Radioautographical and Biochemical Studies on Nucleoplasmic Glycoproteins

A thesis submitted to the

Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree

of Doctor of Philosophy

by

Richard John Hemming

Department of Anatomy
McGill University
Montreal, Canada

December 1992

ISBN 0-315-91835-7

Short Title: Radioautographical and biochemical studies on nuclear glycoproteins

Abstract

EM radioautography was used to examine the tissue distribution of cells exhibiting nucleoplasmic labeling after being exposed to ^{3}H-sugars or ^{35}S-sulphate to indicate the general extent of the occurrence of nucleoplasmic glycoproteins within animal cells. The observation of some degree of such labeling in virtually all cells in tissues of three animal species suggests that nucleoplasmic glycoproteins are a common cellular feature. To better define the distribution and nature of the putative labeled nucleoplasmic glycoproteins, cultured cells were used as a model cell type for both quantitative EM radioautographic and biochemical studies. After exposure to ^{3}H-sugars, all three lines of cultured cells examined exhibited significant nucleoplasmic reaction in which the euchromatin, heterochromatin and nucleoli were all labeled to some extent. Studies on isolated, envelope-depleted nuclei from myeloma cells confirmed that the molecules in the nucleoplasm itself were the source of the radioautographic reaction observed over nuclei. Biochemical analyses of fractions of isolated nuclei indicated that much of the label resided in nuclear matrix glycoproteins of different molecular weights. Lectin binding studies on nuclear matrix fractions revealed the presence of galactose, fucose, and/or sialic acid residues in proteins. Glycosidase experiments indicated that some but not all of these glycoproteins had N-linked sidechains.

Résumé

La distribution dans les tissus de cellules montrant un marquage du nucléoplasme après exposition à des sucres marqués au tritium (^{3}H) ou au sulfate (^{35}S), à été examinée par radioautographie en microscopie électronique, ceci dans le but de déterminer si ce marquage s'observe ou non dans tous les types cellulaires. Nos résultats montrent un certain marquage dans virtuellement toutes les cellules des tissus de trois espèces animales. Ceci indique la présence de glycoprotéines intranucléaires dans pratiquement toutes les cellules animales. Pour préciser la nature et la distribution des molécules marquées intranucléaires, des cellules en culture ont servi de modèle pur une étude quantitative en microscopie électronique et biochimique. Après une exposition à des sucres radioactifs les trois lignées de cellules en culture montrent une réaction radioautographique marquée du nucléoplasme dans le quel l' euchromatine, l'hétérochromatine, et les nucléoles sont tous marqués à divers degrés. L'examen de noyaux de cellules de myélomes, isolés et séparés de leur membrane confirme que des molécules du nucléoplasme sont à la source de la réaction radioautographique. L'analyse biochimique de fractions nucléaires démontre également que le traceur réside principalement dans les glycoprotéines de la matrice nucléaire. Ces glycoprotéines ont divers poids moléculaires. L'utilisation de diverses lectines, dans un étude cyto-chimique des sucres du noyau, souligne la présence de

galactose, de fucose et d'acide sialique dans les glycoprotéines intranucléaires. Des expériences, faisant appel à la glycosidase montrent enfin que plusieurs glycoprotéines, mais non pas toutes, possèdent des chaines glycosidiques liées à l'azote (N) présent dans les chaines polypeptidiques.

To my Family

Acknowledgements

There are so many people to thank for their contributions during the course of these studies, some in technical and/or advisory capacities pertaining to the project itself and others by just being there for me during the highs and lows which are part of the process.

I would like to first express my gratitude to my supervisor, Dr. Gary Bennett, for giving me the opportunity to pursue this project and who provided support throughout my studies.

For their insightful discussions and miscellaneous (but important) contributions, I thank the entire academic staff of the Dept. of Anatomy under the chairmanship of Dr. Dennis Osmond, especially Dr. Gary Wild, Dr. Yves Clermont and Dr. Mike Lalli.

