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# Chromosomal Nonhistone Proteins

Volume II

*Edited by*

**Lubomir S. Hnilica**



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# Chromosomal Nonhistone Proteins

## Volume II Immunology

Editor

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## INTRODUCTION

The second volume of the *Chromosomal Nonhistone Proteins* treatise is dedicated in its entirety to the immunobiology of these proteins. Since immunological methods are adding rapidly to our knowledge of the specificity and intranuclear localization of chromosomal nonhistone proteins, a collection of chapters dealing with various aspects of this important area was judged appropriate. The reader can select a variety of topics dealing with techniques, cell and tissue specificity, autoimmune disease, virus associated nuclear antigens, and interesting localizations of select chromosomal proteins to transcriptionally active sites on polytene chromosomes. It is certain that immunological methods will contribute immensely to future inquiries into the complex puzzle of chromatin structure and function.

## THE EDITOR

**Lubomir S. Hnilica** is a professor of biochemistry and pathology at the Vanderbilt University School of Medicine, Nashville, Tennessee.

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Chapter I

IMMUNOCHEMICAL METHODS IN NONHISTONE PROTEIN  
RESEARCH

William F. Glass, II and Robert C. Briggs

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## I. INTRODUCTION: HETEROGENEITY OF NONHISTONE PROTEINS

Chromosomal proteins have been assigned to two major categories, histones and nonhistones. The five common histones are relatively small basic proteins which are almost universally found in chromatin, and exhibit limited heterogeneity between all eukaryotic cells.<sup>1-3</sup> Yet only recently have their roles in chromatin structure become

very well understood.<sup>3-4</sup> The nonhistone chromosomal proteins are generally larger and neutral or acidic in overall charge. They are also far more heterogeneous in quantities and numbers than are the histones. Further, only a few specific functions have been assigned to any of them; rather they are understood largely in terms of their associations with nuclear structures and fractions.

Though nearly all eukaryotic cells contain histones in about a 1:1 ratio by mass with DNA,<sup>2</sup> total quantities of nonhistone protein range widely from as little as 0.25:1 in chicken erythrocyte chromatin,<sup>5</sup> to greater than 2.4:1, in HeLa chromatin.<sup>2</sup> Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) may reveal only 20 to 115 bands of nonhistone proteins,<sup>1</sup> but expansion to two dimensions, isoelectric focusing followed by SDS-PAGE, has allowed visualization of 450 nonhistone protein species in HeLa chromatin.<sup>6</sup>

The great heterogeneity of the nonhistone proteins and the inability to readily determine their biological activities has made it difficult to identify and follow individual proteins with respect to distributions in different tissues and states of differentiation. Also difficult to ascertain is whether proteins that migrate similarly in electrophoretic separations and are found in different nuclear fractions are actually the same or different proteins. In some cases the presence of specific nuclear proteins only in certain cell types and differentiation states has been used to suggest their probable involvement in cellular differentiation.<sup>7</sup> Proteins common to all or many classes of cells may have roles in regulation of commonly expressed genes or shared nuclear structures and functions.

To study an individual protein in a complex mixture of proteins one must have a means of identifying that protein. The best properties by which to identify individual proteins are intrinsic enzyme or binding activities unique to each. However, many nuclear proteins may be of interest although their unique intrinsic properties are unknown. Two approaches widely used to study such proteins have been to use polyacrylamide gel electrophoresis<sup>6,8,9</sup> and immunological methods.<sup>7</sup> The former method has the disadvantage that numerous proteins in crude preparations can have similar mobilities, particularly in a single dimension. Two-dimensional methods involving isoelectric focusing have greatly expanded the resolving power of electrophoretic methods. One disadvantage in its application is that the mobility of a single protein can be shifted by modifications, thereby complicating the interpretation of experimental results where new proteins may also appear or some may be lost. Although two-dimensional gels may be used to identify interesting and specific proteins, comparison of spots between many preparations may be rather tedious and time consuming in routine small scale experimentation.

Immunological methods provide means by which specific proteins can be identified, characterized, and localized in the presence of a multitude of other proteins. In spite of the fact that identification of the intrinsic activities associated with interesting proteins may not be immediately possible, marker activities specific to each can be "created" by eliciting antibodies to them. The most frequently used immunological methods include

1. Identification of antigenic proteins separated by polyacrylamide gel electrophoresis
2. Complement fixation (Cf)
3. Radioimmunoassay (RIA)
4. Immunocytochemical localization
5. Immunodiffusion

The first category is particularly useful for identifying antigens using antisera prepared

to heterogeneous fractions of proteins. It can be used to obtain information about the distributions between subcellular fractions and tissues of numerous antigens simultaneously. If a standard preparation of a given antigen is available it can also be used for quantitation.

Complement fixation is dependent on the arrangement in space of bound antibodies and is therefore useful for studying antigen conformation. This plus the fact that it is also dependent on antibody affinity make it useful for studying antigenic differences between similar proteins.

Radioimmunoassay has been the method of choice for antigen quantitation for many years; however, until the recent development of solid phase methods it was not applicable to chromosomal proteins. Because of its dependence on antibody affinity this test is also able to distinguish between related antigens.

Immunocytochemical localization methods are widely used to study the subcellular localizations of antigenic proteins. However, these methods are difficult to control and do not easily provide for quantitation.

Immunodiffusion methods have limited applicability to chromosomal protein research because of the difficult solubility characteristics of most chromosomal proteins and the observation that antibodies to certain fractions often do not immunoprecipitate with antigen.<sup>10</sup>

Each of these methods and their applications to chromatin research is reviewed in this chapter. Certain of the methods, which we commonly use, are described in detail. Preparative methods vary greatly between different investigators and since this may have relevance to the observed results, those used by us are also described in detail.

## II. IDENTIFICATION OF ANTIGENS SEPARATED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Although these methods are among the most recently developed they are discussed first because they allow the detection of different antigens simultaneously in crude preparations. Some physical properties, such as apparent molecular weight in sodium dodecyl sulfate (SDS) or pI are immediately available for individual antigens. The ability to identify electrophoretically separated antigens is particularly valuable if one wishes to screen an antiserum against a crude preparation of proteins to determine if any activities display interesting correlations.

Recently, identification of antigenic proteins separated by polyacrylamide gel electrophoresis has been greatly simplified. Most of the older methods involved lengthy periods of diffusing antisera into and out of the gels. However, the newer methods involve replicate transfer of the separated proteins from gels onto nitrocellulose sheets<sup>11</sup> or diazobenzylomethyl-paper<sup>12</sup> (DBM-paper) followed by immunochemical detection of antigenic species. Several variations of the "immunotransfer" methods exist; however, all of these greatly shorten the time required to identify antigenic proteins.

