

A MODIFICATION OF THE METHOD OF HEWISH AND BURGOYNE
FOR THE ISOLATION OF RAT LIVER NUCLEI

by

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TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	v
LIST OF TABLES	vi
ABSTRACT	vii
1. INTRODUCTION	1
2. MATERIALS AND METHODS	10
Animals	10
Buffers and Solutions	10
Isolation of Nuclei	11
DNA, RNA and Protein Determination	12
In Vitro Transcription	12
Alkaline Sucrose Gradients	13
Phase Contrast Micrographs	15
3. RESULTS	16
A Schematic Comparison of the Hewish and Burgoyne Procedure with the Modified Procedure	16
Biochemical Properties of Nuclei Isolated by Four Different Procedures	19
In Vitro Transcription by the Endogenous RNA Polymerases	21
Alkaline Sucrose Gradients of DNA from Isolated Nuclei	23
Phase Contrast Micrographs of the Isolated Nuclei	26
4. DISCUSSION	31
REFERENCES	37

LIST OF ILLUSTRATIONS

Figure		Page
1.	A Schematic Comparison of the Hewish and Burgoyne Procedure with the Modified Procedure	17
2.	Time Course of <u>In Vitro</u> Transcription by Four Different Preparations of Rat Liver Nuclei	22
3.	Alkaline Sucrose Gradients of Rat Liver Nuclear DNA	24
4.	Alkaline Sucrose Gradients of Rat Liver Nuclear DNA Exposed to $MnCl_2$	25
5.	Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Blobel and Potter (1966)	27
6.	Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Chaveau et al. (1956)	28
7.	Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Hewish and Burgoyne (1973)	29
8.	Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Modified Method	30

LIST OF TABLES

Table	Page
1. Biochemical Properties of Rat Liver Nuclei Isolated by Various Procedures	20

ABSTRACT

This study describes a rapid and convenient modification of the established procedure of Hewish and Burgoyne (1973) for isolating rat liver nuclei. Substitution of the polyamines spermine and spermidine for divalent metal cations in the isolation buffers distinguishes this technique from other well-known methods. In addition, this method utilizes the chelators EDTA and EGTA to remove any endogenous divalent cations. This modification utilizes a single, high-speed centrifugation step for separating nuclei from the homogenate by sedimentation through a dense sucrose solution. This modification is based on the nuclear isolation scheme of Blobel and Potter (1966). An important advantage of the new method is that it produces a high yield of purified nuclei, as measured by DNA content, which contain significantly more protein and RNA. These nuclei also contain more endogenous RNA polymerase activities than nuclei obtained by other well-known methods and may contain larger amounts of other nuclear enzymes. In addition, the DNA from these nuclei is highly polymerized when compared to that from nuclei isolated by other established procedures which utilize divalent cations.

It is suggested that the modified nuclear isolation procedure described produces more intact nuclei which will form the basis for beginning a reinvestigation of the nucleus as an isolated eukaryotic organelle and allow the further characterization of the endogenous enzymology in situ.

CHAPTER 1

INTRODUCTION

Mammalian cell nuclei were first identified by Robert Brown in 1830. A function was vaguely assigned to them as the governor of the cell. Subsequent interest in the organelle developed when observable changes in nuclear morphology could be correlated with various pathologic and disease states (see Smuckler et al. 1976). Nuclei were first isolated to examine their chemical composition and metabolic capabilities (see Roodyn 1969). However, the anatomical organization of functional components of cell nuclei have not been fully coordinated with its macromolecular dynamics.

Elucidation of the roles of nuclear structural and enzymatic components can best be achieved by separating nuclei from other cellular constituents. Ideally, when nuclei are separated from other cellular components, all cytoplasmic contaminants will be eliminated from the final nuclear fraction and the desired nuclear structure and enzymatic activities preserved. However, many nuclear isolation techniques are either too destructive or do not effectively separate nuclei from other cellular components and debris (for review see Roodyn 1969, Busch and Smetana 1970, Smuckler et al. 1976).

Nuclei are isolated from various tissue sources for reasons which are almost too innumerable to list. Originally, nuclei were isolated to study their chemical composition, metabolic capabilities,

and as a source of nucleic acids. Isolated nuclei also are a source of specific nuclear proteins (histones, nucleases, nonhistone chromosomal proteins, and other nuclear enzymes), chromatin, and ribonucleo-protein particles. They also can function as model systems when examining in situ activities of chromatin bound enzymes such as DNA and RNA polymerases, nuclear protein kinases, and histone acetylases.

Several important factors must be considered when isolating nuclei. According to Smuckler et al. (1976), they are:

1) The Homogeneity of the Starting Material

The homogeneity of the resulting nuclei will depend on the degree of cellular and nuclear heterogeneity of the starting material. For example, rat liver consists of 90% hepatocytes by volume and only 70% by cell count (Hertzfeld et al. 1973).

2) The Mechanism of Tissue Disruption

Some methods are so drastic that severe damage can occur to nuclear structures, but milder methods may not effect complete tissue homogenization and cellular disruption so that poor nuclear yield with significant cytoplasmic contamination may result. The Polytron and Waring blender can cause severe damage to nuclei when they are used for homogenizing liver tissue, whereas the Dounce-type Teflon homogenizer is ideal for homogenizing liver tissue for isolation of nuclei. However, it is unsuitable for homogenizing fibrous heart tissue because efficient cell breakage can only be achieved by using the more effective Polytron (Widnell et al. 1967, Vorbeck et al. 1976).

3) The Composition of the Isolation Medium

The composition of the isolation medium can affect the structure and function of the resulting nuclei; nonphysiologic activation or inactivation of enzymatic components can result and various subnuclear structures may have their normal morphology altered. The addition of divalent metal ions such as Ca^{++} and Mg^{++} have been used in the past to preserve nuclear structure but have a deleterious effect on nucleic acids (Hewish and Burgoyne 1973, Burgoyne and Hewish 1978). Citric acid solutions have been used in the isolation of nuclei that contain large amounts of intact RNA (Dounce 1950). Sucrose, Ficoll, and glycerol also have been used because they are considered beneficial for osmotic stability (Roodyn 1969).

4) Separation of Nuclei

Contamination of isolated nuclei with other cellular components can affect the quality of isolated nuclei. This is particularly important because some cytoplasmic enzymes, which can degrade nuclear protein, DNA, and RNA, may be released during tissue disruption. Other contaminating cytoplasmic enzyme activities also may mask the activities of nuclear enzymes being studied or prevent an accurate assessment of enzyme distribution between the nucleus and cytoplasm. Nuclei can be grossly viewed for particulate contamination with a phase contrast microscope. However, cytoplasmic enzymes may adhere to isolated nuclei and remain undetected.

There are a variety of approaches for isolating mammalian cell nuclei. Some of the more important methods are: 1) citric acid, 2) calcium, 3) nonaqueous, and 4) dense sucrose isolation methods.

One of the first nuclear isolation methods was the citric acid method. In 1856, Francis Guerny Smith observed nuclei from skin and tumor cells were released by exposure to citric acid (see Roodyn 1969). Later, Dounce (1943, 1950) exploited this phenomenon by using a solution containing citric acid for the homogenization of rat liver and centrifugation of the resulting homogenate to isolate relatively "pure" nuclei quantitatively. Nuclei isolated in the presence of citrate contain a relatively high amount of RNA. Presumably, citrate acts as a chelator for divalent metal ions such as Ca^{++} which may be present and thus protects nucleic acids from digestion (Busch and Smetana 1970). Unfortunately, the low pH (2-5) of solutions required for significant nuclear yield caused inactivation of many nuclear enzymes (Roodyn 1969, Busch and Smetana 1970) and disruption of subnuclear structures (Smuckler et al. 1976).

