ISOLATION OF SKELETAL MUSCLE NUCLEI

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ABSTRACT

A method employing aqueous media for isolation of nuclei from rat skeletal muscle is described. The technique involves (*a*) mincing and then homogenizing in a 0.32 M sucrose-salt solution with a Potter-Elvehjem type homogenizer using a Delrin (an acetal resin) pestle and a carefully controlled, relatively large pestle-to-glass clearance, (*b*) filtering through fiberglass and stainless steel screens of predetermined mesh size to remove myofibrils and connective tissue, and (*c*) centrifuging in a 2.15 M sucrose-salt solution containing 0.7 mM ATP. Electron and phase-contrast microscopic observations show that the nuclei are intact, unencumbered by cytoplasmic tags, and possess well preserved distinct nucleoli, nucleoplasm, and nuclear membranes. Cytoplasmic contamination is minimal and mainly mitochondrial. Chemical assays of the nuclear fraction show that the DNA/protein and RNA/DNA ratios are comparable to those obtained in other tissues. These ratios, as well as the low specific activity obtained for cytochrome c oxidase and the virtual absence of myofibrillar ATPase, indicate a high degree of purity with minimal mitochondrial and myofibrillar contamination. The steps comprising the technique and the reasons for their selection are discussed.

INTRODUCTION

The isolation, from liver (1–8), thymus (6, 9), kidney (6, 10–12), brain (13, 14) and spleen (15, 16), of nuclei of varying degrees of homogeneity and state of preservation has been accomplished. However, we have been unable to find any reports of the successful isolation of nuclei from striated muscle. The abundance and infiltrative nature of the surrounding connective tissue, the peripheral location of the nuclei that could lead to their damage or ultimate destruction during homogenization, and the relatively large amount of structurally organized, firm cytoplasmic material (*e.g.*, the myofibrils) are characteristics which compound the difficulties in isolating nuclei from striated muscle.

The following report describes a method for isolating nuclei from rat striated muscle. The techniques employed involve homogenization at controlled and relatively large pestle-to-glass clearance, filtration through screens of predetermined mesh size to remove larger structures and connective tissue, and centrifugation in high-density media to float all remaining structures except nuclei. The nuclei obtained by these procedures were studied and evaluated as to morphology, preservation, and homogeneity by use of phase-contrast microscopy, electron microscopy, chemical and enzymatic assay procedures.

MATERIALS AND METHODS

Solutions

1. 0.32 M SUCROSE-SALT SOLUTION: 0.32 M sucrose, 1 mM $MgCl_2$, 0.2 mM K_2HPO_4, and 0.6 mM KH_2PO_4, pH 6.7–6.8.
2. 2.15 M SUCROSE-SALT SOLUTION: 2.15 M sucrose, 1.0 mM $MgCl_2$, and 3.5 mM K_2HPO_4, pH 6.7–6.8.

Adenosinetriphosphatase Determination

Myofibrillar ATPase was assayed in a medium containing 0.05 M Tris(hydroxymethyl)aminomethane, pH 7.6, 0.025 M KCl, 5 mM ATP (Na^+). After adding the tissue preparation, the mixture was incubated at 28°C, and the reaction stopped by the addition of a solution of cold trichloracetic acid (TCA). Cold TCA was added until its concentration was 5 per cent, the resulting precipitate was removed by centrifugation, and the supernate was then assayed for inorganic phosphate (17). The results were expressed in terms of units (one unit was equal to 1 μg P_i liberated per hour). Homogenates were incubated for 5 minutes and nuclear preparations for 30 minutes.

Ferrocytochrome C Oxidase Determination

This enzyme was assayed by the spectrophotometric method of Cooperstein and Lazarow (19). The chemically reduced cytochrome c was freed of excess reducing agent and other related products with Dowex 2 (20). A unit of ferrocytochrome c oxidase is defined as follows: if $\triangle$ log [ferrocytochrome c]/minute = 1, then one unit of activity is present (13).

DNA and RNA Determination

DNA and RNA were isolated from tissue fractions by hot trichloracetic acid extraction of material previously washed in cold trichloracetic acid and ethyl alcohol (18). The extract was then assayed for DNA by the colorimetric method of Dische (21) and for RNA by the method of Mejbaum (22), employing a correction factor for the amount of DNA present. Calf thymus DNA and yeast RNA (Sigma Chemical Company, St. Louis, Missouri) were used as standards.

Protein was determined by the method of Lowry *et al.* (23) using serum as standard. Inorganic and total phosphorus were determined by the method of Fiske and Subbarow (17). ATP (Na) and cytochrome c (type III) were purchased from the Sigma Chemical Company. All other chemicals employed were reagent grade.

Electron Microscopy

A 2 per cent solution of osmium tetroxide at pH 7.4 in Veronal buffer containing 27 mM K^+ was added to an equal volume of resuspended nuclear sediment. After an initial fixation period of 5 to 15 minutes, the mixture was centrifuged at 0 to 4°C at 1200 *g* for 10 to 15 minutes. The resulting pellet was rapidly dehydrated with increasing concentrations of ethanol and embedded in Araldite as described by Glauert and Glauert (24).

Sections were cut with a Porter-Blum ultramicrotome, placed on collodion carbon-shadowed grids, stained with lead hydroxide, and examined with the Akashi TRS 80 electron microscope.

Procedure for Isolation of Rat Skeletal Muscle Nuclei

Sprague-Dawley female rats weighing 225 to 250 gm are fasted for 24 hours, decapitated, and the intact femoral musculature is quickly removed and pretreated in one of two ways: (*a*) *Series A* No pretreatment. (*b*) *Series B* The intact musculature is incubated for 30 minutes in Krebs-Ringer bicarbonate medium containing 1.5 mg/ml glucose and 4 μg/ml I^{131}-insulin and gassed with a 95/5 per cent O_2/CO_2 mixture. Approximately 1 gm of tissue is used for each ml of incubation medium. The muscle is then exhaustively washed in isotonic saline, bathed in 10^{-3} M *N*-ethylmaleimide for 5 minutes, and then rewashed in a Krebs-Ringer bicarbonate solution and finally in isotonic saline. Results of the insulin distribution in striated muscle will be reported in a subsequent paper (25). After rinsing procedures, the tissue is fractionated for nuclei in the following manner.

