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Fractionation of Rat Ventricular Nuclei

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Myocardial cells were isolated after treatment with collagenase (0.05%) and hyaluronidase (0.1%) by discontinuous-gradient centrifugation on 3% Ficoll. Nuclei derived from these myocardial cells were then fractionated on a discontinuous sucrose density gradient with the following steps: (I) 2.0M/2.3M, (II) 2.3M/2.4M, (III) 2.4M/2.5M, (IV) 2.5M/2.6M and (V) 2.6M/2.85M. The myocardial nuclei were sedimented in the interfaces of gradient fractions (II) and (III). Nuclei from whole ventricles that had been treated with the enzymes before isolation sedimented into five major subsets of nuclei. These findings suggest that nuclei sedimented in the isopycnic gradient at fractions (II) and (III) are most probably derived from myocardial cells. However, this procedure is laborious and lengthy, and the recovery of myocardial-cell nuclei is low. An alternative method was developed to isolate an enriched fraction of myocardial-cell nuclei from whole ventricular tissue without exposing the tissues to enzyme digestion. These ventricular nuclei could be fractionated into five nuclear subsets by using the same discontinuous sucrose density gradient as that described above. The content of DNA, RNA and protein per nucleus for each band was determined. Although the DNA content per nucleus was constant (10pg), that of RNA varied from 1.5 to 4.5pg and that of protein from 16 to 24pg. Nuclei from each band were examined by light-microscopy: large nuclei occurred in the lighter regions whereas smaller nuclei were found in the denser regions of the gradient. From the size distribution pattern of myocardial-cell nuclei compared with that of total ventricular nuclei, it was found that nuclear subsets (II), (III) and (IV) were similar to myocardial nuclei. Electrophoretic analyses of the proteins solubilized in sodium dodecyl sulphate/phenol or Tris/EDTA/2-mercaptoethanol/phenol obtained from each nuclear subset indicate that these fractions are similar, with limited qualitative differences. These findings indicate that isolation of an enriched fraction of myocardial-cell nuclei could be achieved by discontinuous-sucrose-density-gradient centrifugation.

The eukaryotic-cell nucleus, which is the major site of RNA synthesis, plays a dominant role in differential regulation of gene expression. Many investigators, using the experimental model of hypertrophy (Morkin & Ashford, 1968; Sasaki *et al.*, 1970*a*,*b*; Nair *et al.*, 1974) and the animal models (Beznak *et al.*, 1974; Liew & Sole, 1978*a*,*b*) (e.g. cardiomyopathy), have demonstrated alterations in nuclear activity expressed as changes in the content of nuclear proteins, RNA and DNA. However, studies of unfractionated nuclei are difficult to interpret since the heart is composed of heterogeneous cell types (Harary & Farley, 1963; De Haan, 1967; Morkin & Ashford, 1968). In general, two major classes of cells can be defined: the non-muscle cells, consisting of interstitial and endothelial types, and muscle cells (i.e. myocardial cells).

In normal adult rat heart, non-muscle nuclei comprise approx. 73% of the total nuclei observed in the left ventricle, whereas myocardial-cell nuclei represent only 27% of the total (Enesico & Puddy, 1964; Morkin & Ashford, 1968; Zak, 1974). It therefore follows that, in order to elucidate the biochemistry of nuclear events, one must either

Abbreviation used: SDS, sodium dodecyl sulphate.

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employ techniques that result in a pure cell preparation or develop methods to fractionate total heart nuclei. Enyzmes have been employed to dissociate the different cell types (Berry *et al.*, 1970; Glick *et al.*, 1974; Cutilletta *et al.*, 1977; Farmer *et al.*, 1977; Grosso *et al.*, 1977). However, after enzymic digestion the isolated myocardial cells were resistant to mechanical disruption. Only after treatment with a hypo-osmotic buffer and sonication could a reasonable quantity of intact nuclei be recovered. These nuclei became predominantly spheroid and were no longer oblong in shape, indicating that the nuclei had gone through some morphological change during their isolation.

In recent years numerous investigators have used continuous- and discontinuous-sucrose-density-gradient centrifugation to fractionate nuclei of different cell types: liver (Johnston *et al.*, 1968*a*,*b*; Haines *et al.*, 1969; Gonzalez-Mujica & Mathias, 1973; Neal *et al.*, 1976), brain (Løvtrup-Rein & McEwen, 1966; Austoker *et al.*, 1972; Mathias & Wynter, 1973; Stambolova *et al.*, 1973) and colonic epithelial cells (Boffa *et al.*, 1976). For example, Mathias and his co-workers (Austoker *et al.*, 1972; Mathias & Wynter, 1973; Stambolova *et al.*, 1973) have used discontinuous-sucrose-density-gradient centrifugation to fractionate the nuclei of different cell types (neuronal, astrocytic, glial) from whole brain by exploiting small differences in density. The present paper is the first report of the utilization of this technique to fractionate nuclei of heart tissue into various subsets, on the basis of their buoyant density, and to characterize them further by their chemical composition.

Materials and Methods

(*a*) *Isolation of heart nuclei*

Male albino rats (Wistar strain) of body wt. 140–150g were used. They were fed with Purina chow *ad libitum*. Food was removed overnight before decapitation of the animals. All subsequent operations were carried out at 4°C. The procedure for the isolation of nuclei was essentially the same as that of Liew *et al.* (1972). The hearts were removed, rinsed in ice-cold MA buffer [0.25M-sucrose/10mM-Tris/HCl buffer (pH 7.4) / 3mM-$MgCl_2$ / 0.1mM-phenylmethanesulphonyl fluoride]. The aorta and atria were removed. The ventricles were weighed and minced with scissors. The minced tissue was rinsed three times with MA buffer followed by homogenization in 10vol. of MA buffer in a Polytron homogenizer (Brinkman) at position 4 for 10s. This homogenate was centrifuged at 1000*g* for 10min. The pellet was then resuspended in 10vol. of MA buffer and homogenized further in a Potter–Elvehjem tube with a rotating Teflon pestle [clearance 0.51–0.64mm (0.020–0.025in)]. After six strokes of the pestle the homogenate was filtered through two layers of nylon cloth and a nylon sieve of mesh size 200 (75μm). The filtrate was then centrifuged at 1000*g* for 10min. The pellet was resuspended in MB buffer (0.1% Triton X-100 in MA buffer). The crude nuclear pellet was recovered by centrifugation at 1000*g* for 10min. The pellet was resuspended in MC buffer [2.2M-sucrose/10mM-Tris/HCl buffer (pH 7.4)/1mM-$MgCl_2$/0.1mM-phenylmethanesulphonyl fluoride] and underlaid with 5ml of MC buffer before centrifugation at 113000*g* for 60min in a Beckman SW 27 rotor.