The use of calcium-sucrose solutions for isolating nuclei has always been popular (Hogeboom et al. 1952, Maver et al. 1952, Roodyn 1956, Allfrey et al. 1957, Maggio et al. 1963). Schneider and Petermann (1950) and Wilbur and Anderson (1951) noted that low concentrations of Ca^{++} and other divalent metal cations stabilized nuclei from fragmentation during homogenization. In addition, sucrose is included in buffered solutions to provide osmotic stability. Nuclei isolated in the presence of Ca^{++} and sucrose were visualized as sharp and clear by phase contrast and electron microscopy. In addition, these nuclei did not tend to clump and lyse like nuclei isolated by other techniques. The yield of protein, DNA and RNA from nuclei isolated in the presence of Ca^{++} was much greater when compared to

citric acid procedures, while the amount of cytoplasmic contamination was minimized (Busch and Smetana 1970). However, nuclei isolated in the presence of Ca^{++} were hardened and fragile and many nuclear enzymes such as the RNA polymerases (Busch and Smetana 1970) and DNA (Hewish and Burgoyne 1973) are adversely affected by the presence of this cation.

Many investigators have been concerned with the loss of soluble nuclear components. During nuclear isolation, nuclei may be diluted several times and soluble nuclear components and enzymes may leach out of nuclei (Lindell 1975). Isolated nuclei which are suspended in certain buffers on ice also have been shown to lose various enzymes, such as RNA polymerase, into the supernatant (Liao et al. 1968). Certain detergents and divalent metal ions are also damaging to nuclei and nucleic acids and can effect an efflux of material from nuclei (Blobel and Potter 1966, Smuckler et al. 1976).

Nonaqueous solutions composed of organic solvent mixtures to extract nuclei from freeze-dried tissue were used to alleviate this problem. The method of Dounce (1950) and others utilized several different solutions of benzene and carbon tetrachloride which varied in specific gravity for a series of several extractions. Nuclei isolated by this method retained their soluble enzymes because they were never exposed to aqueous solutions. Nuclear DNA is also obtained in a highly polymerized state by this method. Other nonaqueous methods using different solvent combinations have been developed by others (Allfrey et al. 1951, Kirch et al. 1970). However, these methods are very tedious because of the many steps in each procedure which are

very time-consuming. In addition, many enzymes were nonphysiologically activated or inactivated (Roodyn 1969).

Nuclei can be easily separated from other cellular components by centrifugation because of their high density. When tissue homogenates are subjected to ultracentrifugation through a dense solution such as Ficoll or sucrose of high molarity, they are sedimented under conditions in which other cellular organelles and intact cells float. Chaveau et al. (1956) first exploited this phenomenon as the basis of their technique to isolate highly purified rat liver nuclei. Rat liver was directly homogenized in 2.2 M sucrose and subjected to ultracentrifugation at about 40,000 x g. Previous methods which employed sucrose solutions had not used more than 1.0 M sucrose and had centrifuged homogenates at speeds no greater than 1000 x g (Dounce 1943, Dounce 1950, Schneider and Petermann 1950, Wilbur and Anderson 1951, Hogeboom et al. 1952, Maver et al. 1952, Roodyn 1956). However, the use of dense sucrose solutions can present a problem since at 4⁰ dense sucrose solutions (greater than 1.9 M) used for centrifugation become extremely viscous (Roodyn 1969). When tissue homogenates are subjected to ultracentrifugation in these dense sucrose buffers, the various subcellular organelles sediment to the bottom of the centrifuge tube at a rate proportional to the density of the organelle. This allows nuclei to sediment because their density is considerably greater than other subcellular organelles. Nuclei prepared by this method were considered the very first highly purified nuclei. However, there are two major problems with the original Chaveau et al. method: 1) low nuclear yield because of incomplete cell disruption, and 2) nuclei can become exposed

to deleterious sucrose contaminants (such as Ca^{++}) which may reach significantly high concentrations in the homogenization-centrifugation solution because of the high molarity of sucrose employed (Busch and Smetana 1970, Smuckler et al. 1976).

An improvement of the method of Chaveau et al. (1956) was developed by Blobel and Potter (1966). They homogenized rat liver in a solution of isotonic sucrose having low density to achieve complete cell disruption and then mixed the homogenate with two volumes of 2.3 M sucrose solution. The resulting mixture was dense enough so that cellular debris, lipids and other organelles float, while nuclei sediment to the bottom of a centrifuge tube through a cushion of 2.3 M sucrose after ultracentrifugation at 125,000 x g. In addition, the isolation solutions they employed used Mg^{++} instead of Ca^{++} and were buffered at constant pH with Tris-HCl. Nuclei isolated by the method of Blobel and Potter were obtained in a 3-fold greater yield than the parent method of Chaveau et al. (1956). The nuclei were also enzymatically active and morphologically identical to nuclei observed in intact tissues. Nuclei isolated by the Blobel and Potter method are also excellent starting material for isolating nucleoli (Busch and Smetana 1970, Roeder and Rutter 1970).

Hewish and Burgoyne (1973) developed a method to isolate enzymatically active rat liver nuclei which contained highly polymerized DNA. Rat liver was homogenized in a low density sucrose solution and nuclei were separated by high-speed ultracentrifugation through dense sucrose in buffers that contained Tris-HCl, polyamines, EDTA and EGTA. The chelators EDTA and EGTA removed all divalent metal cations

present in the sucrose and tissue homogenate. Nuclei were stabilized by the polyamines, spermidine and spermine, instead of Ca^{++} or Mg^{++} ions. These nuclei have normal in vivo morphology, highly polymerized DNA and active chromatin bound enzymes. When Ca^{++} or Mg^{++} were added to these nuclei, the majority of the DNA was extensively degraded by a specific Ca^{++} - Mg^{++} -dependent endonuclease (Burgoyne et al. 1970, Burgoyne and Hewish 1978). The only disadvantage of nuclei prepared by this method is that the DNA is subject to instantaneous digestion when any divalent metal cations are present. However, nuclei isolated by this method yield highly active intact chromatin with highly polymerized DNA and active engaged enzyme complexes such as template-bound DNA polymerases.

Many of the available nuclear isolation procedures were successful in their primary objectives during periods of popular use. But, due to the fact that each has significant disadvantages, no ideal method exists.

The objective was to develop a convenient preparative technique for isolating eukaryote nuclei, which closely resemble nuclei found in vivo. These nuclei would be a better source of highly polymerized DNA and various nuclear enzymes. In addition, these nuclei might represent an improved system to study in situ nuclear enzymology, particularly the DNA template-bound RNA polymerases.

Hewish and Burgoyne (1973) had demonstrated that rat liver nuclei isolated from fresh tissue in the presence of polyamines and divalent metal cation chelators had highly polymerized DNA while still maintaining an active nuclear enzymology. Blobel and Potter (1966)

had previously demonstrated an efficient time saving method to prepare very pure nuclei in high yield in one step from rat liver. The plan was to hybridize the desirable aspects of these two methods to derive an improved nuclear isolation procedure whereby: 1) DNA and chromatin are preserved by omission of deleterious Ca^{++} and Mg^{++} ions; 2) convenience and rapidity are afforded by following the simple Blobel-Potter scheme; and 3) Hewish-Burgoyne buffers would be added to Blobel-Potter sucrose solutions.

The modified method for isolating nuclei from rat liver will be described in the following text. The properties of these nuclei will be described and compared to several parent nuclear isolation methods.

CHAPTER 2

MATERIALS AND METHODS

Animals

Male and female rats (Sprague-Dawley) weighing approximately 150 g, about 10 weeks old, were obtained from a breeding colony maintained by the Division of Animal Resources at the Arizona Health Sciences Center.

Buffers and Solutions

Modified Homogenization Solution

This solution contained 0.25 M sucrose (Schwartz-Mann enzyme grade), 2 mM Na₂EDTA and 0.5 mM Na₂EGTA (Sigma), 120 mM KCl (Mallinckrodt), 30 mM NaCl (Mallinckrodt), 0.3 mM spermine (Sigma), 1 mM spermidine (Sigma), and 30 mM Tris-HCl (Sigma), pH 7.4.

Hewish and Burgoyne 2.1 M Centrifugation Solution

This solution contained 2.1 M sucrose, 0.1 mM Na₂EDTA, 0.1 mM Na₂EGTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine-HCl, 0.5 mM spermidine-HCl, and 15 mM Tris-HCl, pH 7.4.

Modified Final Solution

This solution contained 25% glycerol (v/v), 1 mM Na₂EDTA, and 50 mM Tris-HCl, pH 7.9.

NOTE: All nuclear isolation solutions contained 15 mM β -mercapto-ethanol (B-MSH, Sigma) and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma) which were added to the solutions immediately before their use.