All operations are carried out at 2°C. Muscle is dissected from the bone and the pieces passed through a Latapie mincer (Arthur H. Thomas Co., Philadelphia, Pennsylvania) containing 1 mm diameter perforations in the metal disc. The mince is weighed then homogenized in a 0.32 M sucrose-salt solution, pH 6.8, (2 ml of sucrose solution per gm of mince), with 5 passes of a Potter-Elvehjem homogenizer made of pyrex glass with an inside diameter of 1 inch and a pestle velocity of 600 RPM. Larger yields of nuclei are obtained by employing a pestle made of Delrin (an acetal resin produced by the Dupont Co., Wilmington, Delaware) which has a slightly higher coefficient of friction but a lower thermal coefficient of linear expansion and greater durability than Teflon. The clearance of the pestle is critical; the greatest yields of nuclei are obtained when the pestle clearance is 0.02 to 0.025 inches at 2°C. The homogenate is filtered through fiberglass (pore size, 1.0 mm) netting, and the material which does not pass the filter is rehomogenized with 2 passes of the same

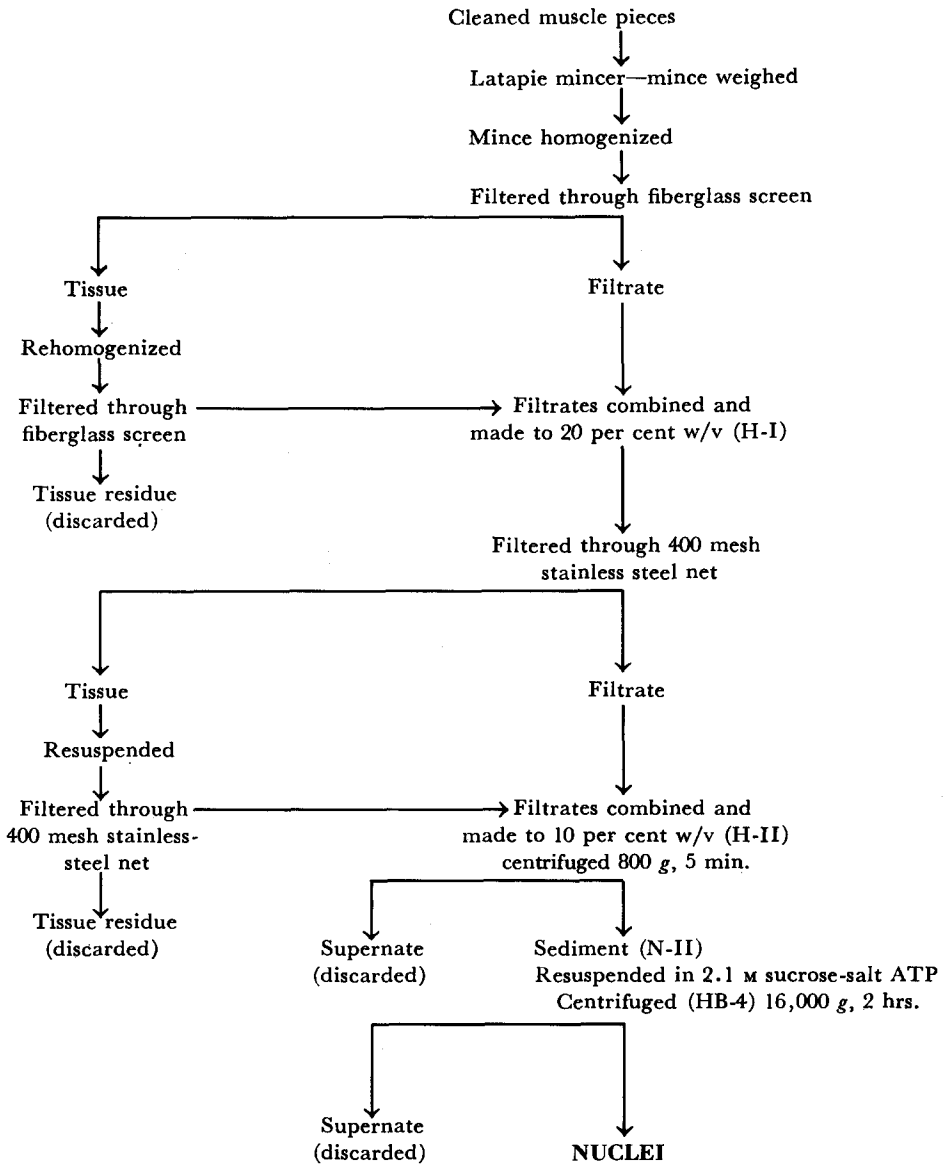


FIGURE 1 Outline of the procedure for preparation of striated muscle nuclei.

homogenizer but with ½ the amount of 0.32 M sucrose-salt solution. Following filtration the two filtrates are combined, and the homogenate made to 20 per cent (w/v) with sucrose solution (H-I in Fig. 1). The material which still does not pass the screen is discarded.