(*b*) *Fractionation of nuclei by density-gradient centrifugation*

Purified nuclei obtained by centrifugation through 2.2M-sucrose were resuspended in 2.0M-sucrose in TM buffer [10mM-Tris/HCl buffer (pH 7.4)/1mM-$MgCl_2$/0.1mM-phenylmethanesulphonyl fluoride] at a concentration of 7.0 A_{260}units/ml. This suspension was layered over a discontinuous sucrose density gradient prepared as follows: a cushion of 2.85M-sucrose in TM buffer (3ml) followed by 3ml layers of decreasing sucrose concentration in TM buffer in the following order: 2.6, 2.5, 2.4 and 2.3M. The gradient was left for 3h at 4°C before use. Fractionation of the nuclei was achieved by centrifugation in a Beckman SW 27.1 rotor for 118000*g* for 120min. The gradient was unloaded by introducing a 50μl micro-sampling pipette to the bottom of the gradient tube and connecting it to a variable-speed-drive Masterflex tubing pump (Cole–Parmer Instrument Co.). The effluent under suction was passed through a flow cell of 1cm path length in a Gilford 300-N micro-sample spectrophotometer. The effluent was collected in 0.5ml fractions, and the sucrose concentration in each fraction was checked by using a refractometer. The number of nuclei in each zone was determined with a haemocytometer or with a Coulter counter (model ZF) with a 100μm orifice (aperture current $\frac{1}{2}$, amplification 0.707, threshold 14). Size distribution of nuclei was determined with a Coulter Channelyzer (model C-1000).

The chemical composition of the fractionated nuclei in each band was determined by excising the individual bands with a gradient tube slicer. Each excised band was diluted by the addition of 0.25M-sucrose/10mM-Tris/HCl buffer (pH 7.4)/1mM-$MgCl_2$/0.1mM-phenylmethanesulphonyl fluoride to 40ml. The nuclei were collected by centrifugation at 1000*g* for 20min.

(*c*) *Chemical analysis*

The nucleic acid content of each band was determined by suspending the nuclear pellet in 0.5M-$HClO_4$ containing 2mM-EDTA. Nucleic acids in the resulting precipitate were separated by the

method of Shibko *et al.* (1967). DNA was determined by the method of Burton (1956). RNA was measured by the orcinol reaction (Mejbaum, 1939) or by reading u.v. absorbance at 260nm (with yeast RNA as a standard; 1 A_{260} unit $\equiv 50\mu g$ of RNA/ml).

The protein content of nuclei was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

(d) Microscopy

Purified nuclei and those from the individual fractions were suspended in 0.05% Toluidine Blue (in MA buffer). Light-micrographs were taken at a magnification of 400×.

(e) Extraction of total nuclear protein

Extraction with SDS/phenol. Nuclei isolated from each fraction of the gradient were washed twice with 0.14M-NaCl/0.1mM-phenylmethanesulphonyl fluoride and then suspended in 1ml of 2% SDS/20mM-EDTA/20mM-Tris/HCl buffer (pH 8.2)/0.1% 2-mercaptoethanol and extracted with phenol by the method of LeStourgeon & Beyer (1977). The SDS/phenol-solubilized nuclear proteins were dialysed against 0.25M-sucrose/0.1% SDS/6mM-Tris/HCl buffer (pH 6.8)/0.1% 2-mercaptoethanol.

Extraction with TEM buffer/phenol. Nuclei were washed twice with 0.14M-NaCl/0.1mM-phenylmethanesulphonyl fluoride and then suspended in TEM buffer [0.1M-Tris/HCl buffer (pH 8.4)/10mM-EDTA/0.14M-2-mercaptoethanol] and extracted with phenol as described previously (Liew & Sole, 1978*a*,*b*).

(f) Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis (15% acrylamide). SDS/polyacrylamide-gel electrophoresis was performed as described previously (Chan & Liew, 1977). Stained gels were scanned at 570nm. Histones (H1, H2a, H2b, H3 and H4) were identified by comparison with calf thymus histones.

Two-dimensional isoelectric focusing–SDS/polyacrylamide-gel electrophoresis. Two-dimensional isoelectric focusing–SDS/polyacrylamide-gel electrophoresis was performed as described previously (Liew & Sole, 1978*a*). Molecular weights of the nuclear proteins were estimated by using phosphorylase *a* (mol.wt. 91000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000) and carbonic anhydrase (mol.wt. 29000) as standards.

(g) Isolation of myocardial cells

Adult male rats (body wt. 140–150g) were killed by decapitation. Myocardial cells were isolated and separated from other cell types essentially by the procedure of Glick *et al.* (1974). Hearts were removed and the ventricles were immediately cut into chunks measuring approx. 2mm on a side. The chunks were incubated with phosphate buffer, pH 7.4 (Glick *et al.*, 1974), containing 0.05% collagenase (type 1 from *Clostridia*; Worthington Biochemical Corp.), 0.1% hyaluronidase (Sigma Chemical Co.) and 1% bovine serum albumin (Sigma Chemical Co.), and gently agitated in this buffer at 100cycles/min for 20min at 37°C on a Dubnoff metabolic shaker. At the end of this 20min period, 15ml of phosphate buffer was added to the flask and the suspension of heart cells was filtered through one layer of nylon cloth (100 mesh) into a cold test tube. The chunks that did not pass through the filter were returned to the flask and 4ml of enzyme/phosphate was added. The dissociation was continued for another 20min. This process was repeated three more times after filtration. The dispersed cells in the supernatant were sedimented by low-speed centrifugation (60*g*) for 3min. The cells were then gently resuspended in 10ml of phosphate buffer and layered atop of 20ml of ice-cold 3% (w/w) Ficoll in phosphate buffer, and pelleted at 60*g* for 3min. Viability of the myocardial cells as measured by Trypan Blue exclusion (Pretlow *et al.*, 1972) indicates that more than 90% of the cells exclude the dye and of these 80% are also beating. The intact muscle cells, isolated as described above, were washed repeatedly with phosphate buffer before the isolation of myocardial nuclei.