The immunotransfer method that we use to identify nonhistone protein antigens<sup>13</sup> is adapted from Towbin et al.<sup>11</sup> Proteins are separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose sheets. Remaining protein binding capacity on the nitrocellulose is saturated with bovine serum albumin (BSA) and other serum proteins. Antigens are immunochemically localized by the peroxidase anti-peroxidase (PAP) method.<sup>14</sup> The preparative and immunological methods are described first and the applications follow. The other immunotransfer methods and their application to nonhistone protein research are then discussed. Finally, the older approaches to identifying antigens separated by polyacrylamide gel electrophoresis are briefly reviewed.

## A. Preparative Methods

Our experience has been that clean purified nuclei are essential for obtaining tissue specific antisera to nuclear proteins. For this reason the methods used to prepare de-histonized chromatin for immunization of rabbits are detailed here.

### 1. Purification of Nuclei

The methods available for the purification of nuclei vary somewhat with cell type. However, the early methods of Blobel and Potter<sup>15</sup> and Chauvea et al.<sup>16</sup> developed for the isolation of rat liver nuclei can be adapted, with minor changes, to prepare nuclei from most cell types with the exception of some blood cells.<sup>17-20</sup> The changes are primarily in the conditions required for disrupting cells.

#### a. Materials

The apparatus used for homogenizations consists of a 200 ml glass-Teflon® homogenizer (Glenco Scientific, Inc.) powered by a 3/8 in. electric drill mounted on a drill press (Sears Craftsman® Model No. 335.25926). The press is secured to the bench with one or more lead bricks. The drill speed is regulated with a variable transformer. The clearance of the tight pestle should be 0.01 in. and that of the loose pestle 0.03 in. The homogenizer should be cooled by placing it into a plastic container of ice and water.

The choice of buffer varies with cell type. The buffer chosen depends on the relative fragilities of the whole cells compared to their nuclei. Normal rat livers (also applicable to most other organs) are minced and homogenized in 0.25 M, 10 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl<sub>2</sub> termed STKM buffer. 13762 MAT-B cells (and many other tissue culture cell lines) yield intact nuclei in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5). Novikoff ascites hepatoma cells, and many other tumor cells, are particularly resistant to disruption and so require homogenization in 10 mM Tris-HCl (pH 7.5). Immediately before use, 100 µl of 100 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol is added to each 100 ml of the homogenization buffers.

Heavy sucrose is prepared by dissolving sucrose and salts with low heat on a magnetic stirrer to give 2.3 M sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl<sub>2</sub>. To obtain intact nuclei, free of debris, most cell types, particularly Novikoff hepatoma cells, must be fresh (never frozen). Further, preparation of nuclei needs to be monitored continuously by phase contrast microscopy to achieve good yields of purified nuclei.

#### b. Procedure

Cells or minced tissues are washed in 10 mM sodium phosphate (pH 7.2) buffered saline (PBS). The washed cells are disrupted in 10 volumes of the appropriate homogenization buffer by several passes of the tight pestle in the homogenizer. Extent of cell disruption is determined by phase contrast microscopy; homogenizations are continued until at least 80% (routinely greater than 95%) of the nuclei are released from the cells. Homogenizations should be discontinued if nuclei begin to break or clump to a significant degree. Tissue homogenates are filtered through several layers of cheesecloth to remove connective tissue. The homogenates are centrifuged at 1000 × g for 10 min. Pellets from tissues homogenized in STKM are homogenized directly into heavy sucrose; pellets prepared in other buffers are generally resuspended in STKM and pelleted again. The pellets are homogenized in about five times the original packed cell volume (or tissue weight) of heavy sucrose with a loose pestle. Homogenizations should be continued until nuclei and cells are well dispersed. The homogenates are poured into cellulose nitrate centrifuge tubes to about two thirds total volume. Formation of sharp interfaces, which could trap nuclei, are avoided by pouring fresh heavy sucrose



through the homogenates to fill and balance the tubes. The purified nuclear pellets are obtained by centrifugation in a swinging bucket rotor (SW27 or SW28, Beckman Instruments) at  $100,000 \times g$  for 1 hr.

Some tissues or cells require special treatments to obtain good yields of clean nuclei. In general, tissues of more fibrous nature require an initial homogenization with blades to mince the connective tissue and break the cells. When this is done in 10 volumes of STKM, the nuclei usually remain intact. We have found the Virtis® homogenizer the most effective blade type instrument. Although conditions vary with material, usually about half maximal power output for 1 to 3 min is sufficient treatment. The extent of breakage is monitored in the phase contrast microscope after every 15 sec of homogenization. The treatment is stopped once the majority of nuclei are freed from cells and before the nuclei show signs of damage.

The homogenate is filtered and nuclei purified by centrifugation through heavy sucrose as described above.

Tissue culture cells that grow in suspension require some modifications to achieve good levels of cell breakage without disrupting the nuclei. Homogenization in STKM was not found to be effective in breaking the cells as it is in the case of liver tissue. We have found that these cells are easily lysed in 0.25 *M* sucrose 10 *mM* Tris-HCl pH 7.5. However, the nuclei are very fragile and also break along with the cells. Once this occurs the chromatin and cytoplasm are not easily separated. A more efficient method involves washing (vortex mixing) the cells two times with 60 volumes of STKM or 0.25 *M* sucrose, 5 *mM* MgCl<sub>2</sub>. These cells are then suspended in 30 volumes of 10 *mM* Tris-HCl pH 7.5, 1.0 *mM* MgCl<sub>2</sub> by vortex mixing. Many cells usually break at this stage and nearly complete breakage is achieved with additional incubation on ice (30 min) combined with more vortex mixing. Under these conditions, nuclei and cytoplasmic materials are well dispersed. Nuclei should remain intact, and if whole cells or nuclei with contamination remain, the material can be mechanically homogenized with a loose fitting pestle. If nuclei tend to break during the procedure, the Mg<sup>++</sup> level can be increased slightly, but it has never required more than 2 *mM* MgCl<sub>2</sub>. Once nuclei are clean, as determined by phase contrast microscopy, an equal volume of 2.2 *M* sucrose, 5 *mM* MgCl<sub>2</sub> is mixed into the homogenate. The nuclei are then purified by centrifugation through fresh heavy sucrose as described above. Nuclei should not be concentrated by low speed centrifugation while they are in 10 *mM* Tris-HCl, 1 *mM* MgCl<sub>2</sub> as this causes them to break and allows the chromatin to aggregate with cytoplasmic materials. If this occurs it is not possible to resuspend the material well enough to achieve a good separation in a subsequent heavy sucrose step.