Isolation of Nuclei

Rat liver nuclei, isolated by the methods of Chaveau et al. (1956), Blobel and Potter (1966), and Hewish and Burgoyne (1973), were suspended in 1 M sucrose solution, 1 M TMS solution (Blobel and Potter 1966), and the final solution described by Hewish and Burgoyne (1973), respectively. Rat liver nuclei were also prepared by a modification of the method of Hewish and Burgoyne (1973) as described below. Rats were sacrificed by spinal dislocation and the livers were quickly surgically removed and directly placed in an ice water slurry to cool them as rapidly as possible. Individual livers were then blotted dry and weighed.

In the modified procedure, the ice-cold livers were minced with scissors and one volume of the modified homogenization solution per gram liver wet weight (v/v) was added to the finely chopped liver. The mixture was poured into a chilled Potter-Elvehjem glass (Thomas - size C) homogenizing vessel and homogenized 6 strokes with a tear-drop shaped Teflon Dounce pestle by hand. The homogenate was then poured through 2 layers of cheesecloth into another Potter-Elvehjem vessel of the same size and homogenized 10 strokes (up and down) with a motor-driven Teflon pestle. The homogenate was mixed with 2 volumes of the Hewish and Burgoyne 2.1 M centrifugation solution, mixed well by stirring, poured into nitrocellulose ultracentrifuge tubes (SW-27,

35 ml, Beckman), and underlayered with 10 ml of the same 2.1 M sucrose centrifugation solution. This was accomplished using a 50-ml syringe fitted with a blunt-ended number 10 needle. The tubes were centrifuged in a Beckman ultracentrifuge at 25,000 rpm (110,000 x g) for 45 min at 4°. The supernatant was discarded and the resulting nuclear pellet resuspended in the modified final solution (one-half volume per gram original wet liver weight) and briefly homogenized in the glass-Teflon homogenizer by hand. The suspended nuclei were used immediately for in vitro transcription or were frozen by dropwise addition into liquid nitrogen where they then were stored in plastic vials.

DNA, RNA and Protein Determination

DNA and protein were determined according to the methods of Burton (1956) and Lowry et al. (1951), respectively. RNA was determined by a slight modification of the alkaline hydrolysis method of Fleck and Munro (1962), where samples were neutralized with one volume of 5% TCA instead of one volume of 10 N PCA. Percent yield of nuclei was determined by comparing the total amount of DNA in the original liver homogenate to the total DNA content of nuclei recovered.

In Vitro Transcription

Transcription in isolated nuclei in vitro was performed by the method of Roeder and Rutter (1970) with some modifications as detailed below. Ammonium sulfate was omitted from the assay because the presence of salt caused aggregation of the nuclei during in vitro transcription. The reactions were initiated by the addition of nuclei to the complete assay mixture, and 25- μ L aliquots were removed from

the reaction mixture (in duplicate) at 1, 2, 5, 10, 20, and 30 min to determine the time-course of the transcription in vitro. Aliquots of nuclei were pipetted onto 2.3-cm DE-81 filters (Whatman), and the filters were immediately placed into 5% Na_2HPO_4 and washed by swirling in 5% Na_2HPO_4 7 times (4-mL filter), distilled water twice, 95% ethanol twice, and diethyl ether twice. The filters were dried overnight and then placed in scintillation vials with 5 mL of toluene containing NCS and Omnifluor (New England Nuclear) (Lindell 1970). Tritium was counted in a Tracor Mark III liquid scintillation counter.

Alkaline Sucrose Gradients

Alkaline sucrose gradients were prepared in 17-mL SW-27 nitro-cellulose centrifuge tubes (Beckman) with 15.6 mL of 5-20% sucrose (Schwarz-Mann gradient grade) containing 0.4 M NaOH, 0.1 M Na_2EDTA , and 0.1% sarkosyl (Sigma). A 1-mL underlay of 40% sucrose with the same constituents was placed in the bottom of the tube. Nuclei were removed from liquid nitrogen, thawed, and diluted 3-fold with 50 mM Tris-HCl and 1 mM Na_2EDTA , pH 7.9, to a final DNA concentration of 0.4-0.5 mg/mL. One hundred-fifty μL of diluted nuclei (about 65-70 μg DNA) were then pipetted onto the top of the gradient which contained 300 μL of 2.5 mg/mL heparin and 2% sarkosyl as described by Walters and Hildebrand (1975). After 15 min at room temperature, 450 μL of 0.8 M NaOH and 0.2 M Na_2EDTA were added to lyse the nuclei. After standing an additional 45 min, the gradients were centrifuged at 20° in a Beckman SW-27 rotor at 10,000 rpm (22,000 x g) for 25 h. The gradients were fractionated by pumping 40% sucrose from the bottom and collecting 22 0.8-mL fractions from the top.

DNA in the fractions was quantitated by fluorimetry after reacting each fraction with 3,5-diaminobenzoic acid (DABA) as described by Kisane and Robins (1958) with some modification as described. Bovine serum albumin (300 μ g, Sigma) was added to each fraction followed by 0.3 mL of 33% TCA. Samples were then stored at 4^o overnight. The precipitate was collected by centrifugation in a Brinkman microfuge for 2 min at room temperature and washed first with 0.5 mL cold 5% TCA and secondly with 0.3 mL cold 1 N HCl. The precipitate was then solubilized with 50 μ L of 1 N NaOH, and 50 μ L of 1.5 M DABA (Aldrich) was added. Samples were incubated for 30 min at 60^o and then cooled. Nine hundred μ L of 1 N HCl was added to samples, which were then mixed and centrifuged as above to sediment any insoluble material. The amount of DNA in each sample was determined with an Aminco-Bowman fluorimeter using 420 nm wavelength excitation and 520 nm wavelength emission. The amount of DNA corresponding to percent transmission of fluorescence was determined from a standard curve using 0.5, 1, 2, 3, and 5 μ g of calf thymus DNA (Sigma, type 1), carried through the same precipitation procedure above. Standard curves routinely gave a value of 20% transmittance per μ g DNA.

The relative size of the DNA in the alkaline sucrose gradients was compared with Chinese hamster ovary (CHO) DNA as a marker which sediments at 150 S (Walters and Hildebrand 1975).

Chinese hamster ovary cells were obtained from Dr. Eugene Gerner and Mr. David Holmes of this institution.

Phase Contrast Micrographs

Two μ l of nuclei were spotted on a glass microscope slide with 2 μ l of concentrated Gimsa stain (a gift from Dr. John Davis and Mr. Robert Penny), and the cover slip was carefully placed on top. A drop of emersion oil was placed on top of the coverslip. The slides were viewed at a magnification of 1800 x and micrographs were taken with a Zeiss phase contrast microscope equipped with a Zeiss, C35, top mounting camera. Kodak Panatomic-X (ASA 32) was used for black and white prints, and Kodak Ektachrome (ASA 160) was used for color slides.

CHAPTER 3

RESULTS

A Schematic Comparison of the Hewish and Burgoyne Procedure with the Modified Procedure

In describing the modified method for the isolation of rat liver nuclei, it is necessary to compare and contrast it with the parent method of Hewish and Burgoyne (1973). The schematic outline of both methods is shown in Figure 1. The Hewish-Burgoyne method as shown here requires two centrifugation steps to yield purified nuclei from rat liver homogenate. (The washing of the final nuclear pellet suggested by Hewish and Burgoyne is optional and is not shown here.) Only one quick centrifugation step is required in the modified method. This scheme is very similar to that of Blobel and Potter (1966). Clearly, the modified procedure is more time efficient, requiring about one hour as compared to the Hewish and Burgoyne procedure which requires about three hours.

The solutions utilized in the modified method are very similar to those described in the Hewish and Burgoyne method. The 2.1 M sucrose ultracentrifugation solution is the same as described by Hewish and Burgoyne, but the homogenization and final solutions are slightly different in the modified method.