(h) Isolation of myocardial nuclei

Many attempts employing mechanical disruption to release nuclei from myocardial cells have met with limited success. The most suitable method to release the myocardial nuclei from the cells was the hypo-osmotic and sonication technique resembling that described by Cutilletta *et al.* (1977), which involved suspending the myocardial cells in hypo-osmotic buffer [10mM-Tris/HCl buffer (pH 7.4)/1mM-$MgCl_2$/10mM-NaCl/5mM-$CaCl_2$] for 30min at 4°C. The cells were then sedimented at 1000*g* for 10min, and then resuspended in 20ml of hypo-osmotic buffer and sonicated for 40s at setting 2 in a Branson sonifier W-350. Triton X-100 was added to the sonicated preparation to a final concentration of 0.1% and then centrifuged at 1000*g* or 10min. The resulting nuclear pellet was resuspended in 13ml of MC buffer and underlaid with 2.5ml of MC buffer, and centrifuged at 118000*g* for 60min in a Beckman SW 27.1 rotor. Fractionation of myocardial nuclei was performed in similar fashion to that of ventricular nuclei, except that a small discontinuous sucrose density gradient (5ml) consisting of 0.8ml/fraction was used. Centrifugation was performed at 149000*g* for 70min in a Beckman SW 50.1 rotor.

Separation of the myocardial-cell-enriched nuclear subsets (II+III+IV) from the non-myocardial-cell nuclear subset (V). A one-step procedure was developed to separate myocardial-cell-enriched nuclear subsets from the non-myocardial-cell nuclear subset. Crude ventricular nuclei isolated as described in section (*a*) were resuspended in 25 ml of 2.2 M-sucrose in TM buffer and layered on a discontinuous sucrose density gradient, consisting of 5 ml of 2.3 M-sucrose in TM buffer layered over a 5 ml cushion of 2.7 M-sucrose in TM buffer. This gradient was then spun at 113000 *g* for 60 min in a Beckman SW 27 rotor.

Nuclei banding at the 2.3 M-/2.7 M-sucrose interface (fraction A) were collected by the addition of 0.25 M-sucrose in TM buffer to 40 ml and centrifuged at 1000 *g* for 20 min. The nuclei pelleting through the 2.7 M-sucrose (fraction B) were resuspended in 0.25 M-sucrose in TM buffer and centrifuged at 1000 *g* for 20 min.

Results

Isolation of ventricular nuclei

The recovery of Triton X-100-treated nuclei from adult rat heart on the basis of DNA was found to be approximately 25% of the total DNA in the homogenate. The amounts of DNA and nuclear protein recovered from 10 g of heart tissue are shown in Table 1. The protein/DNA ratio was 2.9:1. Purity of the nuclei on the basis of biochemical criteria and electron microscopy as reported previously (Liew & Sole, 1978*a*; Liew *et al.*, 1972) indicated very little myofibrillar or cytoplasmic contamination. As shown in Fig. 1(*a*), total ventricular heart nuclei were heterogeneous in size and shape. Some of the nuclei were oblong in shape (at least 70% of the population) and contained one or two well-shaped nucleoli against a pale chromatin network, and some of the spheroid-shaped nuclei contained a dense and heavy-staining chromatin.

Isolation of myocardial nuclei

Several methods have been reported for producing myocytes essentially free from the non-muscle components of the heart by enzyme digestion followed by separation through a Ficoll gradient (Vahouny *et al.*, 1970; Pretlow *et al.*, 1972; Glick *et al.*, 1974; Moustafa *et al.*, 1976; Cutilletta *et al.*, 1977; Farmer *et al.*, 1977; Grosso *et al.*, 1977). These methods resulted in a highly purified myocardial population, but the yield of such cells was low and the procedures were very laborious and lengthy. Furthermore, the yield of myocardial nuclei was very low. As shown in Fig. 2, isolated myocardial cells from adult rat ventricles appear to be cylindrical in shape with step-like ends. The myocardial cells are predominantly binucleate. The nuclei were oblong in shape, situated near the centre of the cell and oriented along its long axis. Myocardial nuclei derived from the myocardial cells tend to be heterogeneous in size and shape (Fig. 1*b*). The nuclei, once isolated from the myocardial cells, are no longer predominantly oblong in shape but appear to be more spherical. By light-micrograph inspection a much higher degree of myofibrillar

Table 1. *Chemical composition of total heart nuclei*
Experimental details are given in the text. A 10 g (wet wt.) portion of heart tissue was used, and recovery of heart nuclei on the basis of DNA is equal to 24.85 ± 4.0%. Results are expressed as means ± S.D. for six separate experiments.

DNA (mg)	Protein (mg)	Protein/DNA ratio
2.58 ± 0.12	7.54 ± 0.41	2.91 ± 0.02

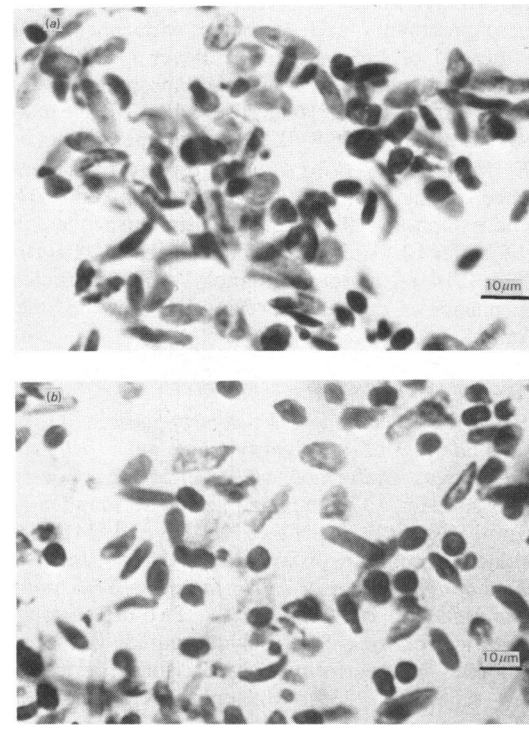

Fig. 1. *Light-micrographs of isolated ventricular and myocardial-cell nuclei*
(*a*) Typical isolation of adult ventricular nuclei as described in the Materials and Methods section. (*b*) Typical isolation of myocardial nuclei from adult myocardial cells as described in the Materials and Methods section. Nuclei were suspended in 0.05% Toluidine Blue in 0.25 M-sucrose/3 mM-$MgCl_2$/10 mM-Tris/HCl buffer, pH 7.4, and light-micrographs were taken at a magnification of 400×.