The blood cells have required completely different approaches to obtain good yields of clean nuclei. Chicken erythroid cell nuclei have been especially difficult to isolate free from physical cytoplasmic contamination. The most effective approach involves the use of nitrogen cavitation.<sup>17</sup> About 80 ml of blood can be drawn from a normal chicken by heart puncture into a syringe containing 20 ml of SSC, 0.01% heparin. When the drawing is completed, the blood is diluted to 200 ml with SSC, 0.01% heparin. After centrifugation ( $700 \times g$ , 10 min) the buffy coat is removed by aspiration and discarded.

The erythroid cells are washed two additional times in saline, 5 *mM* KCl, 7.5 *mM* MgCl<sub>2</sub> and additional buffy coat aspirated off. The erythroid cells are diluted to 400 ml in saline, 1 *mM* CaCl<sub>2</sub> and placed in a cell disruption bomb (Parr Instruments Co., Moline, Ill.). All operations are performed at 4°C and the cavitation should be done in a cold room. The cells are allowed to equilibrate at 1250 psi for 30 min with continuous stirring. The cell suspension is released from the bomb through the outlet, and nuclei and whole cells are collected by centrifugation in a conical tube ( $700 \times g$ , 10 min). The whole cells form a red layer on the bottom of the pellet and the nuclei form a

white band above it. The nuclei can be aspirated off from the whole cell layer. Washing in saline, 5 mM KCl, 7.5 mM MgCl<sub>2</sub> and aspiration of the nuclear layer is continued until no whole cell contamination is visible before proceeding to the wash with Triton® X-100 as described below. It should be noted that we have had little success in applying this method to cell types other than chicken erythroid.

The human neutrophilic peripheral blood granulocyte was an unusually difficult cell nucleus to isolate. In this case, the main source of contamination was not microscopically visible as in the chicken erythrocyte, but originated from soluble proteins binding to nuclear materials during isolation. With these cells it was not only necessary to isolate clean nuclei, but the cells required pretreatments with particular fixatives in order to obtain nuclei free from cytoplasmic proteins.<sup>20</sup> The granulocytes were isolated from units of freshly collected citrate anticoagulated whole blood by dextran sedimentation of red cells and removal of the mononuclear cells by Isopaque-Ficoll separation. The granulocytes were washed in PBS and collected by low speed centrifugation (700 × g, 10 min). The cell pellets were freeze-dried and then treated with 5 volumes of formaldehyde(4%)-acetone for 1 min. The fixative was poured off and cells dispersed in PBS using a loose fitting Teflon® pestle in a glass homogenizer. The cells were collected by centrifugation (200 × g, 10 min) and this wash was repeated. The cell pellets were washed twice in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5 and then dispersed in 200 volumes of 0.2% CPC (cetylpyridinium chloride). Normally at this point, nuclei are freed intact from an unfixed cell during the process of being dispersed in this solution of cationic detergent. However, cells that are fixed required sonication (usually 30 to 60 sec of sonication, administered at 10-sec intervals) to achieve release of clean intact nuclei. The CPC method has been successfully employed with many different cells that grow in a suspension (suspension cultures, ascites cells and blood cells). One limitation to more general application of this method in our laboratory has been the inability to use the complement fixation test with nuclear materials exposed to CPC. Chromatins prepared from CPC isolated nuclei consistently exhibit strong anticomplementarity activity. Our use of this cationic detergent with the fixed granulocytes was out of necessity since no other aqueous method was suitable with the fixed cell. The nonaqueous methods were attempted, but nuclei could not be separated from cytoplasmic materials.

With the exception of the chicken erythroid cells and human granulocytes the sucrose method has been generally applicable in our research for isolating clean nuclei in good yields. The main attractive feature of this approach is the ability to use a heavy sucrose step to separate cytoplasmic components, and whole cells from the isolated nuclei. The separation of whole cells from nuclei is not possible when detergent methods have been used for breaking cells. This type of contamination is especially important to avoid in immunological studies of chromatin.

## 2. Preparation of Chromatin

The procedure for preparing chromatin is based on methods previously described.<sup>21</sup> Although the extents to which some preparations need to be homogenized may vary, the procedure is the same for all cell types studied.

### a. Materials

A smaller glass-Teflon® homogenizer is used to prepare chromatins instead of the 200 ml one used to prepare nuclei. The pestle clearances should be 0.008 and 0.02 in. Buffers are 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 0.5% Triton® X-100 (STT); 10 mM Tris-HCl (pH 7.5); 80 mM NaCl, 20 mM EDTA at pH 6.3 (SE); and 1.4 mM NaCl, 0.14 mM Na<sub>3</sub> citrate at pH 7.0 (1/100 SSC). Each buffer should contain 0.1 mM PMSF added just before use.

**b. Procedure**

Purified nuclei are suspended in 5 volumes (volume, milliliter, per original tissue volume, milliliter, or weight, gram) of STT and homogenized with a tight pestle until well dispersed. The Triton® X-100-extracted nuclei are centrifuged at  $100 \times g$  for 10 min and suspended in the same volume with 10 mM Tris-HCl (pH 7.5). The nuclei should be homogenized until they begin to rupture releasing chromatin. The number of passes of the pestle is highly variable with the source of the nuclei. The swollen, broken, and clumped nuclei are centrifuged at  $3000 \times g$ . The pellets are suspended in SE buffer to a volume equal to that of STT buffer used before, and thoroughly homogenized. In this buffer nuclei become much less swollen and usually begin to break and clump extensively. The nuclei are centrifuged at  $3000 \times g$  for 10 min and resuspended in SE buffer (one half the previous volume) and thoroughly homogenized again. After centrifugation at  $7000 \times g$  for 10 min the pellets are once more resuspended in SE buffer (with a further reduction of volume by one half), homogenized, and pelleted at  $15,000 \times g$  for 10 min. Chromatins are suspended in 1/100 SSC to a volume equal to the first SE wash and allowed to hydrate on ice for 10 min. The hydrated chromatins are homogenized by several passes of the tighter pestle and centrifuged at  $30,000 \times g$  for 20 min. Chromatins should be resuspended to concentrations of about 1 mg/ml as DNA. This can be estimated by dissolving 0.1 ml of chromatin into 1.9 ml of 5 M urea and determining the absorbance at 260 nm (chromatin at 1 mg DNA/ml under these conditions would give an absorbance reading of 1.0).

**3. Dehistonization of Chromatin**

Histones and some of the nonhistone proteins are extracted from chromatin with 5 M urea, 2.5 M NaCl, at either pH 5.0 or 6.0. The buffers that have been employed include: 0.05 M sodium acetate pH 6.0,<sup>21</sup> 10 mM potassium phosphate pH 6.0,<sup>22</sup> 50 mM sodium phosphate buffer pH 6.0,<sup>23</sup> sodium succinate buffer, pH 5.0.<sup>24</sup> The buffer most commonly employed for these studies is 10 mM  $K_2HPO_4$  pH 6.0 with the addition of 0.067 mM  $MgCl_2$ .<sup>22</sup> A certain major group of nonhistone proteins is not dissociated from chromatin<sup>24</sup> under these conditions. This may either be due to precipitation of some proteins at this reduced pH or to a tight DNA binding mechanism operating directly on DNA or through other DNA bound proteins.