For their excellent technical assistance in the processing of the EM radioautographical experiments, I thank Ms. Jeannie Mui, Ms. Matilda Cheung, and Ms. Pat Hales from the EM lab. I also thank Mr. Fernando Evaristo, Dr. Beatrix Kopriwa, and Dr. Muhammed El-Alfy for their work in developing the radioautographs.

For their technical advice in some of the biochemical aspects of the work, I thank the whole crew from the laboratory of Dr. John Bergeron, especially Ali Fazel and Pam Cameron.

For his photographical work, I thank Mr. Tony Graham from our department and also Mr. Hodge from the Montreal Neurological Institute.

For their invaluable administrative assistance, I thank Ms. Karen Halse and Ms. Audrey Innes.

Lastly, there are people that I have gotten to know who have suffered the same "slings and arrows of outrageous fortune" inherent to this kind of work, who have lent a shoulder to lean on when it was needed. I thank all my coworkers for putting up with me through the years but especially Carol and Iain, Elaine, Tom, Sophie, Lucy, Lenny and Jat.

Table of Contents

List of Tables

List of Abbreviations Used in Text

Sugars

Glc - Glucose
Man - Mannose
Gal - Galactose
Fuc - Fucose
GlcNAc - N-acetylglucosamine
ManNAc - N-acetylmannosamine
NeuAc - N-acetylneuraminic acid

Lectins Cited (and basic specificity)

AAA - Aleuria aurantia - fuc
APA - Abrus precatorius - gal
BSA - Bandeiraea simplicifolica - gal, galNAc
Con A - Concannavalin A - glc, man
DSA - Datura stramonium - gal-glc, glcNAc-Ser/thr
LFA - Limax flavis - sialic acid
LPA - Limulus polyphemus - sialic acid
LTA - Lotus tetragonolobus - fuc
MAA - Maackia amurensis - sialic acid
PHA - Phaseolus vulgaris - gal-glcNAc
PNA - Arachis hypogaea (from peanut) - gal-galNAc
PSA - Pisum sativum - man
RCA - Ricinis Communis (I+II) - gal, galNAc
TTA- Tachypleus tridentatus - sialic acid
UEA - Ulex Europeus - fuc
WFA - Wisteria floribunda - galNAc
WGA - Triticum vulgaris (from wheat germ) - glcNAc, sialic acid

Terms

LM - Light microscope
EM - Electron microscope
GAGs - Glycosaminoglycans
HnRNPs - Heterogenous Ribonucleoproteins

Chemicals

Endo H - Endoglycosidase H enzyme
HRP - Horse radish peroxidase
AP - Alkaline phosphatase
BCIP - 5-Bromo-4-chloro-3-indolyl phoshate
NBT - Nitro Blue Tetrazolium
TCA - Trichloroacetic acid
PAS - Periodic acid Schiff

Chapter 1 - General Introduction

I. Introduction

It has been shown that within nuclei of higher eukaryotic cells there are a vast number of nonrandomly-distributed constituent proteins which make fundamental contributions to nuclear structure and/or function. On the one hand, there is known participation of nucleoplasmic proteins in the regulation of nuclear processes such as DNA replication, RNA transcription, RNA processing and nucleocytoplasmic trafficking of molecules (for reviews see Newport and Forbes, 1987; Nigg et al., 1988; Berezney, 1992). On the other hand, there is also an acknowledged contribution of nucleoplasmic proteins to the basic framework of the nucleus and the topological arrangement of its components.

While many of the nucleoplasmic proteins remain ill-defined or unknown in terms of specific identity and/or function, it is known that these proteins collectively form a very diverse group with reference to their physical properties. This diversity has been demonstrated by our ability to fractionate these proteins into an wide assortment of different groups for biochemical study, based either on similar physico-chemical properties or on the structural associations of the proteins with various nuclear elements. Protein fractions which have now been isolated and studied include the histone chromosomal proteins, nonhistone chromosomal (NHC) proteins, proteins of the putative nuclear matrix, nucleolar proteins, ribonucleoproteins (RNPs), and

proteins associated with the nuclear envelope. As with other cellular proteins, a source of the heterogeny of nuclear proteins is transient or permanent posttranslational covalent modifications, such as phosphorylation, acetylation, and methylation (Gerace and Burke, 1988; Elton and Reeves, 1986; Hunter, 1992). There is also an increasing amount of recent evidence that another type of modification which does occur on at least some of these proteins is glycosylation, and the importance of this modification is only now beginning to be understood.