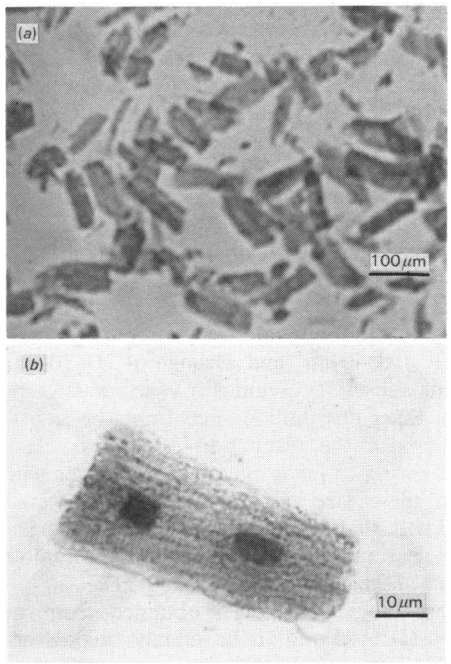

Fig. 2. *Light-micrograph of individual myocardial cells*
Myocardial cells isolated as described in the Materials and Methods section were stained with 0.05% Toluidine Blue in 0.25 M-sucrose/3 mM-$MgCl_2$/10 mM-Tris/HCl buffer, pH 7.4, and light-micrographs were taken at a magnification of 100× (*a*) and 400× (*b*).

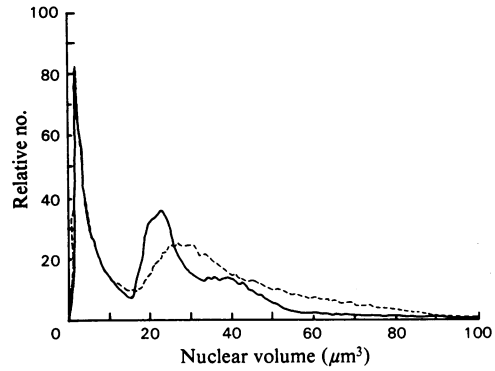

Fig. 3. *Size distribution pattern of ventricular and myocardial nuclei*
Patterns represent typical size distributions obtained from adult rat heart nuclei isolated from ventricles (——) and myocardial cells (– – – –). Ventricular myocardial nuclei were isolated as described in the Materials and Methods section. Nuclei were suspended in an iso-osmotic electrolyte (Isoton) and analysed with a Coulter counter model C-1000 Channelyzer.

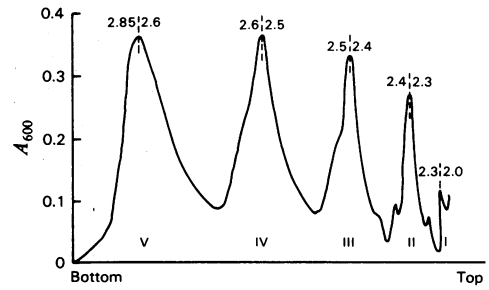

Fig. 4. *Zonal-centrifugation profile of ventricular nuclei*
Highly purified ventricular nuclei, suspended in 2.0 M-sucrose in TM buffer isolated as described in the Materials and Methods section, were centrifuged on a discontinuous sucrose density gradient in a Beckman SW 27.1 rotor (2 g of heart tissue). After centrifugation, the gradient was displaced under suction and passed through a flow cell (1 cm path length) in a Gilford 300-N micro-sample spectrophotometer. Nuclei form a zone at the interfaces of the discontinuous density gradient. Zone (I), nuclei collected at the 2.0 M-/2.3 M-sucrose interface; zone (II), 2.3 M-/2.4 M-sucrose interface; zone (III), 2.4 M-/2.5 M-sucrose interface; zone (IV), 2.5 M-/2.6 M-sucrose interface; zone (V), 2.6 M-/2.85 M-sucrose interface.

contamination was always observed for myocardial nuclei isolated from myocardial cells than for those isolated from total ventricular tissue.

Fig. 3 shows a representative size distribution pattern of both rat ventricular nuclei and myocardial-cell-derived nuclei. Myocardial nuclei appear to give an approximately normal distribution curve with some tailing towards the large-size nuclei (with a mean nuclear volume $\bar{\chi}_1 = 30\mu m^3$ and range of 18–50μm^3). The nuclear distribution pattern from ventricular nuclei tends to consist of two populations of nuclei ranging in volume between 15 and 50μm^3 with population means at $\bar{\chi}_1 = 25\mu m^3$, $\bar{\chi}_2 = 40\mu m^3$ as measured by the Coulter counter.

Fractionation of ventricular nuclei

Nuclei from total heart were fractionated according to their density by using a discontinuous sucrose density gradient. The concentration of sucrose in discrete steps varied from 2.85 M (bottom of the gradient) to 2.0 M (top of the gradient). The light-scattering profile of the gradient (Fig. 4) indicated that five discrete bands of nuclei formed at the interface between each sucrose concentration. The zones obtained are numbered from the top of the gradient to the bottom of the gradient (I, II, III, IV and V). Zone (I) corresponds to the band obtained at the 2.0 M/2.3 M interface, zone (II)

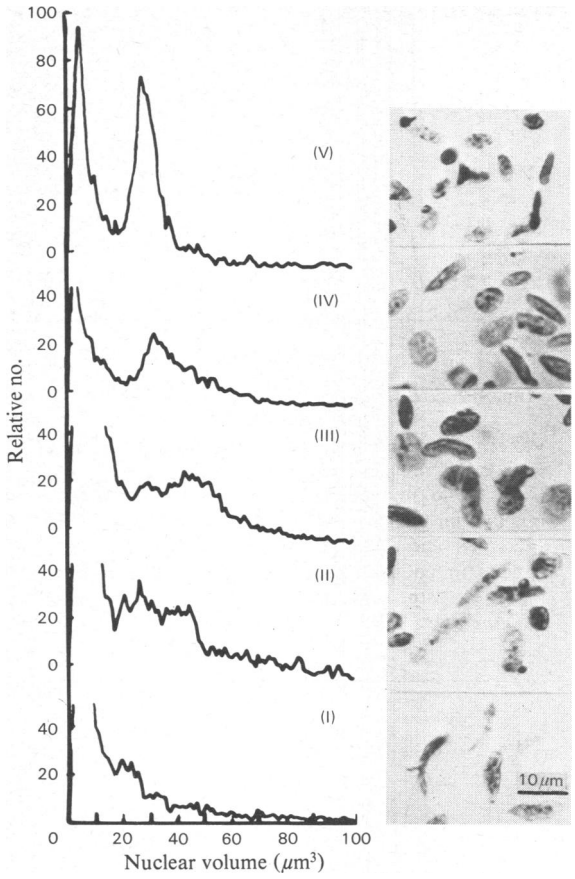

Fig. 5. *Size distribution pattern and morphology of fractionated ventricular nuclei*