**a. Materials**

To dehistonize chromatin a magnetic stirrer and a glass homogenizer with a loose fitting Teflon® pestle (as described above) are used. Stock dehistonization buffer<sup>22</sup> is 3.0 M NaCl, 7.5 M urea, 0.1 mM  $MgCl_2$ , 15 mM  $KH_2PO_4$  at pH 6.0.

**b. Procedure**

Chromatins are diluted to 0.5 or 0.6 mg/ml as DNA with deionized water and mixed with 2 volumes of stock dehistonization buffer. After stirring for 2 to 3 hr at 4°C the dehistonized chromatins are pelleted by centrifugation at  $100,000 \times g$  for 36 hr. The supernatants are poured off and the pellets are gently rinsed with deionized water. The pellets are suspended to 1 to 2 mg/ml in 2 mM Tris-HCl pH 7.5 by homogenization with a loose pestle and stirring overnight at 4°C. Undissolved materials are removed by centrifugation at  $1000 \times g$  for 5 min.

**B. Immunological Methods**

Although the immunological methods detailed in this chapter are applied to a specific case, that of antibody to dehistonized chromatin preparations, the general approach of raising antisera to crude fractions has been used with many other cell or nuclear fractions. Particularly fruitful has been studies on plasma membrane antigens.

Table 1  
IMMUNIZATION SCHEDULES

WK	Adjuvant	Mode	Total dose ( $\mu$ g DNA)	Bleeding
Rabbits A & B				
0	—	—	—	Preimmune
0	Complete	ID,TP	300	—
1	Complete	ID,TP	300	—
2	—	—	—	First immune
2	Incomplete	ID	300	—
3	Incomplete	ID	300	—
4	—	—	—	Second immune
4	Incomplete	ID	300	—
5	Incomplete	IM	300	—
6	—	—	—	Third immune
Rabbits C & D*				
0	—	—	—	Preimmune
0	Complete	ID,TP	300	—
1	Complete	ID,TP	300	—
2	Incomplete	ID	300	—
3	Incomplete	ID	300	—
4	—	—	—	First immune
4	Incomplete	IM	300	—
8	None	IV	300	—
9	—	—	—	Second immune
12	None	IV	300	—
13	—	—	—	Third immune

*Note:* Immunizations were administered according to the above schedule. Intradermal (ID) and intramuscular (IM) injections were delivered as small doses at multiple sites. Toe pad (TP) injections were administered to each of the toes on only one hind foot in a given week. Intravenous injections (IV) were prepared by mixing dehistonized chromatin in deionized water with a 10X saline solution.

Rabbit D was injected according to the same schedule except that the total dose was in each case 600  $\mu$ g of dehistonized chromatin.

### 1. Immunizations

Schedules followed in immunizing rabbits with dehistonized chromatin are shown in Table 1. Injections were prepared by thoroughly emulsifying dehistonized chromatin (1 mg/ml) with complete or incomplete adjuvant. Intravenous injections were prepared by adding 10X saline to dehistonized chromatin to give solutions 1X in saline. Modes of injection included intradermal, toepads of the hind feet, intramuscular, as well as intravenous. Antisera were collected by ear bleeding unless rabbits were to be sacrificed, in which case blood was collected by heart puncture. Sera were separated from cells by allowing coagulation to proceed at room temperature for 30 min then at 4°C overnight followed by centrifugation at 1000  $\times$  g for 10 min.

### 2. Electrophoretic Separation of the Proteins

The discontinuous SDS-PAGE method of Laemmli<sup>25</sup> with samples prepared as described previously<sup>13</sup> or by deoxyribonuclease I (DNase I) digestion provides high resolution separation of chromosomal proteins without extraction of DNA.

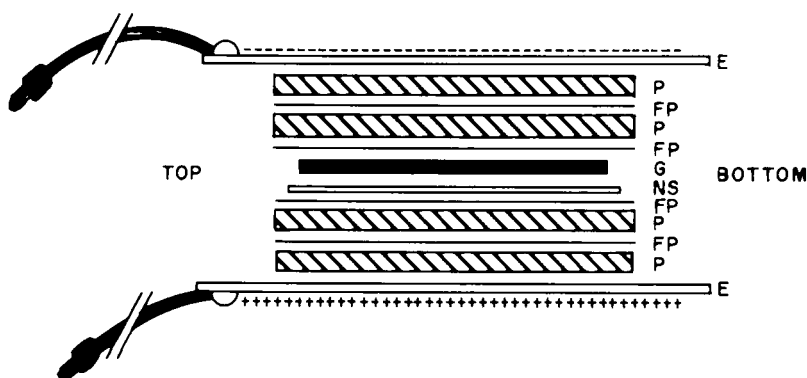


FIGURE 1. Diagram showing the assembled core of the transfer device. The components shown are the (E) electrodes, (P) Scotch Brite® pads, (FP) filter paper, (G) polyacrylamide gel, and (NS) nitrocellulose sheet. (From Glass, W. F., II, Briggs, R. C., and Hnilica, L. S., *Anal Biochem.*, 115, 219, 1981. With permission.)

### a. Materials

A Sonifier Cell Disruptor, Model W185 (Heat Systems — Ultrasonics, Inc.) and a Model SE500 slab electrophoresis apparatus (Hoeffer Scientific Instruments) are used. Stock sample buffer is 0.139 *M* Tris-HCl (pH 6.8), 4.44% SDS, 22.2% glycerol, 25  $\mu\text{g}/\text{ml}$  Pyronin Y.

Polyacrylamide gels are prepared from a stock solution containing 40% acrylamide and a 1.25% *N,N'*-methylene-bis-acrylamide. Other solutions are as described by Laemmli.<sup>25</sup>

### b. Procedure

Chromatin samples in deionized water, 0.1 *mM* PMSF, at 1  $\text{mg}/\text{ml}$  DNA are sonicated 8 times for 10 sec each at 70 W. Samples are cooled on ice for 20-sec intervals between sonications. Samples are mixed with 0.9 volume of stock sample buffer and 0.1 volume of 2-mercaptoethanol. Samples are sonicated again for 90 sec continuously and heated at 100°C for 2 to 3 min. The samples are applied to polyacrylamide slab gels (resolving gel, 1.5 mm thick  $\times$  14 cm wide  $\times$  9.5 cm high; stacking gel, 1 cm high). The proteins are stacked at 10 *mA/gel* and resolved at 20 *mA/gel*.