The 20 per cent homogenate is then passed through a stainless steel wire screen (400 mesh, pore size, 0.037 mm). Stirring facilitates the filtration. The material remaining on the screen is resuspended in a small volume of 0.32 M sucrose-salt solution and refiltered. The combined filtrates are made to a 10 per cent (w/v) homogenate (referred to as H-II in Fig. 1) and centrifuged in a Servall refrigerated centrifuge (2.5 gm of original mince per 50 ml tube) at 800 *g* for 5 minutes. The sediment (a crude nuclear fraction referred to as N-II in Fig. 1) is washed once in a 0.32 M sucrose-salt solution containing 0.7 mM ATP. The sediment in each tube is well drained and resuspended in 40 ml of a 2.15 M sucrose-salt solution containing 0.7 mM ATP by gentle homogenization with a loose-fitting pestle. This suspension is now centrifuged in the swinging bucket head (HB-4) of the Servall refrigerated centrifuge for 2 hours, 16,000

TABLE I

*DNA and Protein Relationships during Fractionation of Rat Skeletal Muscle for Nuclei**

Fraction‡	Protein§		DNA§		DNA/protein × 10³	
	Series A	Series B	Series A	Series B	Series A	Series B
	mg		*mg*			
H-I	1414 ± 105	1386 ± 89	3.02 ± 0.21	—	2.21 ± 0.62	—
H-II	484 ± 25	—	1.45 ± 0.06	—	3.04 ± 0.45	—
N-II	90 ± 12	121 ± 26	1.24 ± 0.07	2.73 ± 0.28	16.2 ± 2.5	25.5 ± 3.5
Nuclei	0.63 ± 0.18	1.59 ± 0.20	0.0857 ± 0.0167	0.447 ± 0.039	165 ± 22	290 ± 20

Series A comprise 8 experiments; series B, 4 experiments. For other differences, see text.

* Results given as mean ± standard error.

‡ For fraction and abbreviation significance, see Fig. 1.

§ Amounts given represent those obtained from 10 gm of wet tissue.

g, at 2°C. At the end of the centrifugation, the head is brought to rest without braking. Mitochondria, myofibrils, and some of the nuclei are found to be at the top, while the pure nuclei are found at the bottom. The top fluid layers are removed by suction, the lower ones by inversion, and the walls of the tube wiped. The nuclei at the bottom are taken up in a convenient volume of 0.32 M sucrose-salt solution containing 0.7 mM ATP. A summary of the procedure is illustrated in Fig. 1.

RESULTS

ISOLATION OF NUCLEI: The recovery of DNA, protein, and the DNA/protein ratios of 12 preparations of skeletal muscle nuclei are shown in Table I. Little breakage of nuclei occurs with the homogenization procedure used, for relatively small losses of DNA occur until the centrifugation in high-density sucrose. Omission of the fine screen filtration causes the high-density centrifugation to be ineffective because of the large aggregates of myofibrillar material sedimented with the nuclei. The use of homogenizers of smaller clearance than those recommended results in microscopically visible and chemically detectable nuclear damage. In these circumstances, solubilization of the nuclear DNA may occur, resulting in poor recoveries of DNA. Many of the visual changes appear only after exposure to high-density sucrose and may be the result of damage to the nuclear membrane with subsequent loss of intranuclear macromolecular constituents (Fig. 6).

PURITY OF THE NUCLEI: The purity and integrity of the nuclei was studied by (*a*) phase-contrast microscopy, (*b*) electron microscopy, (*c*) chemical methods, and (*d*) enzyme assays:

(*a*) Phase-contrast microscopic examination of the nuclei show them to be intact, elongated. and frequently multinucleolated (Figs. 2 and 3), Mitochondria may be seen in the final nuclear fraction, especially those of series A.

(*b*) Electron microscopic examination (Figs. 4 to 6) reveals that the nuclear membranes are generally intact and double-layered, and that the accompanying cytoplasmic contamination appears to be minimal and mainly in the form of mitochondria. The chromatin material appears to be less dense than that found in the nucleus of an intact cell. Bleb-like protrusions of the nuclear membrane as observed in Figs. 4 and 6 have been described in isolated liver nuclei prepared in certain concentrations of sucrose and Mg^{++} or Ca^{++} (26). In the latter case, however, they were large enough to be observed in the light microscope.

(*c*) DNA/protein and RNA/DNA ratios indicate a high level of purity of the nuclei (Tables I and II). The DNA/protein ratio of the nuclear fraction is 100 times that of the homogenate (Table I, series B). This striking difference is simply a reflection of the relative amount of nuclear and cytoplasmic substance characteristic of striated muscle (Table IV). DNA/protein and RNA/DNA ratios of skeletal muscle nuclei do not appear to differ significantly from those observed in rat liver nuclei, guinea pig liver nuclei, or thymus nuclei isolated in sucrose media (for references, see Table IV).

(*d*) The specific activity of cytochrome c oxidase and myofibrillar ATPase present in the nuclear fraction is employed as an assay of nuclear purity. The specific activity of the former enzyme is used to assess the degree of mitochondrial con-