The Figure shows specific size distribution for each nuclear subset differing in buoyant density. Micrographs (taken at 400×) are numbered in order of increasing buoyant density. Zone (I) corresponds to the nuclei banding in the light region of the gradient (2.0 M-/2.3 M-sucrose interface), and zone (V) corresponds to nuclei banding in the heavy region of the gradient (2.6 M-/2.85 M-sucrose interface).

2.3 M/2.4 M interface, zone (III) 2.4 M/2.5 M interface, zone (IV) 2.5 M/2.6 M interface and zone (V) 2.6 M/2.85 M interface. The nuclei banding at zone (I) (being retarded by 2.3 M-sucrose) would have a density at 5°C equivalent to 1.30, zone (II) 1.31, zone (III) 1.32, zone (IV) 1.34 and zone (V) 1.39.

Fig. 5 illustrates the diversity of the size of the nuclei separated according to their buoyant density by this procedure. Large nuclei occur in the lighter regions of the gradient, and the smaller nuclei are recovered from the denser regions. From the size distribution pattern for each fraction of the gradient it can be seen that nuclear subset (V) seems to be enriched in small nuclei and follows a normal distribution with mean of 25 μm^3 and range 15–35 μm^3. In general nuclear subsets (II), (III) and (IV) contain larger nuclei. Nuclear subset (IV) follows a normal distribution that is skewed to larger nuclei with a mean approx. 30 μm^3 and a range of 25–50 μm^3. Nuclear subset (III) consists of a bimodal distribution with means 30 μm^3 and 40 μm^3 and a range of 25–50 μm^3. Nuclear subset (II) also consists of a bimodal distribution with means at 28 μm^3 and 40 μm^3 and a range of 20–50 μm^3, and nuclear subset (I) contains a wide range of nuclear sizes. Size distribution may not be a suitable criterion for the distinction of various classes of nuclei owing to large overall range of nuclear sizes. When these size distribution patterns were compared with that obtained from myocardial cells (Fig. 2), nuclear subsets (II), (III) and (IV) resembled the pattern obtained from myocardial nuclei.

Morphologically, nuclei obtained from nuclear subset (I) appeared to be mainly oblong or oval-shaped with one or two distinct centrally located nucleoli and dispersed very pale chromatin. Nuclear subsets (II) and (III) consisted of nuclei very similar to the type seen in nuclear subset (I) but smaller in size. Nuclear subset (IV) consisted of nuclei that appeared to be predominantly irregular, oval or round in shape, containing centrally located nucleoli. Nuclear subset (V) consisted of a population of nuclei that were mainly smaller in size than those of nuclear subsets (I), (II), (III) and (IV), and appeared to be either round or oval in shape, containing several peripheral nucleoli within a very dark-staining chromatin.

The recovery of nuclei in all five zones accounted for approx. 77% of nuclei applied to the gradient. The remaining 23% of the nuclei were found to be scattered in between each zone. The number of nuclei in each zone (Table 2) indicates that bands (II), (III) and (IV) comprise approx. 79% of the nuclei recovered from the gradient. As shown in Table 3, the homogeneity of each nuclear subset based on the excision and re-centrifugation of nuclear subset (IV) indicated that nuclear subset (IV) was about 70% homogeneous.

The DNA content in each nuclear subset remained constant (10 pg/nucleus), whereas RNA and protein contents decreased with increasing concentration of sucrose. The RNA content varied from 3.8 pg/nucleus at the top of the gradient (I) to 1.5 pg/nucleus at the bottom of the gradient (V). The protein/DNA ratio of the different nuclear zones ranged from 3.55 in zone (I) (top of the gradient) to 2.25 in zone (V) (bottom of the gradient). This difference in protein/DNA ratio was also reflected in the size and buoyant density of the nuclei.

Table 2. *Composition of heart nuclei differing in buoyant density*
Experimental details are given in the text.

Fraction	Density of nuclear subset (g/ml at 5°C)	Concn. of sucrose (M)	Nuclear count	DNA content (pg/nucleus)	RNA content (pg/nucleus)	Protein content (pg/nucleus)	Protein/DNA ratio
(I)	1.2996	2.3	2.2×10^5	10.2	3.8	36.3	3.55
(II)	1.3116	2.4	1.9×10^5	10.5	3.0	34.2	3.25
(III)	1.3237	2.5	8.0×10^5	10.0	1.7	26.9	2.68
(IV)	1.3360	2.6	13.0×10^5	10.3	1.6	23.7	2.30
(V)	1.3933	2.85	4.5×10^5	9.4	1.5	21.3	2.25

Table 3. *Re-centrifugation of nuclear subset (IV)*
Experimental details are given in the text. Nuclear subset (IV) containing the greatest number of nuclei was excised from the discontinuous gradient and resuspended to 2.0M-sucrose and then re-centrifuged through a second discontinuous gradient. The degree of homogeneity of nuclear subset (IV) is expressed as percentage of the total number of nuclei in band (IV).