The homogenizing solution in the modified method differs from that of the Hewish and Burgoyne method in that: 1) the sucrose concentration is reduced from 0.34 M to 0.25 M; 2) the concentration of

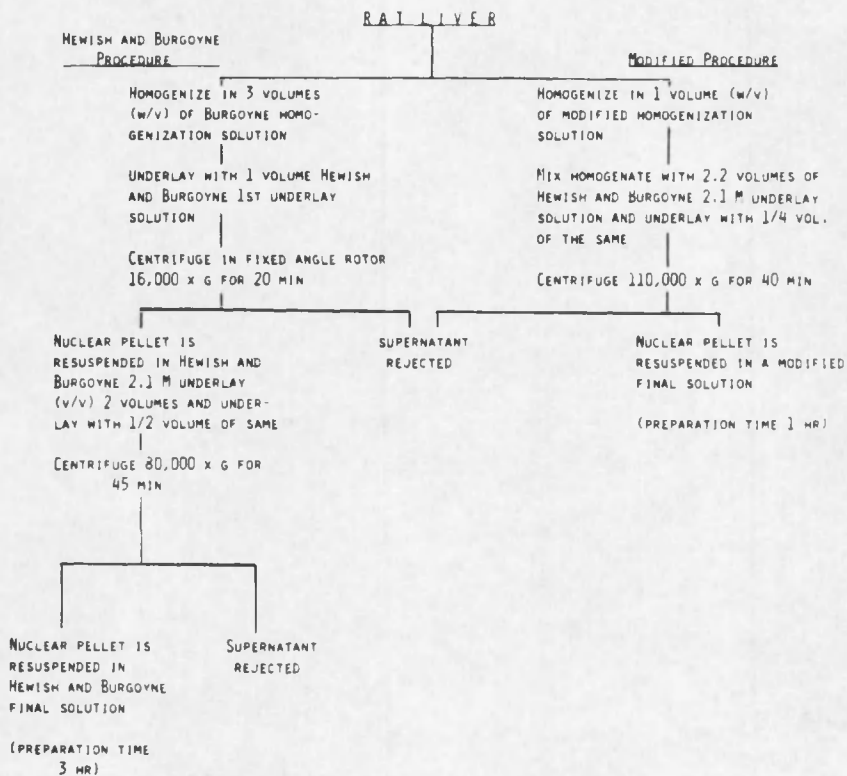


Figure 1. A Schematic Comparison of the Hewish and Burgoyne Procedure with the Modified Procedure.

Na_2EDTA and Na_2EGTA is doubled from 1 mM to 2 mM and 0.3 mM to 0.6 mM, respectively; and 3) the amount of the 10X buffer as described by Hewish and Burgoyne (1973) (which contains Tris-HCl, spermine, spermidine, KCl, and NaCl and is added to most of the solutions) is doubled in the modified method.

The homogenizing solution in the modified method is more concentrated (with the exception of sucrose) than that of Hewish and Burgoyne because the resulting homogenate is more concentrated. More Na_2EDTA and Na_2EGTA are required because the concentration of Ca^{++} and other divalent metal cations will be greater. Because the mass of liver tissue is greater per unit volume in the homogenates, the sucrose concentration is reduced to decrease the viscosity of the homogenate. Also, Blobel and Potter (1966) used a 0.25 M sucrose solution for homogenization. The reason for concentrating this homogenate into a relatively small volume is that a greater mass of material can be prepared at one time and more nuclei can be isolated with a reduction in sample volume and handling time (Lindell 1975). This step alone reduces the volume of homogenate to be centrifuged by one-half (Lindell 1975).

The final solution for suspending the purified nuclei is different from that of Hewish and Burgoyne. They utilized a 0.34 M sucrose solution where this procedure uses a 25% glycerol solution. A 25% glycerol solution freezes at a much lower temperature and more gradually than a 0.34 M sucrose solution. This glycerol-based solution presumably protects the nuclei from microcrystal formation and damage when they are quick-frozen in liquid nitrogen. In addition, 50 mM

Tris-HCl and 1 mM EDTA were employed instead of the Hewish and Burgoyne 10X solution to buffer the nuclei at a constant pH of 7.9.

Two other components are also added, β -mercaptoethanol (B-MSH) and phenylmethylsulfonylfluoride (PMSF), to all of the solutions to preserve nuclear structure and enzymatic activity. PMSF, an inhibitor of serine proteases (Fahrney and Gold 1963), is added to all solutions at a concentration of 1 mM to protect enzymes and protein structures from serine proteases (Lindell 1975). B-MSH is added at 15 mM to all solutions to preserve the activity of enzymes containing sulfhydryl groups and as an antioxidant.

Biochemical Properties of Nuclei Isolated by Four Different Procedures

In the preliminary investigations of nuclei isolated by this modified procedure, nuclei were assayed for DNA, RNA and protein content and then were compared to values obtained from nuclei prepared by three other methods. Table 1 shows the amounts of DNA, RNA, and protein recovered from nuclei isolated by the methods listed, along with RNA/DNA and protein/DNA ratios. In addition, a percent yield has been calculated for each preparation. This value is calculated from the amount of DNA recovered from nuclei divided by the amount of DNA in the original homogenate.

These results show that the average percent yield is about 60% for the modified procedure and is significantly greater than the percent yields for the Blobel-Potter or the Chaveau procedures. The average yield from the Hewish and Burgoyne method is about 10% greater than the modified method. This may be due to removal of interface

Table 1. Biochemical Properties of Rat Liver Nuclei Isolated by Various Procedures.

TYPE OF PREPARATION	EXPERIMENT No.	AMOUNTS (IN MG) RECOVERED FROM 10 G LIVER			PROTEIN/ DNA	RNA/DNA	% YIELD
		DNA	RNA	PROTEIN			
BLOBEL-POTTER	1	13.5	1.67	72.0	5.28	0.182	48.0
	2	12.3	2.04	81.0	6.57	0.166	54.1
HEWISH-BURGOYNE	1	13.8	1.49	73.5	5.31	0.107	69.7
	2	13.6	3.18	80.5	5.92	0.234	68.3
CHAVEAU	1	2.3	0.11	12.8	5.52	0.069	8.0
	2	2.4	0.31	13.2	5.39	0.125	11.2
MODIFIED PROCEDURE	1	10.6	2.87	83.5	7.88	0.270	61.0
	2	11.4	3.45	99.5	8.73	0.302	58.5

forming cellular constituents which trap nuclei in the ultracentrifugation step. In the original Hewish and Burgoyne method there is a preliminary centrifugation and an additional homogenization. However, the nuclear yield from the modified method is significantly high.

One of the most striking differences in these nuclei is the ratios of protein/DNA and RNA/DNA as compared to those derived from nuclei from the other methods. In this method, the ratios are significantly greater than those from the other methods. The increase in RNA and protein with respect to DNA (which is constant) in nuclei isolated by this method could be due to contamination by endoplasmic reticulum (ER), but examination by phase contrast microscopy failed to show any noticeable contamination. Possibly, the nuclei retain more of their soluble protein and RNA because they are isolated more efficiently and quickly.

In Vitro Transcription by the Endogenous RNA Polymerases

This experiment was performed to investigate the utility of isolated nuclei for in vitro transcription by the endogenous template-bound RNA polymerases within nuclei isolated by the modified method and the other methods indicated. Figure 2 shows a comparison of time-course of incorporation of UMP into RNA in various isolated nuclei. The results show that nuclei isolated by the modified method have a greater capability to transcribe RNA when compared to the other nuclei. In addition, the time-course of incorporation in nuclei isolated by the modified procedure is linear for the first ten minutes, whereas the other types of nuclei are not capable of demonstrating linearity of transcription for this length of time.

Figure 2. Time Course of In Vitro Transcription by Four Different Preparations of Rat Liver Nuclei.

A comparison of in vitro transcription of four different preparations of rat liver nuclei. Nuclei were prepared on the same day from the same animals by the methods of (A) Blobel and Potter (1966), and (B) Chaveau et al. (1956); and nuclei which were prepared on another day from the same animals by the methods of (C) Hewish and Burgoyne (1973), and (D) the modified procedure described. Transcription in vitro was performed as described in Materials and Methods. RNA polymerase II activity is represented (▲) and was determined by the difference in [³H]UMP incorporated in the absence and the presence of 0.1 µg/ml α-amanitin; RNA polymerase I and III activities are represented by (●) and were determined from [³H]UMP incorporation in the presence of 0.1 µg/ml α-amanitin.

