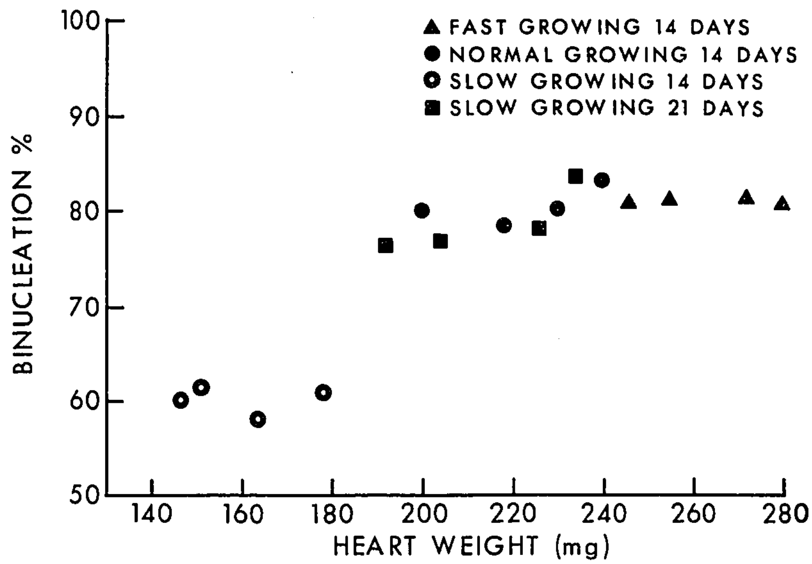

Figure 7. Binucleated myocytes from enzymatically disrupted rat left ventricle. Changes in percent binucleation with respect to heart weight.

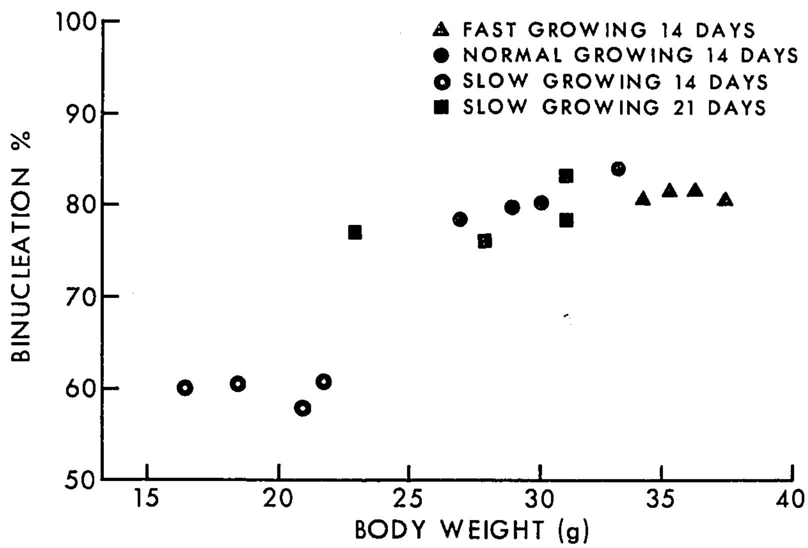

Figure 8. Binucleated myocytes from enzymatically disrupted rat left ventricle. Changes in percent binucleation with respect to body weight.

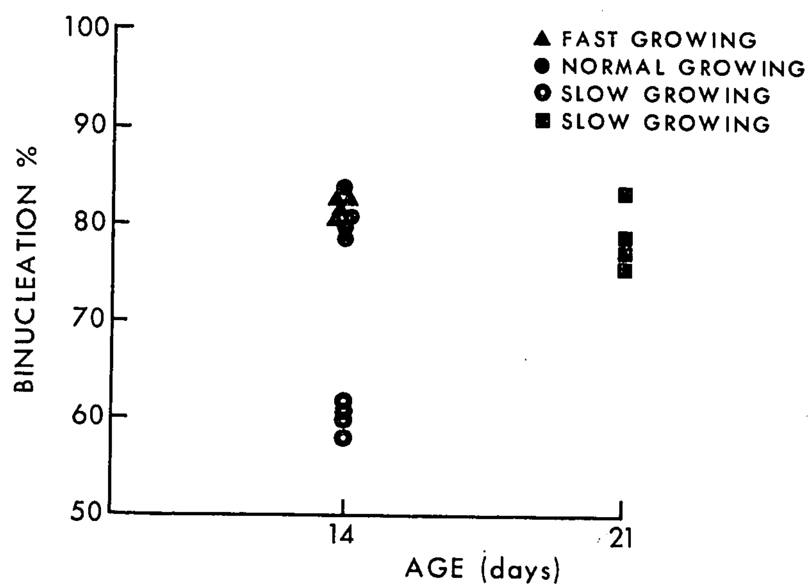

Figure 9. Binucleated myocytes from enzymatically disrupted rat left ventricle. Changes in percent binucleation with respect to age.