Distribution of nuclei		% of total number of nuclei in band (IV)
(I)	3.6×10^4	0.4
(II)	4.8×10^4	0.5
(III)	1.1×10^6	11.4
(IV)	6.6×10^6	67.3
(V)	2.0×10^6	20.4

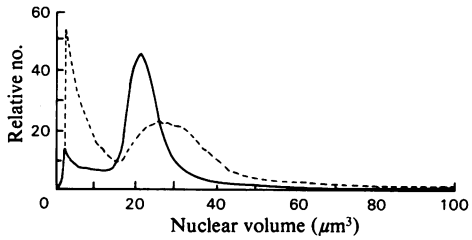

Fig. 6. *Size distribution pattern of myocardial-cell-enriched and non-myocardial nuclear subsets*
Patterns represent typical size distribution obtained from adult rat ventricular nuclei fractionated as described in the Materials and Methods section. Fraction A (----), nuclei banding at the 2.3M-/2.7M-sucrose interface, corresponding to the myocardial-cell-enriched nuclear subsets (II), (III) and (IV). Fraction B (——), nuclei pelleting through 2.7M-sucrose, corresponding to nuclei belonging to the non-myocardial nuclear subset (V).

By using the one-step procedure as described in the Materials and Methods section we were able to fractionate ventricular nuclei into two nuclear subsets, one banding at the 2.3M-/2.7M-sucrose interface and the other subset pelleting through the 2.7M-sucrose. As shown in Fig. 6, fraction A appears to have a size distribution profile very similar to those of subsets (II), (III) and (IV) and superimposable on that obtained from myocardial nuclei (Fig. 3), whereas fraction B had a size distribution profile identical with that of nuclear subset (V). The chemical compositions of fraction A and fraction B as shown in Table 4 indicated that fraction A was comparable with the average of nuclear subsets (II), (III) and (IV) (Table 2) and that fraction B was similar to nuclear subset (V). The chemical composition of fraction A is also very similar to that of myocardial nuclei (Table 4). By using this one-step discontinuous gradient we were able to separate the myocardial-enriched nuclear subsets (II, III and IV) from the non-myocardial-cell fraction (V).

Table 4. *Chemical compositions of myocardial-cell-enriched and non-myocardial nuclear subsets*
Crude ventricular nuclei were fractionated as described in the Materials and Methods section. Fraction-A nuclei banding at 2.3M-/2.7M-sucrose interface correspond to the myocardial-cell-enriched nuclear subsets (II, III and IV). Fraction-B nuclei pelleting through the 2.7M-sucrose correspond to nuclei belonging to the non-myocardial subset (V). Results are expressed as means ± S.D. for three separate experiments. The chemical compositions of nuclei isolated from purified myocardial cells are also shown.

Fractionated nuclei	Protein/DNA ratio	RNA/DNA ratio
A	3.28 ± 0.84 (3)	0.18 ± 0.02 (3)
B	1.95 ± 0.22 (3)	0.13 ± 0.01 (3)
Myocardial nuclei	3.09	0.18

Fractionation of myocardial-cell nuclei

Myocardial nuclei were isolated and fractionated on a small discontinuous sucrose density gradient (5ml). The resulting separation is shown in Fig. 7(*b*). Two prominent bands formed at zones (II) and (III).

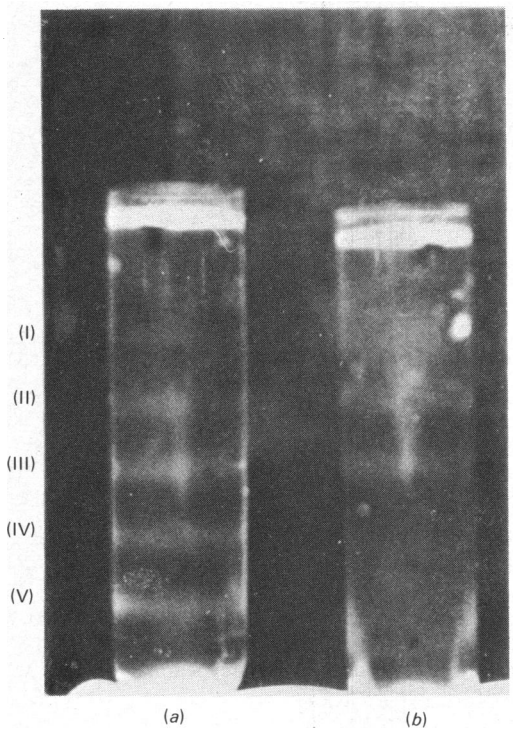

Fig. 7. *Fractionation of myocardial nuclei by using discontinuous-sucrose-density-gradient centrifugation*
Myocardial nuclei isolated as described in the Materials and Methods section were fractionated by using discontinuous-sucrose-density-gradient centrifugation. (*a*) Ventricular nuclei isolated from heart tissue exposed to collagenase and hyaluronidase. (*b*) Purified myocardial nuclei. Roman numerals correspond to the zone of nuclei banding at the sucrose interfaces (I), 2.0 M/2.3 M; (II), 2.3 M/2.4 M; (III), 2.4 M/2.5 M; (IV), 2.5 M/2.6 M; (V), 2.6 M/2.85 M.

As a control, nuclei isolated from heart tissue treated with dissociating enzymes without any cellular separation resulted in five discrete zones (Fig. 7*a*; zones I, II, III, IV and V).

Fractionation of nuclear non-histone proteins by SDS/polyacrylamide-gel electrophoresis

Nuclei recovered from the different densities in the gradient were extracted with SDS/phenol by the method of LeStourgeon & Beyer (1977). This method is able to extract total nuclear proteins in high yield. By using SDS/polyacrylamide-gel electrophoresis (15% acrylamide) we were able to separate total nuclear proteins into two groups, histones (H1, H2a, H2b, H3 and H4), comprising at least 50% of the total nuclear proteins, and the non-histone nuclear proteins, which make up the other half.