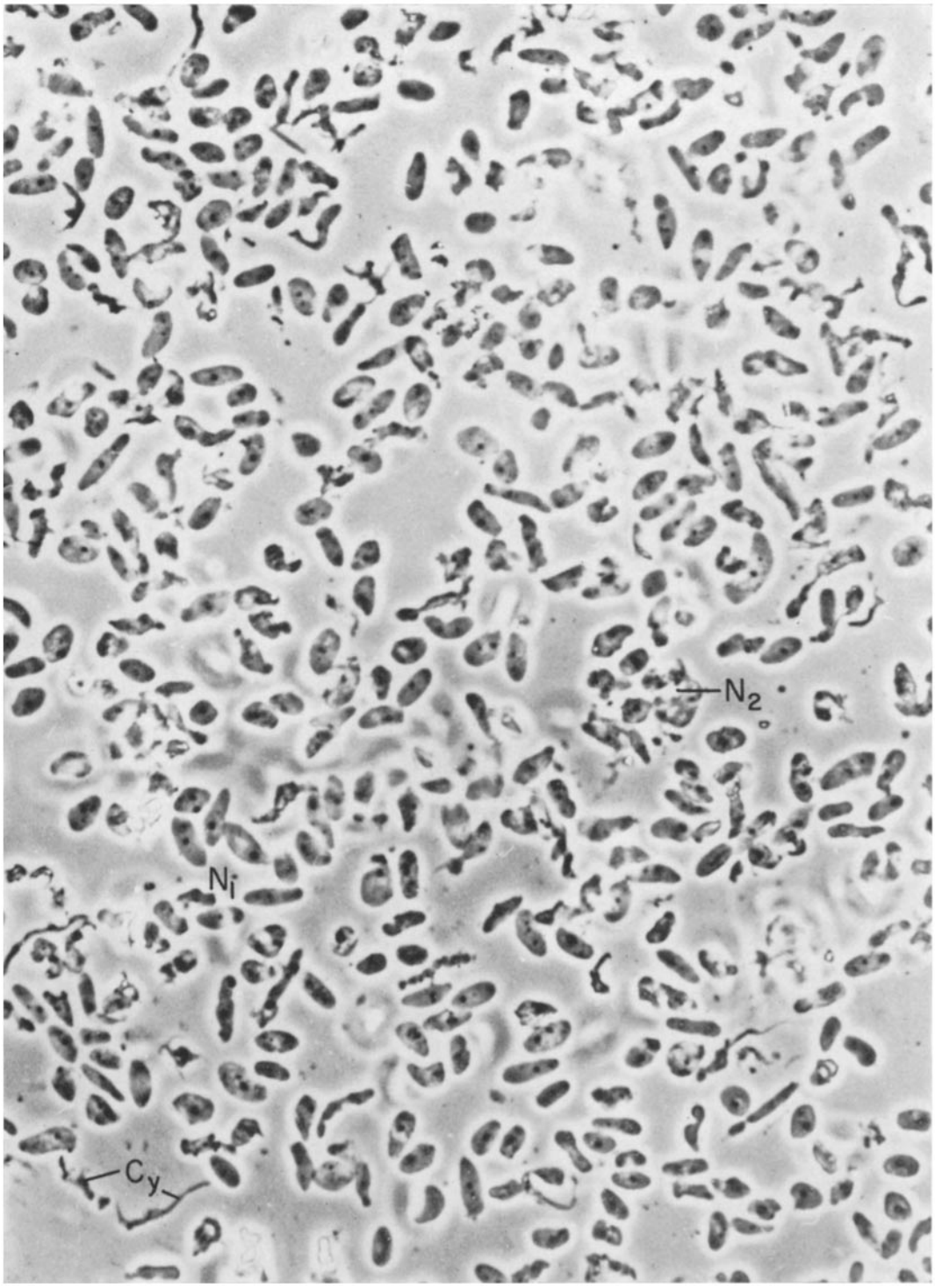


FIGURE 2 Field of isolated nuclei resuspended from the nuclear pellet. The preparation consists of intact (N_1) and partially damaged nuclei (N_2) isolated from striated muscle by the described technique. Broken nuclear components and some cytoplasmic (Cy) contamination in the form of myofibrils may be observed. Phase-contrast micrograph, × 400.

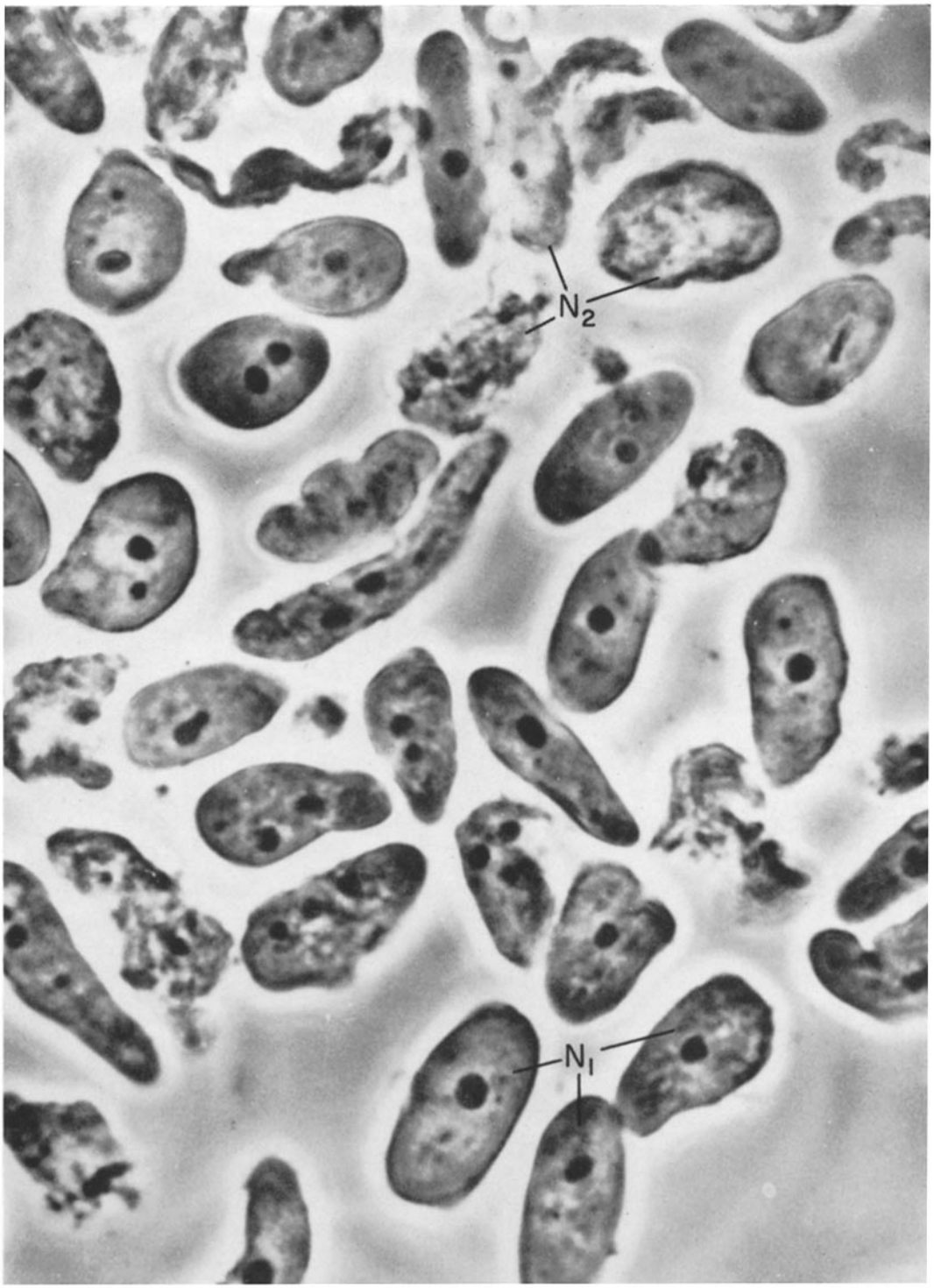


FIGURE 3 Field of intact nuclei (N_1) and partially damaged nuclei (N_2) isolated from striated muscle by the described technique. The majority of the nuclei appear to be intact, elongated, and frequently multinucleolated. Phase-contrast micrograph, × 2000.