Alternatively, samples may be prepared for solubilization by digesting the DNA with DNase I. Chromatin samples in deionized water are mixed with 30 *mM*  $\text{MgCl}_2$ , 100 *mM* Tris-HCl (pH 7.5) in a volume to volume ratio of 10:1. DNase I, final concentration, 20  $\mu\text{g}/\text{ml}$ , is added to the samples and digestion is allowed to proceed at 37°C for 10 to 20 min. Samples are made about 5 *mM* in EDTA by addition of 50 *mM* EDTA (pH 7.0). Chromosomal proteins are solubilized in stock sample buffer as described but without sonication.

## 3. Electrophoretic Transfer of Separated Proteins to Nitrocellulose Sheets

The transfer method described here was adapted from that described by Towbin et al.<sup>11</sup> Some modifications in the design of the apparatus and buffer used for transfer have been made. These modifications allow for one to use commonly available electrophoresis power supplies to accomplish transfers. The Pharmacia Model EPS 500/400 is particularly suitable since it, unlike most other power supplies, has a 400 *mA* capability.

### a. Materials

The transfer apparatus, shown, in Figure 1, is constructed from two stainless steel

plates (13 × 20 cm) with leads attached, Scotch Brite® pads, Whatman® No. 1 filter paper, rubber bands, and a 4-*l* beaker. The transfer buffer (0.0125 *M* Tris, 0.096 *M* glycine, 20% methanol) is reduced in ionic strength by one half compared to that of Towbin et al. to increase the potential across the electrodes at a given amperage. Transferred proteins are visualized by staining with amido black.<sup>11,26</sup>

#### ***b. Procedure***

Immediately after electrophoretic separation of the proteins the apparatus is assembled, as indicated in Figure 1, starting with the positive electrode and proceeding upward. The pads, filter papers, and nitrocellulose sheet (Milipore® HAWP 304 FO, 0.45  $\mu$ m) are wetted in deionized water before being placed, in proper sequence, onto the positive electrode. The nitrocellulose sheet is first floated on the surface of the water before being completely immersed. The device is assembled such that air is not trapped between contacting surfaces of the filter papers, nitrocellulose sheet, and polyacrylamide gel. The assembled apparatus is secured with several rubber bands, drained of excess water, and placed into a 4-*l* beaker containing transfer buffer. The sandwich is lowered into and removed from the buffer several times to thoroughly mix adhering water with buffer. Transfers are accomplished at eight 400 mA for 1 hr or 200 mA for 2 hr depending on the power supply used.

More recently we have acquired an EC Electroblot transfer apparatus. The most significant advantage of this apparatus is that the paladium wire used for the positive electrode does not oxidize to contaminate the nitrocellulose sheets, as can occur with the stainless steel electrode in our device (Figure 1). The instructions supplied with the apparatus are adequate. However, we found that the number of Scotch Brite® pads used was critical and varied somewhat with the age and extent of compaction of the pads. Two gels can be transferred beside each other if pads are trimmed to fit. The number of pads used with the device assembled in this manner is generally five to six per gel. Cooling appears to be more critical with this device than ours. If the transfer is executed at room temperature using the recommended power settings air bubbles apparently form next to the gel surfaces and distort the patterns of transferred proteins. Thus, the recommendation by the manufacturer that transfer be accomplished at 4°C should be strictly followed or power settings should be decreased.

#### ***4. Immunochemical Staining of Antigenic Proteins***

The PAP method of immunochemical localization<sup>14</sup> provides a rapid and sensitive means of detecting antigenic proteins on nitrocellulose sheets.<sup>13</sup>

##### ***a. Materials***

Equipment needed include a 40°C shaking water bath or incubator and rocker platforms at 4°C and room temperature. Buffers are PBS and PBS containing 3% BSA and 10% heat-inactivated calf serum (HCS). Immunological reagents used are goat or sheep anti-rabbit IgG and peroxidase rabbit anti-peroxidase. The appropriate dilutions of these reagents vary with source and must be individually titrated. The dilutions used in the described investigations were 1:40 goat antirabbit IgG (Serasource, Inc.) and 1:200 PAP (Miles Biochemicals) or prepared according to Sternberger.<sup>14</sup> Peroxidase stain is 3,3'-diaminobenzidine (0.3 mg/ml), 0.005% H<sub>2</sub>O<sub>2</sub> in 50 mM Tris-HCl (pH 7.5) prepared just before use.

##### ***b. Procedure***

Nitrocellulose sheets containing transferred proteins are saturated with protein by incubation in PBS containing BSA and HCS at 40°C with shaking for 1 hr. The protein saturated sheets are then gently rocked for either 1 to 2 hr at room temperature or

overnight at 4°C in antisera diluted into the BSA-HCS buffer. If only a few lanes are being immunochemically stained, 2 to 3 ml of diluted antiserum in an appropriate sized container suffices and 10 ml is adequate for a complete transferred slab gel. After exposure to antisera, the sheets are rinsed five times in PBS (each time with three to four times the volume of antiserum used). If the antisera used are prepared in rabbits, then the second incubations are in goat or sheep antisera against rabbit IgG. After 30 min at room temperature the sheets are washed again in PBS and then incubated with diluted PAP produced in rabbits. After 20 to 30 min at room temperature the sheets are rinsed again and finally stained for peroxidase activity. After staining about 5 to 10 min the sheets are rinsed with water and allowed to air dry.

### C. Example Applications

Glass et al.<sup>13</sup> have previously demonstrated the presence in Novikoff hepatoma chromatin of high molecular weight (100,000 to 200,000) nuclear antigens not present in normal rat liver chromatin. These results are reproduced in Figure 2. Experiments since then have centered on determining whether other antisera raised to dehistonized chromatin consistently recognize specific antigens in this molecular weight range and on determining whether these antigens are present in other cell fractions and other neoplasia.

#### *1. Comparison of Several Antisera Prepared Against Novikoff Hepatoma Dehistonized Chromatin*

Two series of antisera were raised in rabbits according to the schedules in Table 1. Sera A and B were sampled at 2-week intervals in order to determine the time course with which antibodies to the nuclear proteins would appear and the extent to which the proteins recognized would vary between rabbits and bleedings. As shown in Figure 3, antibodies observed at 1:100 dilution appeared between 2 to 4 weeks after starting weekly immunizations. At 6 weeks the titers were greater than at 4 weeks and some new activities also appeared. Interestingly, while the lower molecular weight antigens recognized by the two rabbits varied somewhat, both recognized antigens with molecular weights greater than about 100,000 which stain more strongly in Novikoff hepatoma and 13762 MAT-B mammary tumor cell line chromatins than in liver chromatin.