The existence of at least certain types of glycoproteins in the nucleoplasm of cells is now generally acknowledged, but it was only a relatively short time ago that this concept was either viewed with scepticism or dismissed outrightly. In the past, the prevailing belief was that glycoproteins within cells were restricted to the luminal surface of compartments of the central vacuolar system (rough and smooth endoplasmic reticulum, nuclear envelope, Golgi complex, secretory vesicles, endosomes and lysosomes), and were excluded from the cytosol and the nucleoplasm. As recently as 1988, for example, a major textbook of cell biology stated that "The activated oligosaccharide and the specific transferase are located on the lumenal of the ER, accounting for the fact that proteins in the cytosol are not glycosylated" (Stryer, 1988).

This popular belief was derived not from any known physical incompatibility of carbohydrate side chains with the

cytosolic milieu, since glycogen molecules obviously reside in the cytosol. Rather, the belief came from the fact that, at this time, the only known glycosyltransferase enzymes capable of glycosylating cellular proteins were located on the luminal side of the limiting membrane of the rough endoplasmic reticulum and Golgi apparatus (Berger et al, 1982; Kornfeld and Kornfeld, 1985). This meant that oligosaccharide side chains could be added to protein molecules only on the luminal side of this limiting membrane, and being highly hydrophilic, these side chains would not be able to pass through the hydrophobic lipid interior of the limiting membrane to the cytosol. Thus, cytosolic proteins were not thought to be glycosylated. Similarly, since nucleoplasmic proteins are thought to be synthesized exclusively in the cytosol and then delivered to the nucleus, they would also be unglycosylated.

In spite of this prevailing belief, a significant number of scattered reports have appeared in the literature over the last twenty years, suggesting the existence of glycoproteins in both the nucleoplasm and cytosol. This evidence can be broadly classified into three categories: that from biochemical studies, that from cytochemical studies, and other indirect evidence. A summary of these reports is presented below.

II. Review of the Evidence in the Literature for the Existence of Nucleoplasmic and Cytosolic Glycoproteins

A. Evidence from Biochemical Studies

I) Nucleoplasmic Glycoproteins

A) Nonhistone Chromosomal (NHC) Proteins

1) High Mobility Group (HMG) Proteins

The first widely distributed nucleoplasmic proteins to be identified as glycoproteins and characterized in terms of their sugar content were the "high mobility group" (HMG) proteins of Friend Erythroleukemia cells or calf thymus cells (Reeves et al., 1881; Reeves and Chang, 1983; Elton and Reeves, 1986). The initial evidence indicating that these proteins were glycosylated came from the finding that they stained positively with the Periodic acid-Schiff (PAS) reagent. Subsequent colorimetric reactions specific for the detection of various sugars revealed the presence of mannose, N-acetylglucosamine (glcNAc), galactose and fucose. Lectin blotting of HMG proteins adsorbed to nitrocellulose filters with radiolabeled UEA I provided additional evidence for the presence of fucose. Further evidence of glycosylation came from experiments where the HMG proteins were demonstrated to be labeled after cells were exposed to either tritiated mannose, glcNAc, galactose, or fucose, indicating the presence of the corresponding residues in their carbohydrate side chains. Lastly, it was suggested that the oligosaccharide side chains are N-glycosidically linked to the proteins, since the linkage was not sensitive to mild alkaline borohydride

reduction (i.e. ß-elimination).

2) RNA Transcription Factors

There is good evidence that numerous nucleoplasmic proteins belong to a fairly recently discovered class of glycoproteins which have single glcNAc residues O-linked to serine and/or threonine. These glycoproteins were first described by Torres and Hart (1984) and are now known to include a diverse group of cellular proteins (reviewed in Hart et al., 1989). In fractionation studies, moieties containing these O-linked glcNAc residues were shown to exist in several subcellular fractions, including both the nuclear envelope and the envelope-depleted nuclear fractions (Holt and Hart, 1986). Among the proteins found in this latter group are some of the transcription factors for RNA polymerase II.