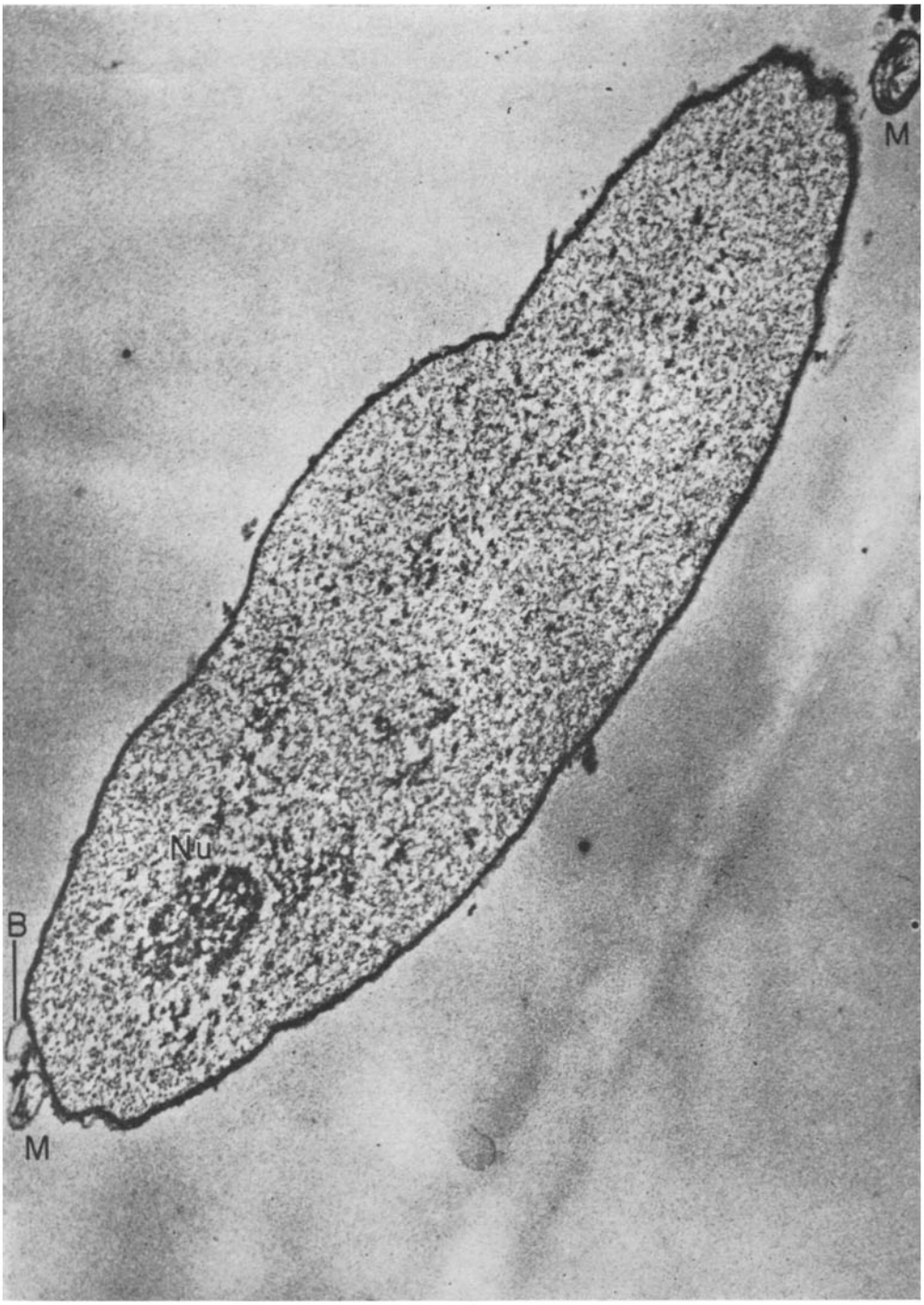
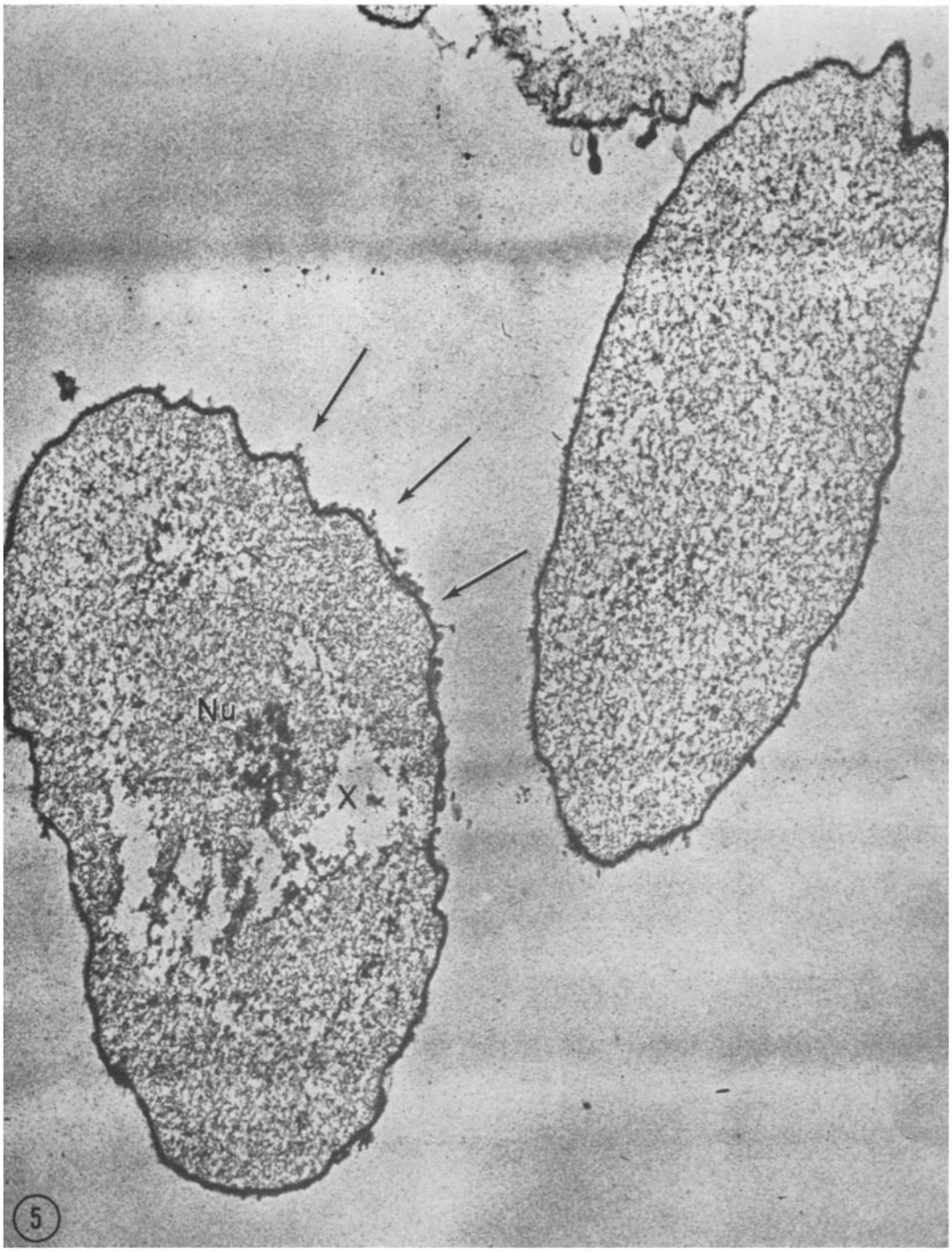