Another set of sera (C and D of Table 1) were also prepared against dehistonized chromatin from Novikoff hepatoma. One rabbit was injected with the same dose of dehistonized chromatin as before while the other rabbit was injected with three times this amount. Sera were collected at 4, 9, and 13 weeks. Figure 4 shows that again, antibodies were present in both sera that recognized numerous high molecular weight antigens in Novikoff hepatoma which were either absent or decreased in normal liver chromatin. Also, as with the other sera most of the variability is confined to the lower molecular weight antigens, although one noticeable exception was observed in series D antisera. Interestingly the earliest bleedings, 4 weeks, appeared to be the most specific.

#### *2. High Molecular Weight Antigens Present in Both Novikoff Hepatoma and a Mammary Tumor Line*

Although there is variation in many of the antigens recognized by sera from the four rabbits, all appear to recognize a group of antigens having apparent molecular weights of about 100,000 to 200,000 which stain more strongly in the cancer lines than in normal rat liver. The darker staining could be due either to quantitative differences in common antigens or to the presence of cancer cell specific antigens. Extensive adsorption of the 4-week bleeding of antiserum D indicates that the latter is the case.<sup>163</sup> As with the previously reported antiserum, seen in Figure 2, this antiserum also recognized

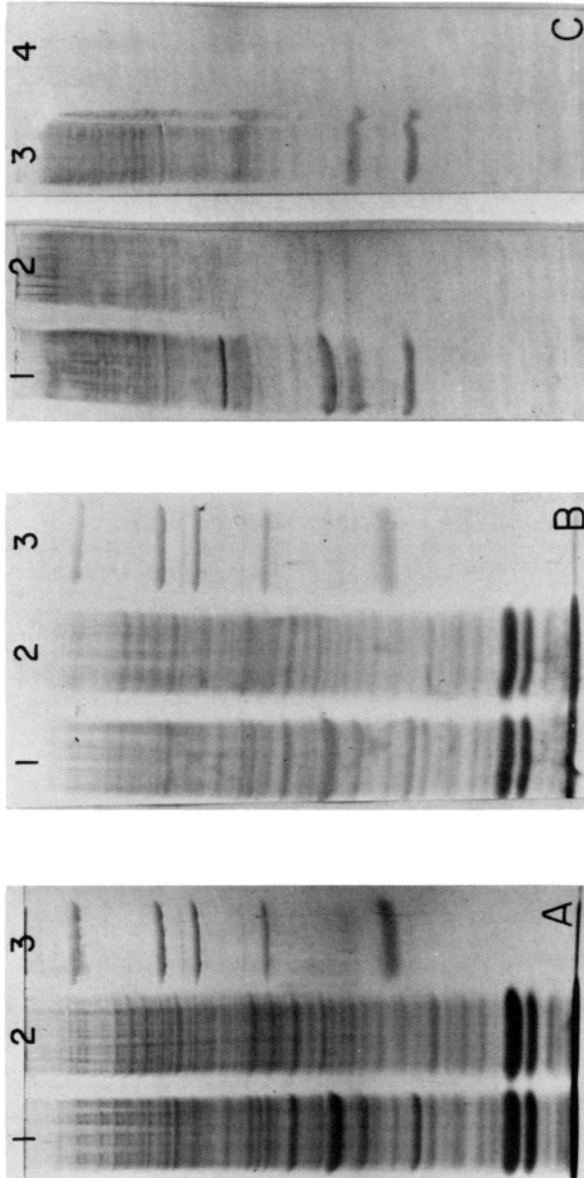


FIGURE 2. (A) Electrophoretic separation in SDS of (lane 1) proteins from Novikoff ascites hepatoma chromatin ( $25 \mu\text{g}$  as DNA), (lane 2) proteins from normal rat liver chromatin ( $25 \mu\text{g}$  as DNA), and (lane 3) molecular weight standards (Bio-Rad) myosin (200,000),  $\beta$ -galactosidase (116,500), phosphorylase B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000). (B) Samples duplicating those in (A), after electrophoretic transfer from a polyacrylamide gel to a nitrocellulose sheet. The two heavily staining bands migrating near the dye front are H1 histones. (C) Immunochemical localization reaction of electrophoretically separated and transferred proteins from (lane 1) Novikoff ascites hepatoma chromatin ( $25 \mu\text{g}$  as DNA) with antiserum to Novikoff dehistonized chromatin diluted 1:200, (lane 2) normal rat liver chromatin ( $25 \mu\text{g}$  as DNA) with antiserum as in lane 1, (lane 3) Novikoff ascites hepatoma chromatin ( $25 \mu\text{g}$  as DNA) with antiserum as in lane 1 adsorbed with normal rat liver chromatin ( $200 \mu\text{g}/\text{mL}$  as DNA), and (lane 4) normal rat liver chromatin ( $25 \mu\text{g}$  as DNA) with antiserum as in lane 3. (From Glass, W. F., II, Briggs, R. C., and Hnilica, L. S., *Science*, 211, 70, 1980. Copyright 1980 by the American Association for the Advancement of Science. With permission.)