The first of these factors that was demonstrated to be glycosylated in this fashion was the human transcription factor Sp1 (Jackson and Tijan, 1988). This protein was shown to become labeled when incubated with UDP-^{3}H-gal in the presence of exogenous galactosyltransferase, indicating the existence of acceptor terminal glcNAc residues. The incorporated radioactivity was then found to be completely released when these molecules were subjected to an alkaline ß-elimination reaction which indicated an O-linkage of carbohydrate to protein. Lastly, biochemical analysis of the released labeled oligosaccharides by paper chromatography showed a migration identical to a galß1-4-glcNAcitol standard.

Other transcriptional factors which have since been shown to be similarly glycosylated are 1) some members of the human AP-1 family of enhancer binding proteins including the c-jun and c-fos proteins, and a "Fos-related antigen", 2) two members of the CTF/NF-1 family of human transcription factors, 3) transcription factor AP-2, 4) the mouse liver-specific transcription factor HNF1 and 5) p67 serum response factor of human HeLa cell nuclei (Tijan and Jackson, 1988; Lichtsteiner and Schibler, 1989; Schroter et al., 1990).

3) DNA Polymerases

In a number of reports studying the effects of lectins and toxins on cell-free DNA replication, Bhattacharya and co-workers provided evidence that at least some DNA polymerases are glycosylated. Working on human IMR-32 neuroblastoma cells, Bhattacharya et al. (1979) found that the lectins Con A and RCA II specifically reduced the activity of isolated DNA polymerase α to 19% and 10% respectively of controls. PHA, a gal-glcNAc-binding lectin with different specificity from RCA II, reduced DNA polymerase ß activity to 16% of controls. This evidence suggested that the effect of the lectins on enzyme activity was due to their binding to mannose and galactose (or galactosamine) associated with the catalytic portion of the DNA polymerase α and ß proteins. In the same cells, Bhattacharya et al. (1981) reported that different isoforms of DNA polymerase α were differentially inhibited in their activity by the presence of RCA II lectin. Their evidence

suggested DNA polymerase α_2 was most affected while polymerase α_3 was least affected. In another study, when DNA polymerase α_2 was isolated from tunicamycin-treated cells, its activity was inhibited by 40-60%, suggesting that the molecules lacked functionally important carbohydrate residues that would have been added in a tunicamycin-sensitive step. Additionally, RCA II had no further effect on the DNA polymerase activity of tunicamycin-treated cells, which indicated that the RCA II binding sites were no longer present (Bhattacharya et al., 1982).

Recently, Hsi et al. (1990) have also provided evidence that human DNA polymerase α is glycosylated in human KB cells by demonstrating RCA I and Con A lectin binding to nitrocellulose membranes containing purified DNA polymerase α. The lectin binding in each case was shown to be abrogated by prior treatment of the protein with the corresponding glycosidases, i.e. ß-galactosidase for RCA I and α-mannosidase for Con A.

It is notable that there has been some controversy as to the predominant intracellular localization of the DNA polymerase α in cells. Preparations of the enzyme are routinely prepared from cytoplasmic extracts of cells and one report using polyclonal antisera from partially purified DNA pol α labeled only the cytoplasm (Brown et al., 1981). Bensch et al. (1982), on the other hand, found that a panel of monoclonal antibodies to DNA pol α almost exclusively labeled the nucleoplasm.

4) Simian Virus 40 (SV 40) Large Tumor (T) Antigen

The SV 40 large T antigen proteins have been localized to the nucleoplasm of SV 40-infected or transformed cells, where they have been found to be associated with DNA (Mann and Hunter, 1979). There is evidence that in transformed TC7 cells these molecules contain glcNAc and galactose residues (and possibly mannose as well) (Jarvis and Butel, 1985, 1988; Schmitt and Mann, 1987). Further, in these studies the incorporation of labeled sugars was not affected by tunicamycin which indicated that the oligosaccharide sidechains are not N-glycosidically linked to the protein.