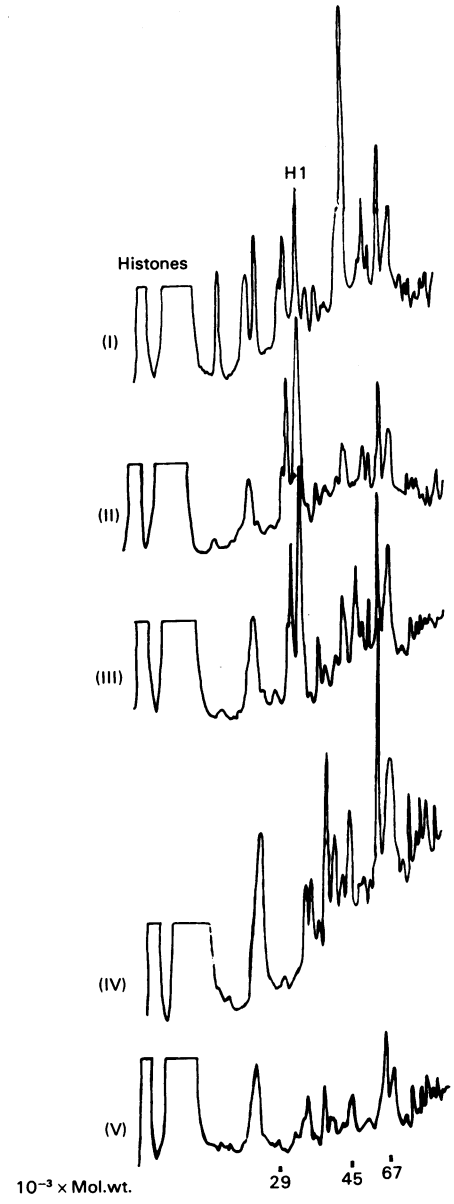

Fig. 8. *Electrophoretic analysis of ventricular nuclear proteins fractionated by using discontinuous-sucrose-density-gradient centrifugation*
Nuclear proteins from each of the ventricular nuclear subsets [nuclei banding at the sucrose interfaces: zone (I), 2.0 M/2.3 M; zone (II), 2.3 M/2.4 M; zone (III), 2.4 M/2.5 M; zone (IV), 2.5 M/2.6 M; zone (V), 2.6 M/2.85 M] were extracted with SDS/phenol as described in the Materials and Methods section. The resulting nuclear proteins were separated by SDS/polyacrylamide-gel electrophoresis (15% acrylamide) as described in the text. The identification of histones was done with standard calf thymus histone, as shown previously (Chan & Liew, 1977).

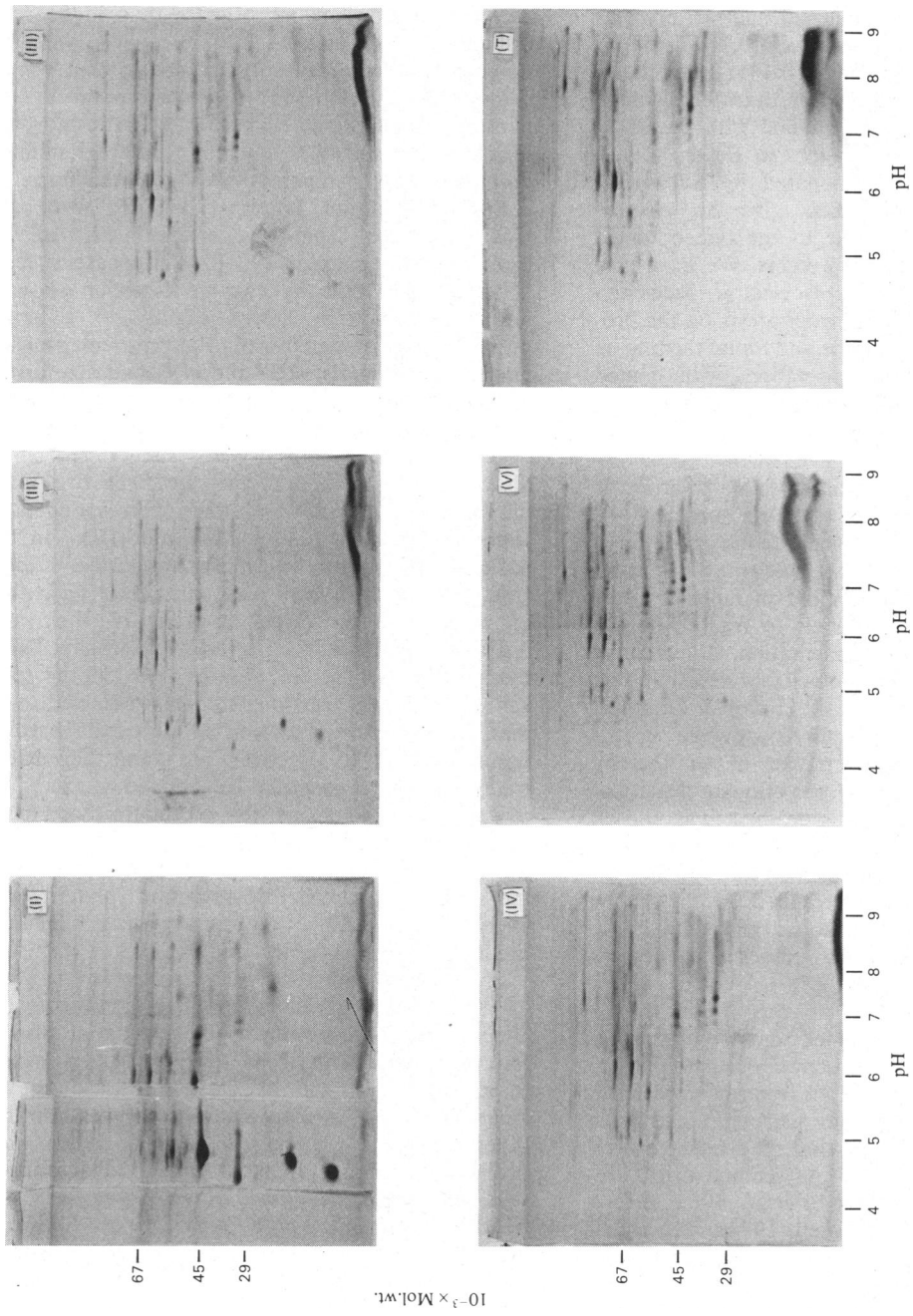

Fig. 9. *Two-dimensional isoelectric focusing–SDS/polyacrylamide-gel-electrophoretic analysis of ventricular non-histone nuclear proteins*
Ventricular nuclei obtained from 50 adult rats (yielding 25 g of ventricular tissue) were fractionated by using sucrose-density-gradient centrifugation as described in the Materials and Methods section. Nuclear proteins from each of the ventricular nuclear subsets (I–V) were extracted with TEM buffer/phenol as described in the Materials and Methods section. The resulting nuclear proteins were fractionated in the first dimension by isoelectric focusing and in the second dimension by SDS/polyacrylamide-gel electrophoresis (10% acrylamide) as described previously (Liew & Sole, 1978*a*). Then 200 μg of nuclear protein was applied to each gel. Roman numerals correspond to the nuclear proteins extracted from the nuclear subsets banding at the sucrose interfaces: (I), 2.0 M/2.3 M; (II), 2.3 M/2.4 M; (III), 2.4 M/2.5 M; (IV), 2.5 M/2.6 M; (V), 2.6 M/2.85 M. (T), Total ventricular nuclear proteins.