FIGURE 4 Electronmicrograph of an isolated nucleus obtained from striated muscle. The nuclear membrane appears to be intact. A nuclear bleb (*B*) may be observed at one pole. The nucleolus (*Nu*) is clearly visible. Mitochondria (*M*) may be seen at each pole. Fixed in osmium tetroxide and embedded in Araldite. × 12,000.



FIGURES 5 and 6 Limited field in a nuclear fraction prepared by the described procedure. Nuclear membranes appear to be intact in the nuclei of Fig. 5 but are undergoing disintegration (*D*) in Fig. 6. A nucleolus (*Nu*) is visible in Fig. 5. A nuclear membrane bleb (*B*), cytoplasmic contamination (*Cy*), and a mitochondrion (*M*) may be observed in Fig. 6. The nucleoplasm in both figures possesses areas which appear devoid of nucleoplasm (*X*). Arrows in Fig. 5 may indicate ribosomes attached to the nuclear membrane (see reference 1). Fixed in osmium tetroxide and embedded in Araldite. Fig. 5, × 10,000; Fig. 6, × 12,000.

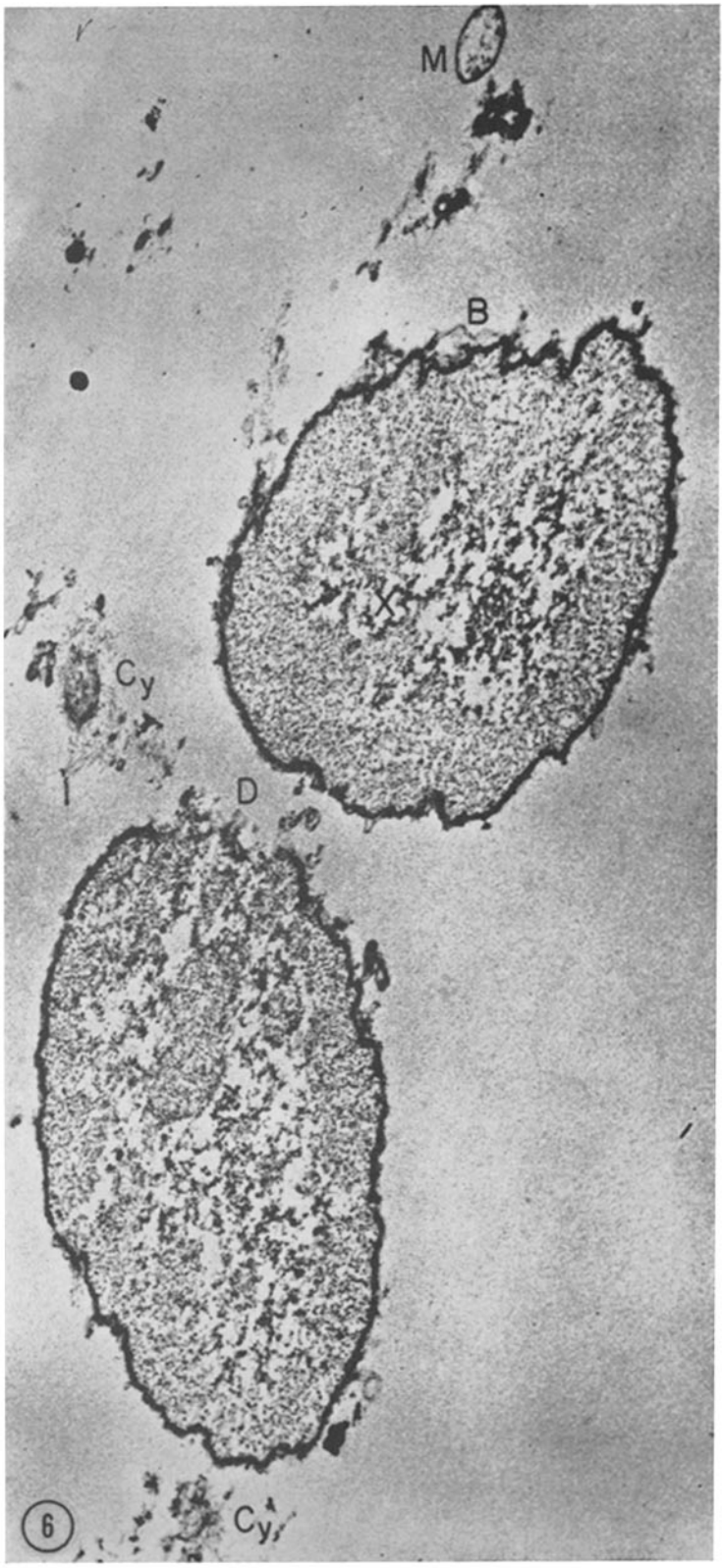

M
B
Cy
D
Cy
6

TABLE II

RNA Content of Rat Skeletal Muscle Fractions and Nuclei

Fraction*	RNA/protein		RNA/DNA	
	Series A‡	Series B‡	Series A‡	Series B‡
H-I	0.00380 ± 0.00041	—	1.73 ± 0.33	—
N-II	0.00738 ± 0.00114	0.0107 ± 0.002	0.520 ± 0.235	0.435 ± 0.044
Nuclei	0.0638 ± 0.0065	0.0290 ± 0.005	0.296 ± 0.061	0.101 ± 0.019

* Fractions are those shown in Fig. 1.

‡ For explanation of series A and series B, please refer to text.

TABLE III

Enzymatic Assay of Rat Skeletal Muscle Homogenates and Nuclei

Fraction*	Cytochrome c oxidase‡		ATPase‡	
	units/mg protein	*units/μg DNA*	*units/mg protein*	*units/μg DNA*
Homogenate (H-I)	13.4 ± 2.1	6.2 ± 1.3	289 (147–366)§	125 (43–191)§
Nuclei	0.431 ± 0.095	0.00289 ± 0.00096	<28.9	<0.216

* Fractions are those indicated in Fig. 1.

‡ Cytochrome *c* oxidase values are the average of 5 experiments; ATPase values are the average of 4 experiments.

§ Values in parentheses indicate the range.

tamination; that of the latter to measure the degree of myofibrillar contamination. These enzyme assays are performed on the less pure nuclear fractions, series A, and not upon the fractions isolated from the *N*-ethylmaleimide– or insulin-treated muscle. In comparing the homogenate to the isolated nuclear fractions, one may note a 30-fold decrease in the specific activity of cytochrome c oxidase on a protein basis; on a DNA basis, the decrease in the specific activity of this enzyme is over 2000-fold (see Table III). It is apparent from the cytochrome c oxidase activity present in the nuclear fraction that there is mitochondrial contamination. On the other hand, ATPase, as measured here, is not detectable in the nuclear fraction because the amount of phosphate obtained may have been below the threshold of sensitivity of the assay. It may be observed, therefore, in Table III that a 10-fold decrease in the specific activity of the ATPase on a protein basis would be undetectable whereas a 30-fold decrease in the specific activity of cytochrome c oxidase is clearly demonstrable.