5) Other NHC Glycoproteins

A number of early pioneering reports describing the existence of nucleoplasmic glycoproteins came from studies on hepatocyte nuclei isolated from normal rat liver or transformed hepatocyte cell lines. Tuan et al. (1973) reported that nonhistone chromosomal proteins fractionated from either rat liver Walker carcinoma cells or normal rat liver contained a considerable amount of carbohydrate (estimated at 32% by the α-napthol reaction). Spangler et al. (1975) reported a significant amount of sialic acid in chromatin fractions of the same cell types.

Yeoman et al. (1976) isolated a 26 kd galactosamine-containing NHC glycoprotein from Novikoff hepatoma cells. Immunologically-related proteins were found to be present in chromatin of 18 day old fetal rat liver, but absent from adult

liver chromatin.

Rizzo and Bustin (1977) reported the presence of three Con A binding NHC glycoproteins in electrophoretically-separated rat liver chromatin samples with apparent molecular weights of 69, 125, and 135 kd.

Goldberg et al. (1978) demonstrated that the chromatin from both normal rat liver hepatocytes and Novikoff hepatoma cells showed similar Periodic Acid-Schiff (PAS)-positive bands (30, 54 and 75 kd) on treated polyacrylamide gels, which indicated the presence of common glycoproteins in those fractions. The Novikoff hepatoma chromatin also contained additional bands of 64, 104 and 127 kd, while a 16 kd band present in normal rat chromatin was much more strongly stained than that from the tumor cells. When samples of these chromatin fractions were subjected to rocket affinoelectrophoresis with various lectins, both normal and tumor cell chromatin fractions were found to bind Con A, WGA, and/or APA indicating the presence of mannose, glcNAc and fucose, respectively. However, there was much greater lectin binding to proteins observed in the tumor cell chromatin versus that of normal adult rat livers suggesting a higher carbohydrate content in tumor cell chromatin.

Stryjecka-Zimmer et al. (1982) reported that proteins of 39, 49, and 56 kd isolated from Novikoff hepatoma chromatin stained positively with PAS in polyacrylamide gels indicating them to be glycoproteins. Proteins of similar molecular weight

in normal rat liver did not stain positively with PAS. Biochemical analysis indicated that all the sugar residues were of the amino type.

In studies on isolated sea urchin embryo nuclei, Sevaljevic and Krtolica (1973) found that some "high mobility fractions" of the nonhistone chromosomal proteins separated by SDS-PAGE stained with the PAS reaction. In subsequent studies on these same nuclei, a number of proteins from both chromatin and nuclear matrix fractions were found to bind Con A, indicating the presence of mannose residues (Sevaljevic et al., 1979; Sevaljevic et al., 1981).

When Stein and co-workers exposed cultured HeLa cells to either ^{3}H-glucosamine or ^{3}H-fucose, there was an observable uptake of label into numerous high and low molecular weight nonhistone nuclear proteins of chromatin fractions (Stein et al., 1975). To establish that the glycoproteins were bona fide nucleoplasmic constituents and not cytoplasmic contaminants resulting from the isolation procedure, trypsin-treated ^{3}H-glucosamine-labeled cellular material or similarly-labelled isolated plasma membrane fractions were added to unlabeled isolated chromatin which was then re-isolated and examined for radioactivity. No significant amount of label was found in the re-isolated chromatin fractions indicating they were not contaminated by cytoplasmic elements.

Gronow et al. (1979) observed a considerable amount of labeled material to be associated with the nonhistone nuclear

protein fraction of ^{14}C-glucosamine-labeled HeLa cells indicating the presence of glucosamine-containing glycoproteins.

Miki et al. (1980) demonstrated specific binding of ^{125}I-labeled Con A to 45 kd and 130 kd proteins of mononucleosome fractions of Ehrlich ascites tumor cells, indicating the presence of mannose residues. The mononucleosomes were also found to become labeled when the Ehrlich cells were incubated with ^{3}H-glucosamine suggesting the presence of glcNAc residues.