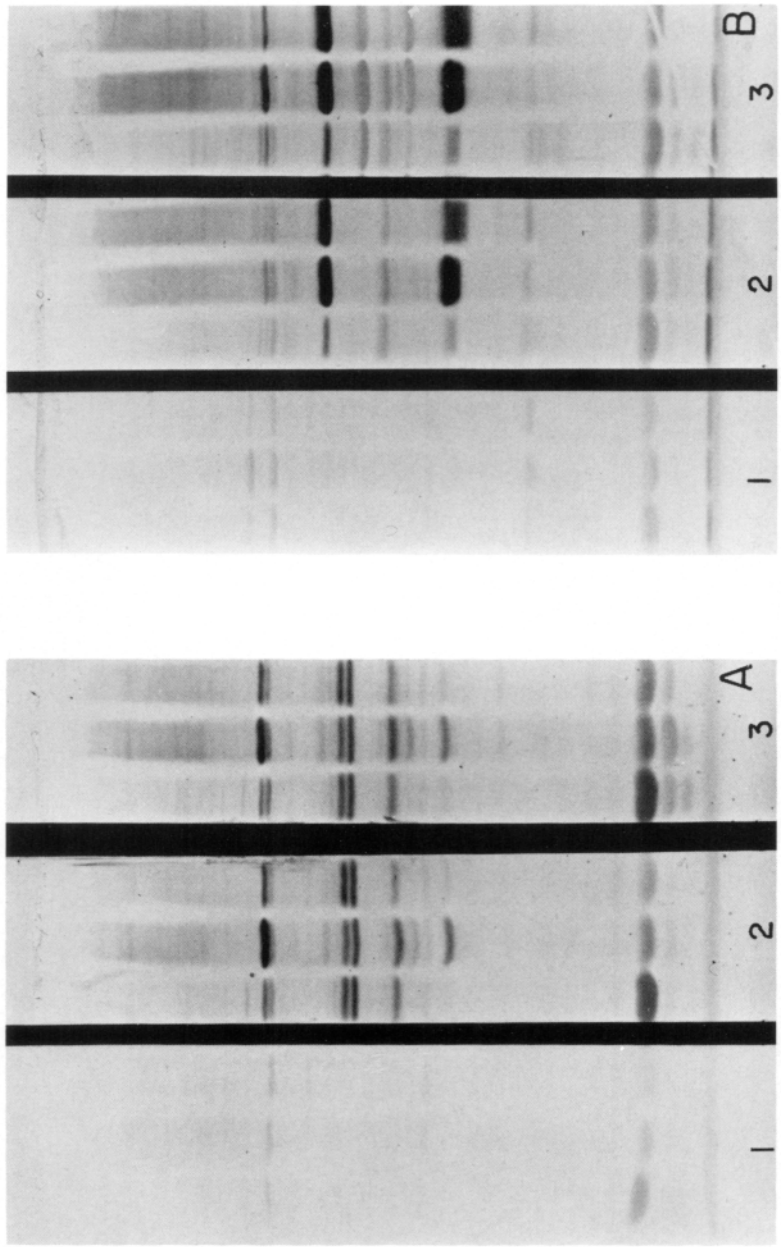


FIGURE 3. Immunotransfer staining of antigens in chromatin (25  $\mu$ g as DNA) from normal rat liver, Novikoff ascites hepatoma and rat mammary tumor 13762 MAT-B, respectively, with (1) the first, (2) the second, and (3) the third immune bleedings of antisera (diluted 1:100) from rabbits A and B described in Table 1. Proteins were separated on a 1.5 mm thick, 7.5% polyacrylamide gel.

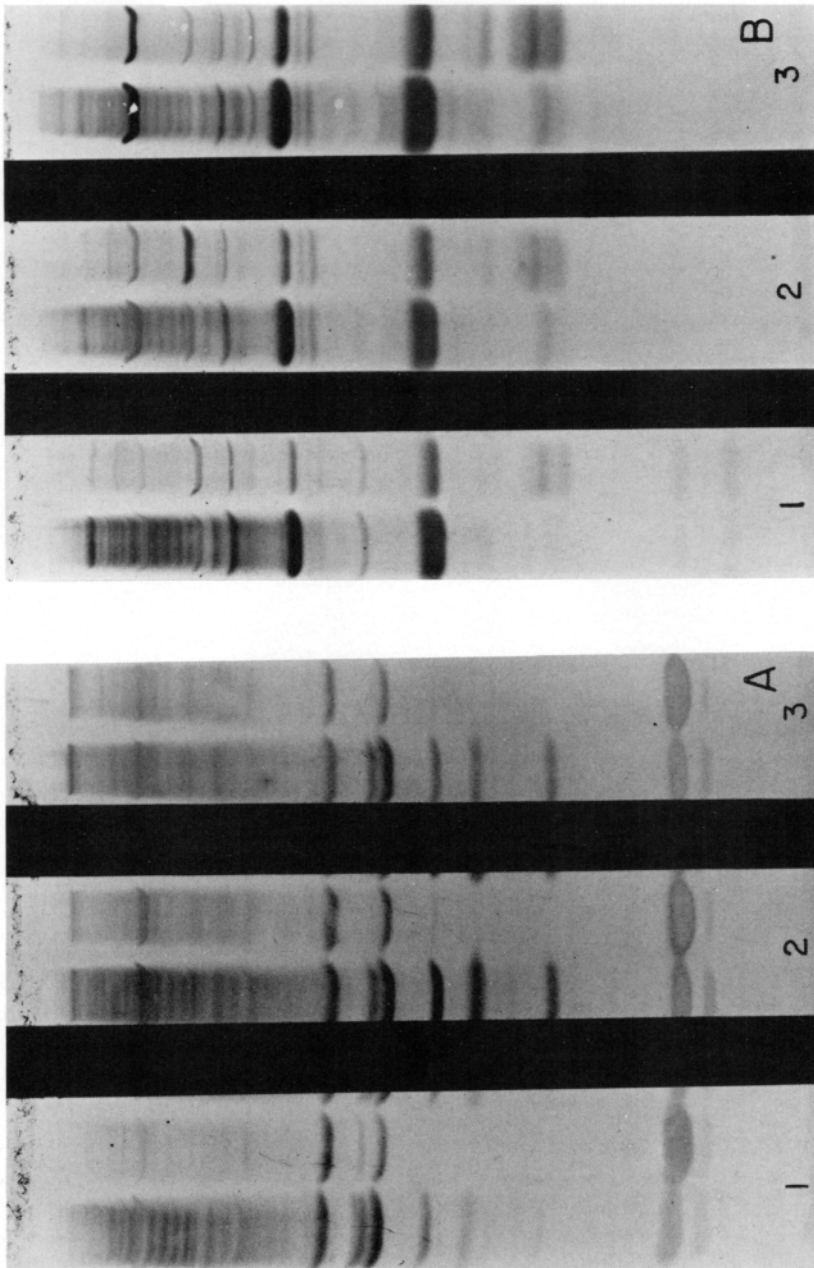


FIGURE 4. Immunotransfer staining of antigens in chromatins ( $25 \mu\text{g}$  as DNA) from Novikoff ascites hepatoma and normal rat liver, respectively, with (1) the first, (2) the second, and (3) the third immune bleedings of antisera (diluted 1:100) from rabbits C and D (A and B, respectively) described in Table 1. Proteins were separated on a 1.5 mm thick, 7.5% polyacrylamide gel.