As described in Figure 10, the mean body weights and the corresponding percentages of binucleated myocytes for the fast, normal and slow growing rats were superimposed on the corresponding results for the animals used in the experiments involving the development of binucleated myocytes with respect to age. In both of these experiments, closely corresponding percentages of binucleated myocytes tended to come from rats with very similar body weights, regardless of age.

When observing the percentage of binucleated myocytes with respect to age, only 60% of the left ventricular myocytes of the slow growing 14 day old rats were binucleated. The percentage of binucleated cells was essentially the same in normal and fast growing 14 day old rats. The results are plotted in Figure 11. It is evident from Figures 7-11 that the suggested adult value of 80% binucleated myocytes is reached in cells of fast and normal growing animals by 14 days of age, whereas slow growing animals appear to reach this level during the subsequent week.

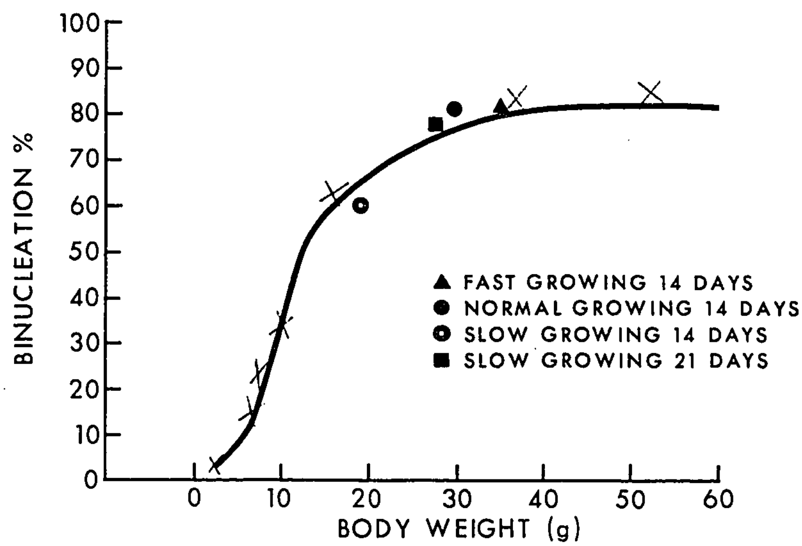

Figure 10. The percentages of binucleation found in normal, fast and slow growing rats are superimposed upon the normal trend of development of binucleation for rats during the early post-natal period. Curvilinear line obtained by fitting the mean values of binucleation for corresponding body weights of normal rats from the experiments involving the appearance of binucleation with respect to age.

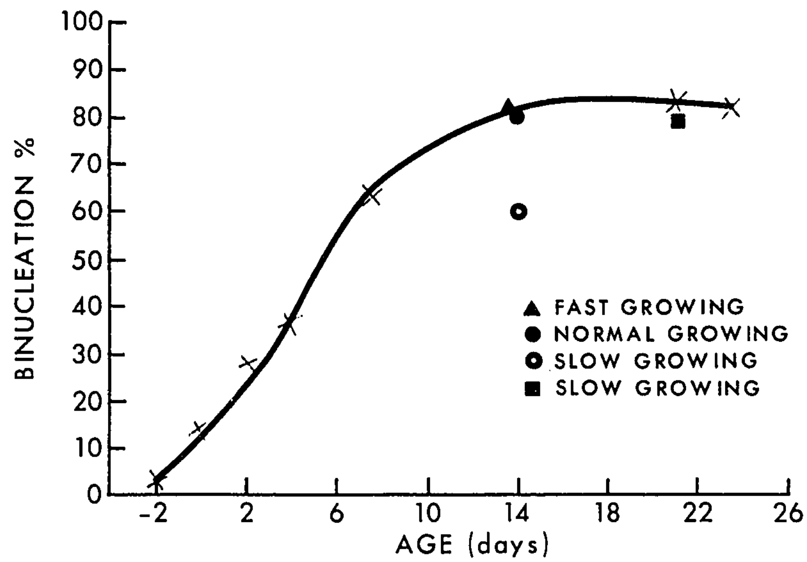

Figure 11. The percentages of binucleation found in normal, fast and slow growing rats are superimposed upon the normal trend of development of binucleation for rats during the early postnatal period. Curvilinear line obtained by fitting the mean values of binucleation for corresponding ages of normal rats from the experiments involving the appearance of binucleation with respect to age.