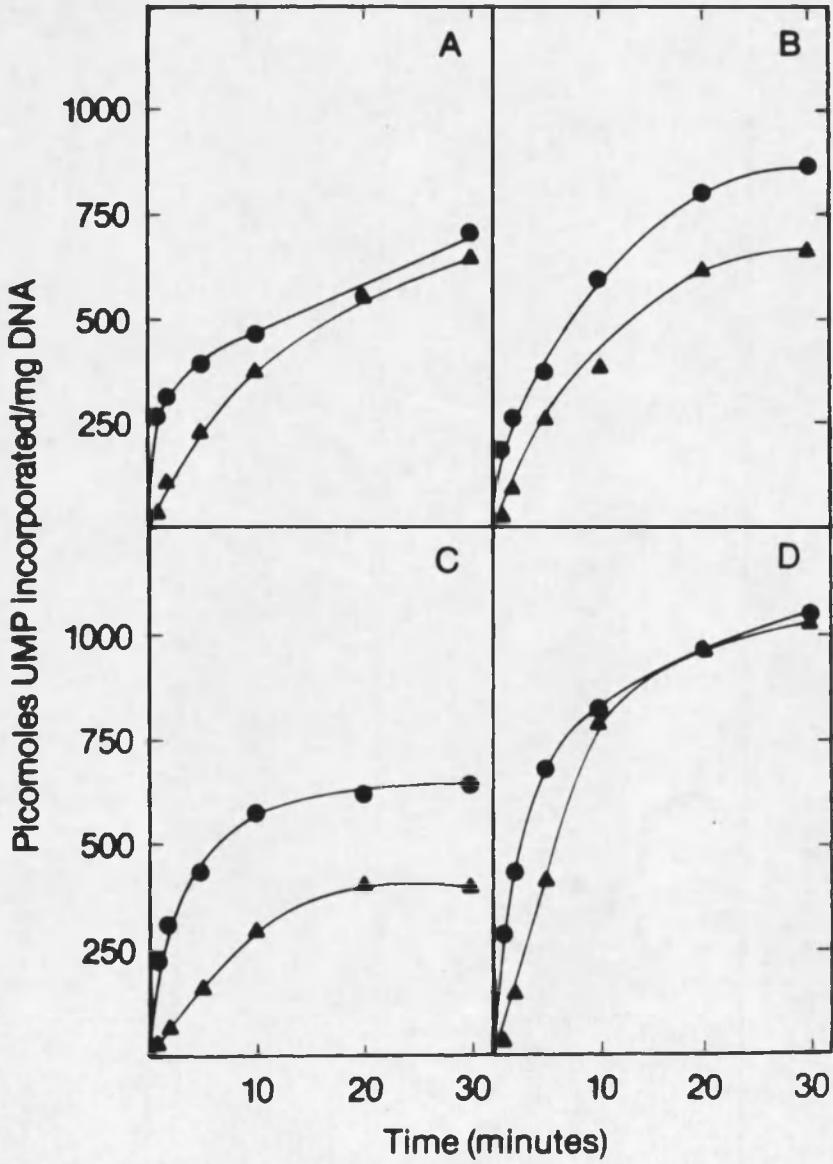


Figure 2. Time Course of *In Vitro* Transcription by Four Different Preparations of Rat Liver Nuclei.

Alkaline Sucrose Gradients of DNA from Isolated Nuclei

Since it was the intent of this investigation to isolate nuclei with highly polymerized "intact" DNA, the experiment described here was to examine the size of DNA from nuclei isolated by the four methods. The nuclei were lysed in situ on the top of 5-20% alkaline sucrose gradient on top of a 40% sucrose cushion. This method has been used before to determine a sedimentation coefficient for DNA from CHO cells (Walters and Hildebrand 1975). Figure 3 shows the gradient profiles of DNA which sedimented after ultracentrifugation for 25 hours. DNA from nuclei prepared according to the methods of Blobel and Potter (1966) and Chaveau et al. (1956) remains at the top of the gradient in one large peak and is of low molecular weight. DNA from nuclei isolated by the method of Hewish and Burgoyne (1973) and the modified method is spread out identically into three peaks, and most of this DNA is of high molecular weight. The second DNA peak from the top of the gradient is around 159S CHO DNA marker peak, and the DNA third peak is closer to the bottom of the gradient and is of higher molecular weight than 159S. The DNA from both Hewish and Burgoyne and modified nuclei is relatively intact and only slightly degraded, if at all.

The next experiment was to examine the effect of the Mn^{++} ion on the size of the DNA from nuclei prepared by our method and that of Hewish and Burgoyne. Mn^{++} is a component of the assay for the RNA polymerases. There was reason to believe it would substitute for Ca^{++} and Mg^{++} to activate endogenous endonucleases. Mn^{++} was added at a concentration of 1.6 mM to suspended nuclei for 5 minutes at 30° . In Figure 4, the results show that the DNA from both types of nuclei was

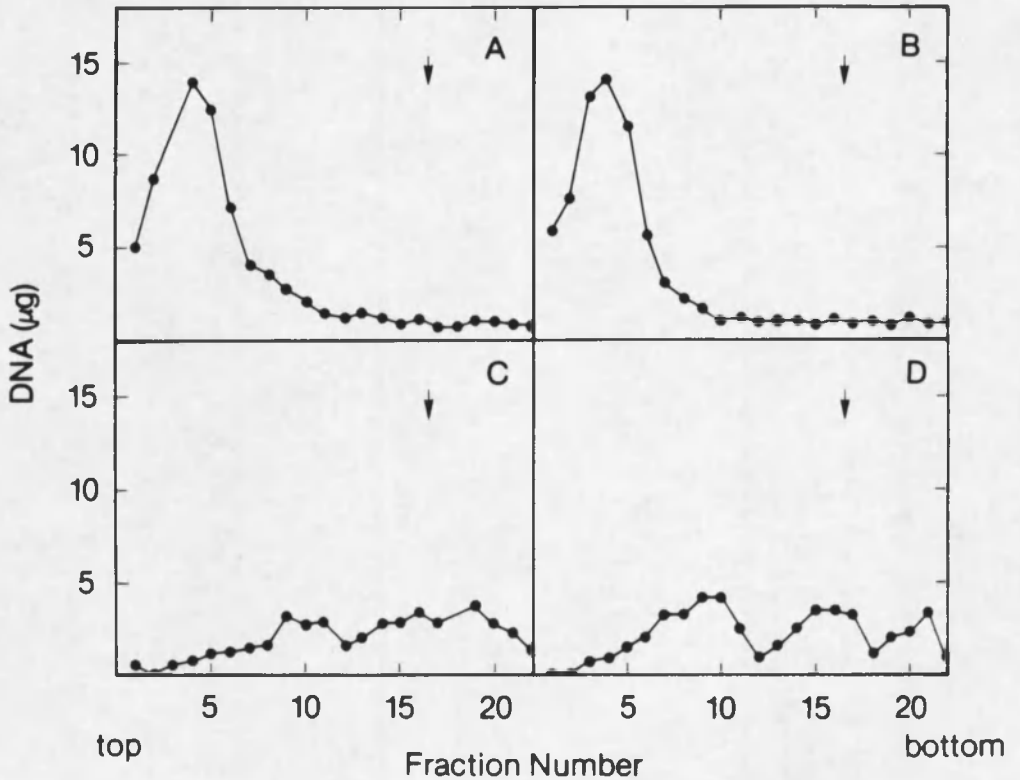


Figure 3. Alkaline Sucrose Gradients of Rat Liver Nuclear DNA.

Alkaline sucrose gradient sedimentation of the DNA from rat liver nuclei isolated by the methods of (A) Blobel and Potter (1966), (B) Chaveau et al. (1956), (C) Hewish and Burgoyne (1973), and (D) the modified procedure described. Nuclei were prepared by the methods described above and were then quickly frozen and stored in liquid nitrogen. Nuclei were thawed and added to the top of the sucrose gradients where they were lysed *in situ* according to the method of Walters and Hildebrand (1975). The gradients were then centrifuged in a Beckman SW 27 rotor at 10,000 rpm (22,000 \times g) for 25 h at 20°. Twenty-two 0.8-ml fractions were collected and assayed for DNA content as described in Materials and Methods. The quantity of DNA added was 80 μ g (A) and 70 μ g (B,C,D). DNA recovery was 89 (A), 60 (B), 44 (C), and 46 g (D), respectively. The arrow is a 159S marker of DNA obtained from CHO (1.64×10^7) cells.