D'Erme et al. (1987) reported that NHC proteins with molecular weights of 24, 34, and 38 kd, which were tightly bound to chromatin loops in pig kidney nuclei, bound Con A and could be stained with dansylhydrazine indicating them to be glycoproteins.

In studies carried out on sea urchin embryonic cells, Kinoshita et al. (1988) showed that fluorescent dye-labeled lectins (Con A, RCA I, SBA, TTA, UEA, WGA) bound to isolated chromatin fractions. These results suggested the presence of mannose, galactose, galNAc, sialic acid, fucose, and glcNAc. For some lectins the extent of binding was found to change depending on the stage of development of the embryos.

Polet and Molnar (1988) incubated isolated human lymophocytes with ^{3}H-mannose, ^{3}H-glcNAc, ^{3}H-galactose or ^{3}H-fucose and showed that, in each case, numerous acidic nonhistone nuclear proteins became labelled. The labeling was

inhibited by tunicamycin, indicating that the sugars were part of N-glycosidically-linked glycoproteins.

The chromatin of Drosophila melanogaster was shown to contain glycoproteins bearing single O-linked glcNAc residues, as evidenced by the finding that they became radiolabeled with UDP-^{3}H-gal in the presence of exogenous bovine galactosyl transferase (Holt et al, 1987). These molecules were not characterized further, but could presumably include transcription factors similar to those described by Tjian and Jackson (1988) (described in a previous section).

Tischendorf et al. (1988) reported the presence of four major nuclear glycoproteins in the Y chromosomal giant lampbrush loops of Drosophila hydei spermatocytes. Proteins of 38, 58, and 98 kd reacted with the lectins Con A and UEA I, indicating the presence of mannose and fucose residues. A 55 kd protein reacted with UEA I, BSA I, DBA, WGA, and PSA, indicating the presence of fucose, galactose, terminal galNAc, glcNAc and/or sialic acid and glucose respectively.

Finally, in studies to investigate the possible existence of nuclear glycosyltransferases, Richard et al. (1975) incubated isolated rat liver nuclei with radiolabeled nucleotide sugars GDP-man, UDP-gal, UDP-glcNAc and UDP-galNAc, and demonstrated that endogenous nucleoplasmic proteins became labeled, thus providing evidence for glycosyltransferases specific for mannose, galactose, glcNAc, and galNAc. In similar studies, chromatin-associated proteins were shown to

become labeled when isolated nuclei from monkey liver were incubated with GDP-^{14}C-mannose or UDP-^{14}C-glcNAc (Bertillier and Got, 1980; Bertillier et al, 1982). Fayet et al. (1987) found that proteins from chromatin of rat liver became labeled when nuclei were incubated with GDP-^{14}C-mannose and UDP-^{14}C-glcNAc. Galland et al. (1988) showed that the transfer of labeled mannose or glcNAC to endogenous protein acceptors in chromatin was largely inhibited by tunicamycin, suggesting that many of the side chains were N-glycosically-linked to protein. In all of the above studies, the location of the glycosyltransferase enzymes was not established, but these works did provide evidence for the presence of nucleoplasmic glycoproteins with mannose, galactose, glcNAc, and galNAc residues.

B) Histones

Levy-Wilson (1983) provided evidence that histone proteins of the macronuclei of Tetrahymena thermophila are glycosylated. All five types of histones were found to be labeled after the cells were exposed to ^{3}H-fucose, and the amount of radioactivity incorporated indicated that at least 1 in every 1000 nucleosomes contained a fucosylated H2A-type histone. The histones also bound radioiodinated UEA I and Con A, providing further evidence for the presence of fucose residues, while suggesting the presence of mannose residues as well. The author speculated that glycosylation of histones could be related to a high degree of genome transcription.

Thus in Tetrahymina macronuclei, where a very high proportion of the genome is transcribed, the degree of glycosylation could be much higher than that occurring in the nuclei of mammalian cells where the proportion of the genome that is transcribed is much lower.