DISCUSSION

The cardiac muscle cell is not the only type of cell in the body that may contain more than one nucleus. Skeletal muscle, most closely related to cardiac muscle, is normally multinucleated (Enesco, 1964). Helwig-Larsen (1952) noted binucleated cells in the thyroid, pancreas and liver. Pseudostratified ciliated columnar epithelial cells of the trachea may also be binucleated (Craigmyle 1975).

(a) Regional Distribution of Binucleated Myocytes in the Heart

We found the percentages of binucleated myocytes in the enzymatically disrupted rat right ventricle and in different regions of the left ventricle, to be quite uniform. In all regions inspected, approximately 80% of the myocytes were binucleated.

As in the rat, there was little deviation found in the percentage of binucleated myocytes in the right ventricle and in the different regions of the left ventricle of the beef heart. Binucleated cells varied from 46.5 - 48.9%.

(b) Validation of the Cell Isolation Technique

The cell isolation technique yields only about one quarter of the heart mass in the form of intact muscle cells. Therefore, on this basis alone, it would be difficult to assume that the results obtained by cell isolation technique are representative for the heart as a whole. However, certain factors should be considered. In both the rat and beef hearts, extremely consistent percentages of binucleated cells were obtained from all regions inspected. This suggests a biological origin, rather than artifact.

The validity of the cell isolation technique is supported by the results of serial sections. According to the cell isolation technique (shaking), newborn rat left ventricles were found to contain 13.6% binucleated myocytes. Serial sections of 3 newborn rat left ventricles were made and in 2 of these hearts 13.3% and 15.3% of the myocytes were found to be binucleated. Therefore, the cell isolation results closely approximate those of the serial sections for newborn rat hearts. This would indicate that results obtained by the cell isolation technique may be reasonably reliable, such that not only low percentages of binucleated myocytes appear in the newborn rat, but also predominantly binucleated myocyte populations exist in the adult rat, as well as in adults of other species.

(c) Appearance of Binucleated Myocytes with Respect to Age

When the development of binucleated myocytes with respect to age was studied, 2 days before birth only about 5% of the left ventricular myocytes contained 2 nuclei in fetal rats, but by 14 days of age around 80% were binucleated. It appears then, that the predominantly binucleated myocyte populations in normal growing rats were complete by the end of the first 2 weeks of postnatal life.

It is recognized that a serious methodological shortcoming of these studies lies in the fact that all hearts in each age group were pooled, in order to obtain a sufficient yield of cells to be counted. This was necessary because of the small size of the organs in young rats. Therefore, the results for these young rats can only be representative from the cell population from which they were obtained. Only the hearts from the 23-24 day old group were separately enzymatically disrupted.

Therefore, the results from the 23-24 day old group may be tested as to whether they are an accurate representation of the binucleated cell population of all enzymatically disrupted rat hearts.

It should be noted that even though the hearts were pooled, all results followed a reasonable trend of cellular binucleation from the fetal hearts to those 14 days of age. The fact that all hearts were equally represented cannot be verified; however, it is probable, in view of the high frequency of binucleated cells, that the results for pooled hearts were in fact representative of each individual heart.

(d) Fast and Slow Growing Animals

Fetal and newborn rats appear to have less than 15% binucleated left ventricular myocytes, yet by 14 days of age, about 80% of the myocytes contain 2 nuclei, as in the adult. From birth to 14 days of age, along with aging, the heart weight of the animal increases tenfold.

To detect whether the appearance of binucleated myocytes is primarily genetically programmed to age or stimulated by growth of the heart, early postnatal body growth (and heart growth) was modified by forming litters of 4, 8 and 16 offsprings. Therefore, members of the smaller litters attain a given heart weight earlier than those in the larger sized litters. These results showed that according to litter size, adult percentages of binucleation were reached between 14 and 21 days of age.

Rakusan et al. (1978) found that 3 week old rats which had faster growth rates from birth and as a result attained greater heart and body weights, also had greater numbers of heart muscle cells than smaller sized rats of the same age.

As indicated in Figures 7-9, members of the fast and normally growing litters attained greater heart and body weights along with adult percentages of binucleation (80%) earlier than rats in the slow growing litters. Therefore, it appears that heart weight and body weight are more important determinants than age in reaching the adult level of 80% binucleated myocytes, as well as increases in the number of muscle cells present.

From our results, it appears that when the body weight of the rat surpasses 23 g. and (wet) heart weight of 190 mg., adult percentages of 80% binucleated left ventricular myocytes will appear regardless of the age of the animal. These values could serve as the "threshold" value of body weight and heart weight in the rat for the appearance of adult percentages of binucleated cardiac myocytes. Thus, along with the cessation of mitotic activity, DNA formation and muscle cell hyperplasia, adult percentages of binucleated cardiac myocytes are attained early in post-natal life in the rat.