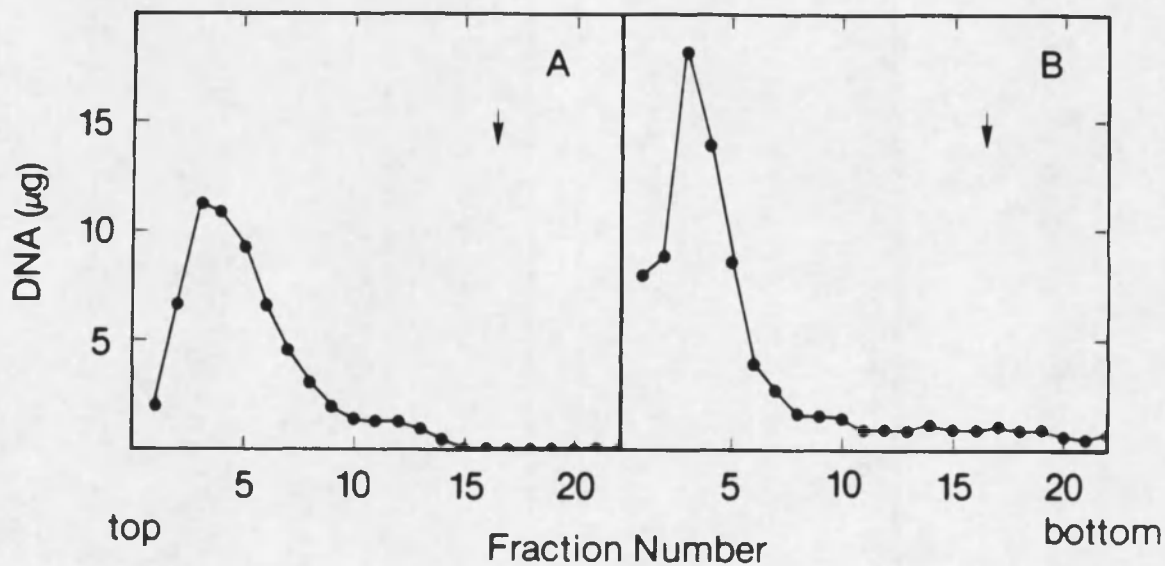


Figure 4. Alkaline Sucrose Gradients of Rat Liver Nuclear DNA Exposed to $MnCl_2$.

Alkaline sucrose gradient analysis of the DNA from rat liver nuclei isolated by the methods of (A) Hewish and Burgoyne (1973) and the (B) modified procedure described. Thawed nuclei were incubated in the presence of 1.6 mM $MnCl_2$ at 30° for 5 min. The gradients were prepared by the same methods and conditions as described in the legend to Figure 3. The quantity of DNA added was 70 (A) and 80 µg (B). DNA recovery was 62 (A) and 80 µg (B). Arrows indicate the 159S CHO cell DNA marker described above.

as extensively degraded in the presence of Mn^{++} as DNA from the Blobel-Potter or Chaveau nuclei. Therefore, the DNA from polyamine-stabilized nuclei is very labile to endonuclease digestion in the presence of Mn^{++} .

Phase Contrast Micrographs of the Isolated Nuclei

Phase contrast micrographs were taken for all of the nuclei prepared to show their relative purity. This way, any cytoplasmic contamination by ER and cellular debris would be documented. The basic nuclear morphology also could be viewed. The micrographs shown here were taken at 1800-fold magnification. The first (Fig. 5) shows nuclei isolated by the Blobel and Potter (1966) method. They appear normal and unfragmented with distinct nucleoli and without any contaminating ER. The nuclei isolated by the method of Chaveau et al. (1956) (Fig. 6) also show good morphology but are sparse and shown significant contamination. Nuclei prepared by the method of Hewish and Burgoyne (Fig. 7) show good morphology with no contamination. Nuclei isolated by the modified method (Fig. 8) are very similar in appearance to those in Figures 5, 6 and 7 and also have excellent morphology. In this micrograph, the euchromatin is highlighted very well against the darker heterochromatin and the nucleoli are very distinct. Rat liver nuclei prepared by the modified method have morphology that is the most distinct of all the nuclei that were photographed.

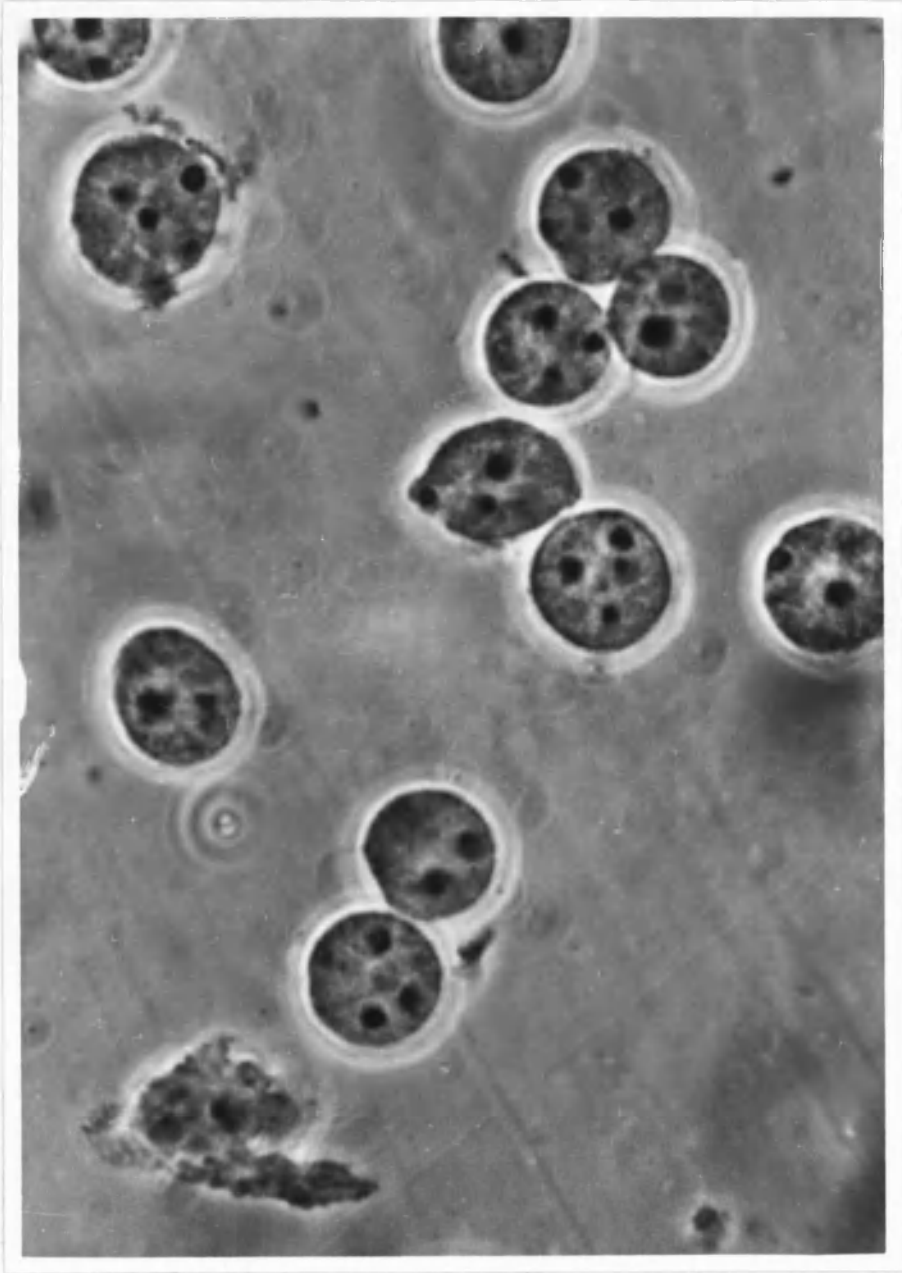


Figure 5. Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Blobel and Potter (1966).

These nuclei are spheroid in appearance. These nuclei are very good starting material for isolating nucleoli which are apparent in this micrograph.



Figure 6. Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Chaveau et al. (1956).