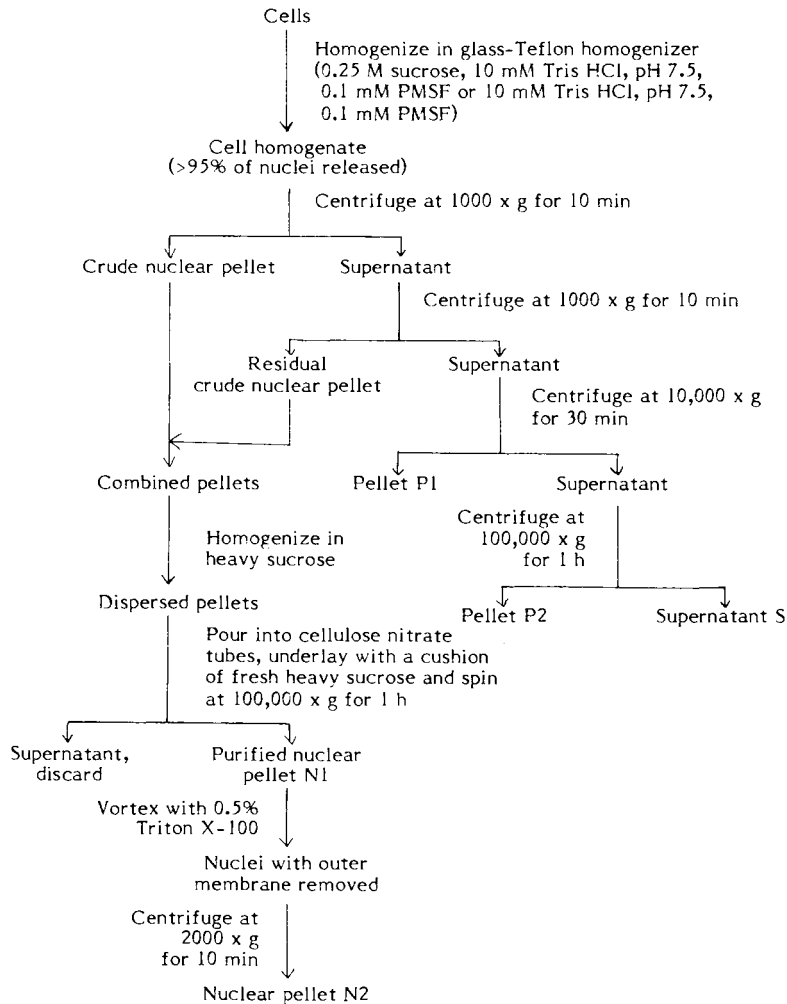


FIGURE 5. Flow diagram of the procedure used to fractionate cells. (From Glass, W. F., II, Briggs, R. C., and Hnilica, L. S., *Anal. Biochem.*, 115, 219, 1981. With permission.)

antigens not absorbed by normal liver chromatin (although activity in liver is adsorbed). Further, equal adsorption with chromatin from Novikoff hepatoma removed activity recognizing these antigens from the serum.<sup>163</sup> Even more interesting is the observation that these antigens were also present in chromatin from the rat mammary tumor line 13762 MAT-B but not in regenerating rat liver.<sup>163</sup>

### 3. Distribution of Antigens in Cell Fractions

Nuclei from the two neoplasms discussed were prepared in the absence of salts and  $Mg^{++}$  in order to rupture the cells whereas that was not the case for normal liver nuclei (consult preparative methods). Thus, the possibility exists that differences in these methods altered the fractionation of these antigens. To investigate the possibility that these antigens may be present in other cellular fractions, normal liver, Novikoff hepatoma and MAT-B cells were fractionated according to the scheme in Figure 5, described previously.<sup>27</sup> Immunotransfer staining of antigens in these fractions with unadsorbed antiserum D, week 4, indicated that these antigens are not present in other

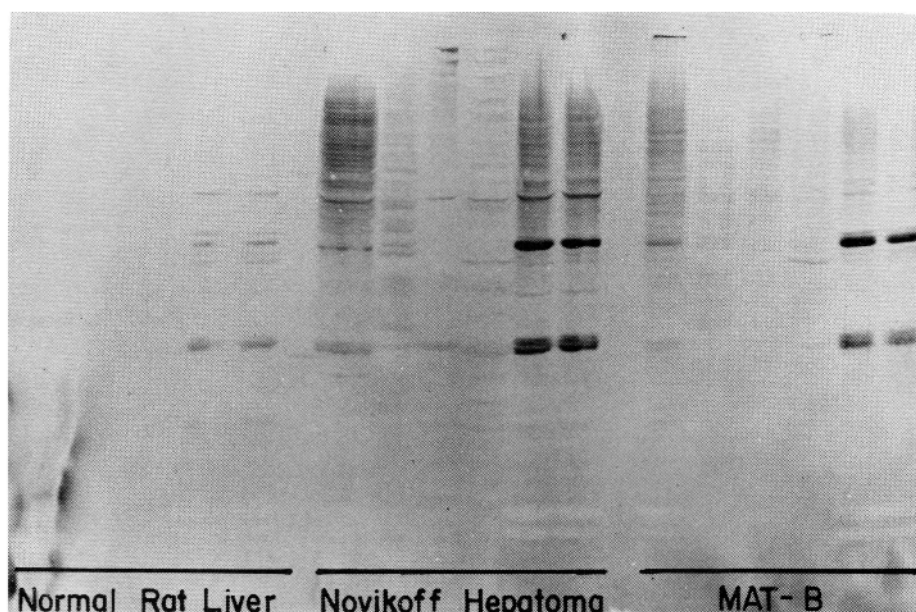


FIGURE 6. Immunotransfer staining of antigens in (C) whole cells ( $2 \times 10^5$  cells each) and (P1, P2, S, N1, and N2, respectively) cell fractions ( $5 \times 10^5$  cells each) obtained as described in Figure 5 from normal rat liver, Novikoff ascites hepatoma and rat mammary tumor 13762 MAT-B. Samples in the lanes between the tissue fractions are molecular weight standards (Bio-Rad®).

liver fractions (shown in Figure 6). Further, these results confirmed that these antigens are primarily nuclear. Results with adsorbed antiserum D also supported this contention.<sup>163</sup>

These experiments illustrate the utility of the immunotransfer method of antigen identification for studies involving antisera to crude preparations. Further, this immunological method is equally applicable to any fraction of the cell making it a valuable tool with which to evaluate cell fractionation procedures.

#### D. Other Immunotransfer Methods

The description of immunotransfer methods has been limited primarily to a modified version of the immunotransfer method of Towbin et al.<sup>11</sup> However, several variations of immunotransfer methods have been reported, some of which have been applied to studies on chromosomal proteins.

Renart et al.<sup>12</sup> were the first to publish an immunotransfer method. They separated simian virus 40 virion proteins by SDS-PAGE in cleavable cross-linked polyacrylamide gels containing agarose. After cleavage of the cross-linker, they transferred the separated proteins onto diazobenzylxymethyl-paper<sup>28</sup> essentially as described by Southern<sup>29</sup> for transfer of DNA to nitrocellulose sheets. The transferred antigens were detected by incubation with antisera followed by  $^{125}\text{I}$ -labeled protein A. They showed that an antiserum-specific for virion protein VP3 and VP2 did not react with VP1. They also found that an antiserum raised in rabbits to simian virus 40-transformed rabbit kidney cells was primarily directed against a periodate sensitive moiety present in tumor (T) antigen from infected or transformed cells, while an antiserum raised in rabbits to large T antigen purified from lytically infected monkey kidney cells was not directed against periodate sensitive determinants. To determine the transfer efficiency they blotted  $^{125}\text{I}$ -labeled standard proteins overnight from composite gels containing 12% acrylamide. The five proteins transferred with efficiencies varying between 11 and 16%.