As can be seen in Fig. 8, the electrophoretic pattern of non-histone nuclear proteins from the different nuclear subsets (I–V) are very similar, whereas the amount of protein in a particular band varied with the nuclear subset.

Recently we have solubilized nuclear proteins directly in TEM buffer/phenol [0.1 M-Tris/HCl buffer (pH 8.4)/10 mM-EDTA/0.14 M-2-mercaptoethanol] with a recovery greater than 90%, which was comparable with that obtained with the SDS/phenol procedure (LeStourgeon & Beyer, 1977). These proteins, when fractionated by SDS/polyacrylamide-gel electrophoresis, give an electrophoretic pattern very similar to the one obtained with SDS/phenol-extracted proteins. We have also performed two-dimensional isoelectric focusing–SDS/polyacrylamide-gel electrophoresis on the proteins of the five nuclear subsets and found them to be similar in electrophoretic separation, with limited qualitative differences, as shown in Fig. 9.

Discussion

We have been able to fractionate total ventricular nuclei from adult rats into five nuclear subsets (I, II, III, IV and V) by using isopycnic centrifugation in a discontinuous sucrose density gradient. Since adult nuclei are predominantly diploid in number (Zak, 1974) and it would be difficult to fractionate the isolated nuclei by rate sedimentation (Johnston *et al.*, 1968*a*) (i.e. the migration velocity depending on the size and shape of the nuclei; Hinton & Dobrota, 1976), we decided to employ isopycnic centrifugation to fractionate ventricular nuclei, thereby exploiting small differences in the buoyant density of these nuclei. A discontinuous gradient was chosen over a continuous gradient ranging from 2.85 M- to 2.3 M-sucrose, since nuclear fractionations attempted on a continuous gradient resulted in the nuclei spreading out into large zones throughout the gradient. In agreement with other investigators (Løvtrup-Rein & McEwen, 1966; Austoker *et al.*, 1972; Cline *et al.*, 1973), it was found that a discontinuous gradient resulted in a better resolution of separation than did a continuous gradient.

Microscopic examination of ventricular cardiac nuclei indicated that there was a strong resemblance to the condition *in situ* and that they retained their characteristic morphology. As demonstrated by Muir (1965) and other (Rhodn *et al.*, 1961; Brown *et al.*, 1968; McNutt & Fawcett, 1974), myocardial cells of adult rat tended to be cylindrical, containing usually two oblong-shaped nuclei that were situated near the centre of the cell and oriented along its long axis. Myocardial nuclei appeared to be oblong in shape, containing one or two prominent nucleoli against a pale-staining interdispersed chromatin. It was reported by Bloom & Cancilla (1969) that the shape of the myocardial nucleus was dependent on whether the myocardial cell was in a relaxed or a contracted state.

On the other hand, it appears that non-myocardial nuclei, which are mainly endothelial cell nuclei, were smaller in size and had a darker-staining chromatin, and were more dense than myocardial cell nuclei (Meerson *et al.*, 1968; Brown *et al.*, 1968; Fawcett & McNutt, 1969). It appears that ventricular nuclei subset (V) may correspond to the non-myocardial nuclei, since they seem morphologically to be smaller and have a darker-staining chromatin when compared with myocardial nuclei.

Size distribution patterns obtained with the Coulter counter from myocardial nuclei isolated from a pure population (90–98% myocardial cells) of myocardial cells by enzyme digestion show a normal distribution skewed slightly to a larger nuclei. When compared with total ventricular nuclei, nuclear subsets (II), (III) and (IV) had size distribution patterns very similar to that obtained for myocardial nuclei. It was also demonstrated that when myocardial nuclei were fractionated on a discontinuous sucrose density gradient two main bands were detected, and these corresponded to ventricular nuclear subsets (II) and (III). On the basis of these studies we believe that nuclear subsets (II), (III) and (IV) from total ventricular nuclei are of myocardial-cell origin in view of their size distribution pattern and their buoyant density. These fractions accounted for approx. 79% of the total ventricular nuclei fractionated. This indicated to us that, by using the Polytron to dissociate cardiac tissue, we may be selectively enriching the nuclear fraction with myocardial-cell-derived nuclei. We further developed a one-step procedure to separate nuclear subsets (II) + (III) + (IV) (fraction A) from nuclear subset (V) (fraction B). We have shown, on the basis of the size distribution pattern and chemical composition, that fraction A was similar to myocardial nuclei (Table 4 and Fig. 6).

It has been known for years (see review by Zak, 1973) that myocardial-cell-proliferating activity declines rapidly during development, so that from a few weeks after birth normal growth of the ventricle to its adult size can be attributed solely to the enlargement of myocardial cells, whereas on the other hand the non-myocardial cells retain their proliferating capacity. On the basis of this information, our preliminary studies on 50-day-old rats indicate that the incorporation of [*Me*-^{3}H]thymidine into DNA was 4 times greater in fraction B than in the myocardial-enriched fraction A. This supports our claim that nuclear subsets (II), (III) and (IV) (i.e. fraction A) are myocardial-enriched whereas nuclear subset (V) (i.e. fraction B) consists mainly of non-myocardial nuclei.

Electrophoretic analyses of proteins obtained from each nuclear subset indicate that these frac-

tions are similar, with limited qualitative differences. This may be due to the fact that most of the non-histone nuclear proteins in each of the nuclear subsets (II), (III) and (IV) are myocardial cell in origin and there is an overlap of each nuclear subset (see Table 3). Therefore our present analysis only allows us to identify most of the quantitative differences in these nuclear subsets.

In summary, by using discontinuous-sucrose-density-gradient centrifugation we were able to fractionate total ventricular nuclei into distinct nuclear subsets and identify myocardial-cell-derived nuclear subsets from non-myocardial nuclear subsets. This method eliminates exposing cardiac tissue to proteolytic enzymes and the laborious and lengthy procedure used to isolate myocardial cells before the isolation of myocardial nuclei. It should be noted that the success of the enzyme procedure is dependent on the batch of collagenase used. Therefore we offer an alternative method, which is relatively rapid and simple, for isolation of an enriched fraction of myocardial nuclei from cardiac tissue.

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