These nuclei appear to have normal morphology. Unfortunately, low nuclear yields are obtained by using this method as indicated by the sparseness of nuclei in this micrograph. Also, it appears that these nuclei are contaminated with artifactual material.

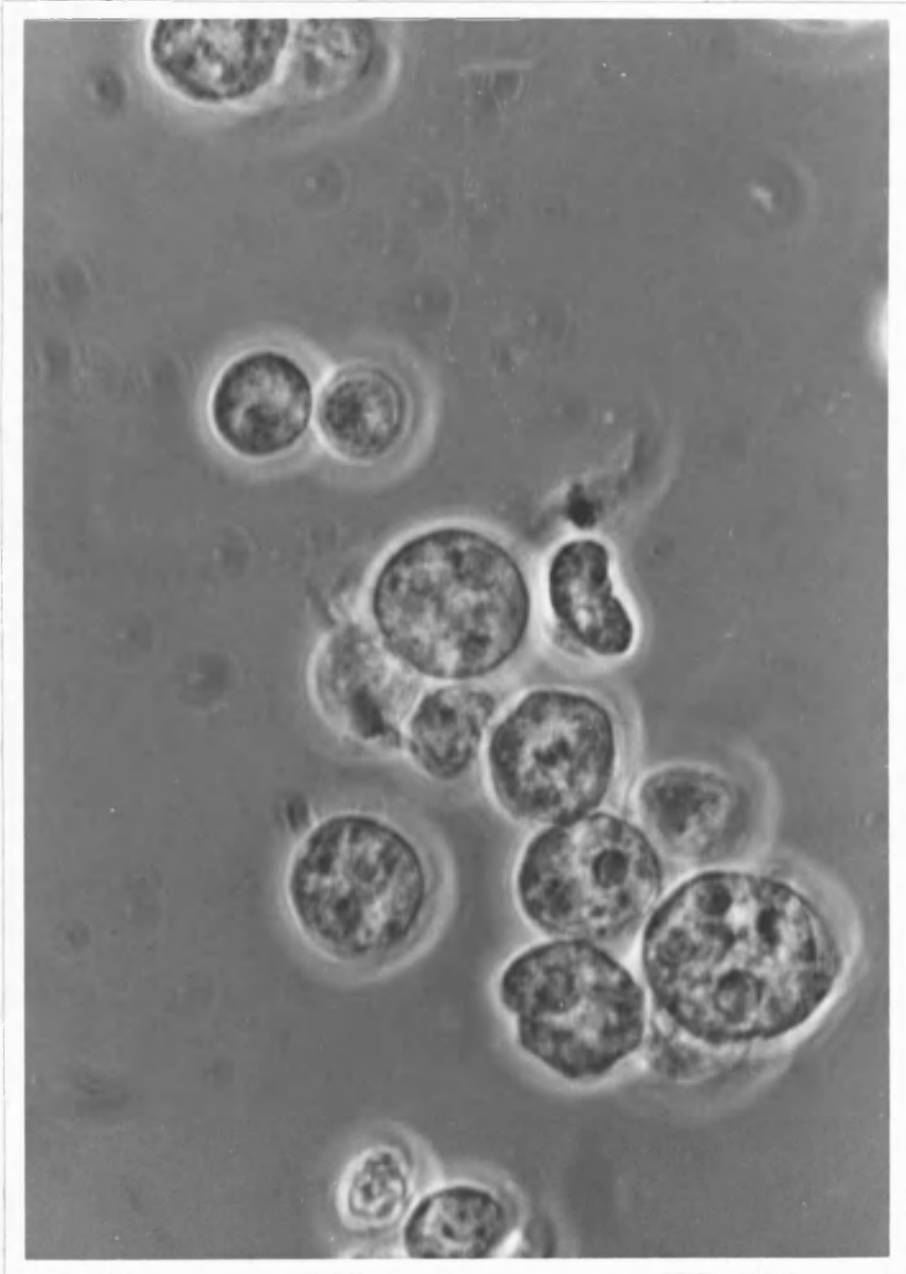


Figure 7. Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Method of Hewish and Burgoyne (1973).

These nuclei have a spheroid appearance. The nucleoli are quite distinct and euchromatin is highlighted against the darker heterochromatin. These nuclei have been observed to clump together.

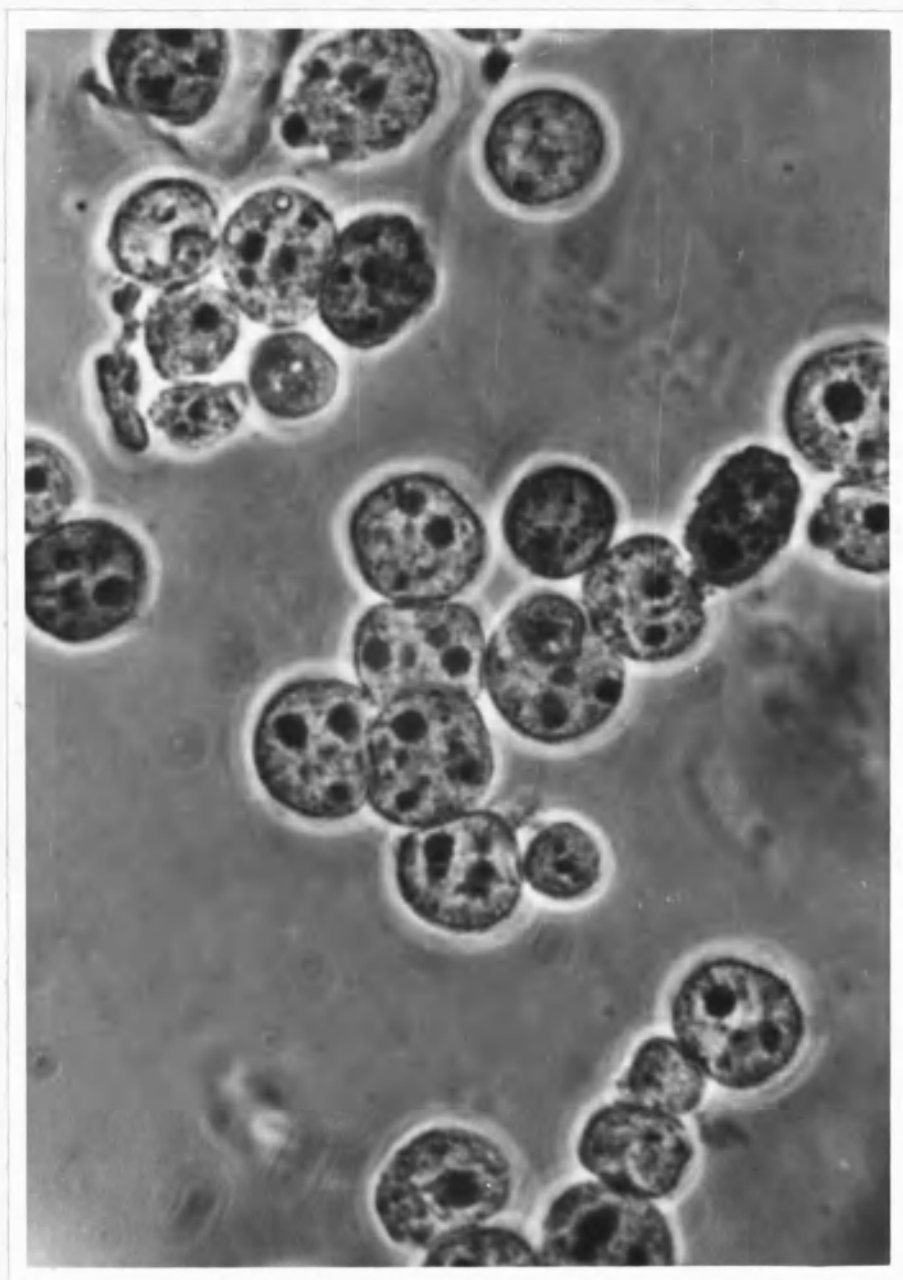


Figure 8. Phase Contrast Micrograph of Rat Liver Nuclei Isolated by the Modified Method.

These nuclei are quite similar in appearance to those seen in Figure 7 except the nucleoli appear a bit darker. Also, there is very little contamination by cytoplasmic materials.