General Observations

The methods employed in producing the nuclei described in series B of Tables I and II result in a greater recovery of nuclei of higher purity as determined by DNA/protein and RNA/DNA ratios, and as directly observed in the phase-contrast microscope (see Discussion).

Sucrose-$CaCl_2$ solutions generally employed to preserve the morphology of isolated nuclei (1, 2, 10, 15) cause extensive damage to the skeletal muscle nuclei. This damage consists of nuclear distortion and rupture, resulting in the release of large amounts of chromatin-like material.

DISCUSSION

A simple technique employing aqueous media is described for the isolation of rat skeletal muscle nuclei. The majority of nuclei produced by this method appear, in both the phase-contrast and electron microscopes, to be morphologically intact, unassociated with contiguous cytoplasmic tags, relatively free of contamination with other

TABLE IV

Summary of Chemical Analysis of Nuclei Isolated by Various Methods

Reference	Tissue	Isolation medium	Fraction	DNA / protein	RNA / protein	RNA/DNA
Schneider and Petermann (15)	Mouse spleen	0.88 M sucrose + Ca^{++}	N	0.29	0.013	0.045
Gurr *et al.* (30)	Rat liver	1 per cent citric acid	N	0.28	—	—
		2.2 M sucrose + Mg^{++} + Na^{+} + glycerol P	N	0.12	—	—
Chauveau *et al.* (7, 8)	Rat liver	2.2 M sucrose	H	0.019	0.047	2.5
			N	0.21	0.034	0.16
Hogeboom *et al.* (2)	Rat liver	0.34 M sucrose + Ca^{++}	H	0.016	0.042	2.5
			N	0.12	0.051	0.38
Hymer and Kuff (42)	Rat liver	0.25 M sucrose + Mg^{++} + Triton-X	H	—	—	3.3
			N	0.29	0.063	0.22
Maggio *et al.* (1)	Guinea pig liver	2.2 M sucrose + Ca^{++}	N	0.31	0.065	0.21
Kay *et al.* (43)	Rabbit thymus	0.05 M citric acid	N	0.51	0.047	0.080
		non-aqueous solvent	N	0.23	0.057	0.21
Allfrey *et al.* (6)	Calf liver	Non-aqueous solvent	N	0.18	—	—
	Horse liver (fasted)	Non-aqueous solvent	N	0.22	—	—
	Calf heart	Non-aqueous solvent	N	0.24	—	—
Siebert (5)	Rat liver	Non-aqueous solvent	H	0.015	0.053	3.5
			N	0.14	0.045	0.32
	Ox thymus	Non-aqueous solvent	H	0.016	0.022	1.4
			N	0.19	0.049	0.25
Edelman *et al.*	Rat skeletal muscle	2.15 M sucrose + K^{+}, Mg^{++}, ATP	H	0.002	0.004	1.7
			N a	0.17	0.064	.30
			b	0.29	0.029	0.10

H, Crude tissue homogenate
N, Purified nuclear fraction
a, Series A
b, Series B

subcellular organelles, and of sufficient yield. The biochemical studies undertaken to assess the purity of the nuclear preparation compare favorably with those obtained from like studies of liver, thymus, and kidney tissues (Table IV).