There are likely to be several factors involved in forming the number and ploidy class of nuclei appearing in a cell. Enesco and Puddy (1964) counted the number of nuclei in different skeletal muscle fibers in the adult rat. They found 1201 nuclei in a biceps brachii muscle fiber weighing 22.4 μg., 2122 nuclei in an extensor carpii fiber weighing 29.9 μg and 3610 nuclei in a gastrocnemius fiber weighing 63.1 μg. At face value, these results would tend to indicate that as the cell mass increases, so will the number of nuclei in the cell. However, this is not always true. An adult human cardiac muscle cell may be 100-150 μ in length and only contain one nucleus, whereas a cardiac muscle cell of a young child which may only be 50 μ in length may contain 2 nuclei (Schneider, Pfitzer, 1972).

There appear to be many factors collectively influencing the number of nuclei within a cell. Some of the more likely ones would be cell metabolism, cell size and surface area. Along with the number of nuclei in a cell, the ploidy class of a nucleus would also be affected by the above mentioned factors.

Before in depth studies are made concerning the factors which influence nuclear properties, it should be noted that great species differences exist which cause differences in the number and ploidy class of nuclei within a cell. A cardiac muscle cell is of similar dimensions and serves the same purpose in both the human and the rat. Most human cardiac myocytes are mononucleated and their nuclei polyploid. However, the majority of adult rat cardiac myocytes are binucleated and their nuclei are diploid (Zak, 1974).

Age may also be a factor. As stated earlier in the Discussion, changes in nuclear configurations occur in the heart muscle cells of the human. In early life, up to 33% of the cells are binucleated along with rather low levels of polyploid nuclei. By adulthood, there are much lower levels of binucleated cells, often less than 10%, but the majority of nuclei have become polyploid. These changes occur with age, but as seen in the rat, these changes may be more closely related to physical factors such as cell, heart and body size.

SUMMARY

1) Approximately 80% of the myocytes isolated from the right ventricle along with the apex, base, subendocardial apex and base, and subepicardial apex and base of the left ventricle of adult rat hearts were found to be binucleated.

2) Between 46-49% of the myocytes isolated from the right ventricle along with the apex, base, subendocardial apex, subendocardial base, subepicardial apex and subepicardial base of the left ventricle of adult beef hearts were found to be binucleated.

3) By 14 days of age, 80% of the myocytes isolated from normal rat left ventricles were binucleated.

4) In 2 serially sectioned newborn rat hearts, 13.3% and 15.3% of the left ventricular myocytes were found to contain 2 nuclei.

5) In the left ventricles of fetal rabbit hearts, less than 20% of the isolated myocytes were binucleated, while about 28% appear in animals 3 days of age.

6) In the left ventricles of fetal dogs, 7 days before birth, 5% of the isolated left ventricular myocytes contained 2 nuclei. In animals 6-60 months, about 46% of the cells were binucleated.

7) In the left ventricles of newborn cats about 12% of the isolated myocytes were binucleated, compared to about 76% in the adult.

8) Development of binucleation in cardiac myocytes in the rat appear to reach adult values of about 80% when the heart weight and body weight surpass 190 mg., and 23 g. respectively.

APPENDIX

Alternative Cell Isolation Techniques

In the perfusion method, 60 ml of the Ringer's solution (Materials and Methods) were constantly circulated in the perfusion apparatus. The heart was then mounted on the perfusion cannula, and the first 20 ml. of perfusate was used to wash out the remaining blood and subsequently discarded. This was replaced by another 20 ml. of Ringer's solution, but this solution contained 0.05% hyaluronidase and 0.025% collagenase. Subsequently, the hearts were perfused through the coronary vessels with 60 ml. of the latter (enzyme) solution at $37^{\circ}C$ in a closed recirculating system for approximately 30 to 40 minutes at a flow rate of 6.5 to 7.0 ml./minute. The pressure within the heart was gauged by a mercury manometer. Pure O_2 was bubbled through the perfusion medium. Two criteria were used in deciding when the heart has been sufficiently broken down: (1) the heart was periodically checked to note the extent of softening; (2) when the perfusion pressure fell below 10 mm. of mercury, the perfusion was stopped.

The heart was removed from the apparatus and all but the left ventricle was discarded. The left ventricle was minced in the Ringer's solution and then transferred to a flask and shaken till a cloudy suspension appeared. When the remaining pieces of tissue settled to the bottom, the suspension was poured into a test-tube and a fraction was observed under the phase contrast microscope. The suspension was then fixed and the cells were stained as described in the Materials and Methods.

The stirring method was identical to that described in the Materials and Methods. However, when the partly digested left ventricle was shaken in the Erlenmeyer flask, in the stirring method the contents were not shaken, rather they were stirred using a glass rod for a period of 10 to 20 minutes.

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