CHAPTER 4

DISCUSSION

It was proposed at the beginning of this project that an efficient method for the isolation of enzymatically active rat liver nuclei could be developed. Nuclei isolated by this method would not only retain in situ activities of particular enzymes but also would retain DNA in a highly polymerized state. These two objectives were to function to achieve the same result: the retention of the DNA template while preserving engaged RNA polymerases in an active form.

Many methods were reviewed for their possible application to this situation. The method of Blobel and Potter (1966) had been the most familiar. Nuclei isolated by this procedure were shown by Lindell (1976) to be a very convenient system for studying the activity of the endogenous RNA polymerases in situ. Nuclei isolated by this method are quickly and easily prepared for immediate use or can also be stored in liquid nitrogen for future use. But when DNA isolated from rat liver nuclei prepared by this method was fractionated on alkaline sucrose gradients, it was discovered to be highly degraded.

Hewish and Burgoyne (1973) developed a method for isolating nuclei from rat liver which was designed to preserve the integrity of the DNA template. They found that Ca^{++} and Mg^{++} caused degradation of the DNA template by a specific endonuclease which they characterized

(see Hewish and Burgoyne 1973). To prevent extensive endonuclease digestion, they substituted the polyamines spermine and spermidine in place of Ca^{++} or Mg^{++} to stabilize nuclear structure. The chelators EDTA and EGTA were added to remove any trace divalent cations present in the liver homogenate. DNA from these nuclei are of extremely high molecular weight as measured by alkaline sucrose gradient centrifugation (Burgoyne and Hewish 1978). In addition, Woll et al. (manuscript in preparation, 1980) have been able to show that the DNA from nuclei isolated by this method is of a much greater molecular weight than DNA from nuclei isolated by the method of Blobel and Potter (1966).

Because the nuclear isolation procedure developed by Hewish and Burgoyne (1973) involved multiple centrifugation steps and was time consuming, it was felt that a more convenient modification of this procedure was needed. In addition, the loss of soluble enzymes (including RNA polymerases) from isolated nuclei has been a problem of great concern. These nuclear enzymes leach out of nuclei (Lindell 1975) even when nuclei are on ice (Liao et al. 1968). Therefore, if nuclei could be isolated faster and without as many steps, it was considered possible that they would contain more endogenous enzymes, and RNA, as well as highly polymerized DNA template.

The modified method of isolation of rat liver nuclei described in this text is a hybridization of the methods of Blobel and Potter (1966) and Hewish and Burgoyne (1973). The convenient scheme of Blobel and Potter (1966) is followed but the buffers used in the modified method are similar to those suggested by Hewish and Burgoyne (1973).

In the procedure described in this text, rat liver is homogenized in 0.25 M sucrose and diluted into two volumes of 2.1 M sucrose. After the homogenate is placed in a centrifuge tube, it is underlayered with 2.1 M sucrose and centrifuged (Fig. 1). Only nuclei are dense enough to penetrate this 2.1 M sucrose cushion.

The nuclei isolated by this method are enzymatically active, contain highly polymerized DNA and retain normal nuclear morphology. Transcription by the three RNA polymerases in nuclei isolated by the modified method described is greater than in nuclei isolated by the methods of Chaveau et al. (1956), Blobel and Potter (1966), and Hewish and Burgoyne (1973) (Fig. 2). In addition, the DNA is highly polymerized in nuclei isolated by the modified method (Fig. 3). These nuclei also contain few impurities when they are observed by phase contrast microscopy (Fig. 8). This may be due to three important components in the nuclear isolation solutions: 1) nuclear structure is stabilized by the polyamines spermine and spermidine instead of Ca^{++} or Mg^{++} ; 2) EDTA and EGTA protect nuclear DNA by chelating deleterious divalent metal ions which can promote endonuclease degradation; and 3) hyperosmolar sucrose and glycerol may allow retention of nuclear enzymes and RNA.

I have shown that rat liver nuclei can be isolated in high yield by the modified method. Despite the fact that a slightly higher yield (70%) can be achieved with the Hewish and Burgoyne nuclear isolation procedure, the percent yield of nuclei which results when using the modified procedure is also significantly high (60%) when compared to the percent yields of the methods listed (Table 1). Since the

modified procedure requires only one-third of the time to isolate nuclei than the Hewish and Burgoyne procedure, this yield was considered acceptable.

Nuclei isolated by my method contain more RNA and protein relative to DNA content. This could be due to either that the nuclei retain more protein and RNA or that nuclei are contaminated by ER, because the ER is directly attached to the nuclear envelope. The possibility of contamination by ER was eliminated by centrifugation of nuclei through a sucrose solution of sufficient density to shear off the ER from nuclei and separate the nuclei from the tissue brie. As anticipated, the isolated nuclei did not contain any attached ER when they were observed by phase contrast microscopy.

As anticipated, the activity of the RNA polymerases in situ was significantly greater in nuclei isolated by the modified method. This is probably because the DNA template is more intact, and also the loss of RNA polymerases from nuclei isolated by this method is minimized. The rate of UMP incorporation into RNA demonstrated in these nuclei is almost constant for the first 10 minutes. This was not found when nuclei isolated by the Blobel-Potter method were assayed (Fig. 2). This might be due to template destruction by the endogenous endonuclease induced by Mg^{++} which is present in the isolation and suspension buffers. The lowered activities of RNA polymerases assayed in the Hewish-Burgoyne nuclei could be due to enzymes leaching out during the many washings which these nuclei underwent during isolation and also due to the time factor involved. Nuclei isolated by the method of Chaveau et al. (1956) incorporate UMP reasonably well. However, this

method did not produce a good nuclear yield (Table 1). These nuclei have been suggested to retain RNA polymerases because of the hyperosmolar environment in which they were isolated and suspended (Yu 1975).

DNA from nuclei isolated by the method of Hewish and Burgoyne (1973) and the modified method, sedimented in an alkaline sucrose gradient (Fig. 3), reveal that the DNA are identical and of high molecular weight. However, it cannot be determined if these two DNAs are sheared or degraded from the size they might have been in vivo prior to isolation. DNA from nuclei isolated by these methods are intact and compare in size to native DNA from CHO cells which were directly lysed on top of alkaline gradients. It is, therefore, concluded that they represent a more intact preparation than other nuclear isolation methods.

The effect of Mn^{++} on the size of DNA from the nuclei isolated by the Hewish-Burgoyne and modified procedures appears to be similar to that observed for Ca^{++} and Mg^{++} (Hewish and Burgoyne 1973) where the DNA is quickly degraded. Mn^{++} may function by the same mechanism as Ca^{++} and Mg^{++} in activating the endogenous endonuclease digestion. However, because the mechanism by which Ca^{++} and Mg^{++} elicit their effect is not known, it would be difficult to speculate how Mn^{++} elicits this phenomenon.

A correlation has been made between the destruction of the DNA template and a loss in the linear time-course of UMP incorporation into RNA during in vitro transcription in Hewish and Burgoyne nuclei (Woll et al., manuscript in preparation, 1980). Since Mn^{++} is a necessary component of the assay of the RNA polymerases in isolated nuclei, these authors demonstrated that destruction of the DNA

template by Mn^{++} was rapid and time-dependent. In addition, this degradation could be correlated with a change in in vitro transcription in isolated nuclei. I speculate that as the template is degraded, RNA polymerases are probably released from the endogenous template.

The addition of divalent metal ions to nuclei isolated by the Hewish and Burgoyne method and the modified method is still a serious problem. During isolation, most of the endogenous divalent metal ions can be removed by EDTA and EGTA, but when in vitro transcription is attempted in these isolated nuclei, degradation of the template occurs in only a few minutes. Possibly, if Mn^{++} could be replaced with a divalent metal cation which does not affect DNA degradation, or with a lanthanide cation which might promote RNA polymerase activity without inducing template destruction and nuclear lysis, the utility of polyamine-stabilized nuclei would render them a valuable system for future study.

In conclusion, a convenient procedure has been developed for the isolation of rat liver nuclei. Nuclei isolated by this method contain highly polymerized DNA, more template-bound RNA polymerase activity, protein, and RNA. They have been observed by phase contrast microscopy to have no significant contamination by ER. I feel these nuclei could represent a system to reevaluate endogenous nuclear enzymology in situ.

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