Experience in preparing sarcolemmae from striated muscle (27) suggested the value of employing fiberglass and stainless steel screens in reducing the amount of contaminating connective tissue, myofibrillae, and relatively intact cells. The use of rigidly controlled pestle-to-cylinder wall clearances was suggested by the morphological characteristics of the tissue and by the experience of Busch *et al.* (28).

Effective pestle-to-glass clearances for the homogenizers were obtained by trial and error. The best clearance was found to be in the 0.020 to 0.025 inch range, and the clearance could be better controlled by using Delrin as material for the homogenizing pestle for the reasons cited above. The homogenizer consisted of precision ground pyrex 1 inch in diameter. The rotational velocity of the pestle was 550 to 600 RPM.

Selection of a specific gravity-flotation method

similar to that of Chauveau *et al.* (7, 8) was made because of our initial interest in the intracellular location of insulin (25). In these experiments, striated muscle pretreated with I^{131}-labeled hormone was fractionated into its subcellular components and the radioactivity of each component determined. The possibility that the employment of an organic solvent in the isolation technique might affect the lipoprotein nuclear membrane and thus vitiate the significance of the determination of the amount of insulin bound to the nucleus precluded its use for our purposes. We elected not to use surface active detergents such as Cemulsol as described by Hubert *et al.* (29) for the same reason, *i.e.*, such agents would be expected to remove the outer nuclear membrane. Also, the use of an isolation technique employing citric acid was precluded because of the effectiveness of this agent in removing the outer nuclear membrane (30); and because our experiences with the effect of pH upon the insulin-sarcolemma complex suggested that an acid medium might reduce the amount of insulin bound to the nucleus (31).

Prior to the fractionation procedure the muscles were handled in one of two ways. The muscles involved in series A received no pretreatment. The muscles employed in series B were pretreated in a Krebs-Ringer bicarbonate medium containing insulin, bathed in 10^{-3} M *N*-ethylmaleimide, and rinsed in Krebs-Ringer bicarbonate solution and saline. The yield of nuclei resulting from the above pretreatment (series B) was nearly 5-fold that of the tissue which received no pretreatment. The nuclei appeared to be much less contaminated by mitochondria and intra- and intercellular debris. The nuclear DNA/protein ratio was higher and the RNA/DNA ratio was lower in the nuclei of series B (Table I and II), and these ratios were comparable to those observed in other tissues (Table IV), suggesting a comparable degree of purity. The lower RNA/DNA ratios of series B suggests to us that the nuclei in series A either adsorbed more cytoplasmic RNA or lost less nuclear RNA, or both, than did the nuclei in series B. It is also possible that pretreatment of series B promoted a loss of nuclear RNA.

Two factors probably play a prominent role in the production of the higher and less contaminated yield obtained in series B: (*a*) the preincubation in a calcium-containing medium, and (*b*) preincubation in a solution of *N*-ethylmaleimide. The role of calcium ion in stabilizing the intracellular matrix and maintaining the integrity of nuclear (15) and cellular membranes is well known. For reviews of this subject see Rinaldini (32) and Weiss (33). *N*-ethylmaleimide has been used for the chemical modification of proteins. It reacts with cysteinyl residues, α-amino groups of peptides, and with the imidazole group of histidine (34, 35). It seems plausible that the use of *N*-ethylmaleimide may have blocked reactive groups on exposed surfaces of subcellular organelles and thereby served to harden or fix them. These proposed alterations would reduce the adhesive properties of the organelles, facilitating their fractionation while contributing to their preservation.

The relatively large diminution in the specific activity of cytochrome c oxidase and the virtual disappearance of ATPase in the nuclear fraction suggets that the nuclear fraction obtained has been highly purified. However, these indications of purity are attenuated by the fact that the metabolism of isolated nuclei has not been sufficiently characterized. The presence of ATPase has been described in rat liver nuclei (38–40), pig kidney cortex nuclei (41) and rat kidney nuclei (10) but was absent in calf kidney nuclei and calf cardiac muscle nuclei (40). The greater than 10-fold decrease in the specific activity of this enzyme which we observed in striated muscle nuclei suggests that it probably is not a component of striated muscle nuclei.

The loss of nuclear protein, RNA, and enzymes during isolation of nuclei in high-density media is an ever-present possibility. However, the small amount of karyolysis and chromatin agglutination obtained, as well as the presence of an abundance of nuclei with distinct nucleoli, suggests that this problem has been minimized. Also, it is noteworthy that the liberated striated muscle nuclei are unencumbered with cytoplasmic tags or satellite membranes, despite their peripheral location in the cell, which at once makes for proximity to the sarcolemma and complicates homogenization.

Isolation of nuclei in high-density solutions always evokes the possibility that one is selecting the densest nuclei. Nuclei of differing density have been found in liver and correlated with the size, DNA content, polyploidy, and tissue source (36). Thus the nuclear fraction obtained by a specific gravity-flotation method could conceivably consist of a non-representative sample of "heavy" nuclei.

The metabolism of these isolated heavy nuclei differs from that of the average nucleus (37). Assuming that the DNA content of the homogenates of series A and B, which were prepared in an identical manner from either incubated or non-incubated tissue, were similar, our recovery was approximately 15 per cent of the nuclei of the homogenate (Table I). Some of the unrecovered nuclei remained suspended in the dense sucrose; however, the majority of them were found on the surface of the dense solution. Mechanical trapping by tissue debris may account for some of the nuclei found on the surface of the high-density sucrose solution. Nevertheless, it is possible that some of these unsedimented nuclei may differ intrinsically in shape, density, and other properties.

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