C) Nuclear Matrix Glycoproteins

Berezny and Coffey (1977) reported the presence of small amounts of carbohydrate in the form of neutral sugars associated with nuclear matrices isolated from rat liver, representing 5.5% of the total matrix composition. The sugars present were identified to be mostly glucose, mannose, and galactose residues. Only a very tiny amount of sialic acid (<0.1%) was detected.

Baglia and Maul (1983) found that ATP-dependent ribonucleoprotein release from nuclei, as well as a nucleoside triphosphatase activity essential for nucleocytoplasmic transport could be inhibited with either Con A or WGA lectins in clam oocytes. Specific antibodies raised against clam lamin B proteins of the nuclear matrix also inhibited these activities, suggesting that lamin B might be a glycoprotein containing mannose and glcNAc residues.

D'Erme et al (1987) found Con A-binding glycoproteins in the chromatin matrix of pig kidney nuclei having molecular masses ranging from 15 to 62 kd. Carma-Fonseca (1988) reported that the major androgen-dependent proteins associated with the nuclear matrix of the rat ventral prostate were glycoproteins

which bound Con A and WGA. This group of 20 kd proteins was found to be dramatically reduced in terms of expression following castration of the animals.

Burrus et al. (1988) found that some tumors of rat liver had Con A- and RCA I-binding glycoproteins (of 55 and 95 kd) in a high salt extracted nuclear matrix fraction suggesting the presence of mannose and galactose residues. These glycoproteins were absent in a corresponding fraction of normal rat liver nuclei.

Ferraro and her co-workers have recently described nuclear matrix glycoproteins in a number of recent reports. Numerous Con A- and UEA I-binding glycoproteins were found in nuclear matrix fractions isolated from pig liver, heart, and kidney tissues, indicating the presence of mannose and fucose residues (Ferrero et al 1989; Cervoni et al, 1990). These investigators have also reported Con A-binding to lamin A molecules (as identified by two-dimentional electro-phoresis) in pig and chicken liver (Ferraro et al., 1989b). Finally, they provided evidence that many of the Con A-binding nuclear matrix proteins of ox, pig and chicken liver have N-glycosylated side chains (sensitive to N-glycosidase F) and bind to DNA (Ferraro et al., 1991).

D) Nuclear Envelope-associated Glycoproteins

1) Nuclear Pore-associated Glycoproteins

Nuclear pore-associated glycoproteins have been identified and studied by a number of investigators. The glycoproteins

thus far described are all of the novel class (described earlier) in which the carbohydrate side chains consist of a single glcNAc residue O-linked to serine or threonine in the protein chain. Schindler and Hogan (1984) provided the first evidence for the existence of proteins containing single glcNac residues in rat liver nuclei. Holt and Hart (1986) found that incubation of various cell fractions with UDP-^{14}C-galactose and exogenous galactosyltransferase resulted in the labeling of a variety of glycoproteins. ß-elimination resulted in the cleavage of labeled side chains which migrated with a ^{14}C-gal-glcNAcol standard in descending paper chromatography, indicating that the original side chains had consisted of single glcNAc residues O-linked to the protein core. There was an enrichment of these O-glcNAc containing proteins in nuclear envelopes. Subsequently, Davis and Blobel (1986) isolated a protein (p62) from rat liver nuclear envelopes and found it to be a constituent of the nuclear pores. It bound WGA but not Con A, indicating that glcNAc but not mannose residues were present. From these results, and the above findings of Holt and Hart, they postulated that this protein might contain O-linked glcNAc residues. In follow up studies, pulse-chase radiolabeling of rat liver cells with ^{35}S-methionine, followed by immunoprecipitation of p62 with a monoclonal antibody, showed that most of the glcNAc residues of this protein were added during the first 5 minutes of its synthesis (Davis et al., 1987).

Studies by Schindler et al. (1987) provided further confirmation of the presence of a 62 kd protein containing O-linked glcNAc side chains in rat liver nuclear envelopes.

Concomitant studies by Holt and coworkers (Holt et al., 1987; Snow et al., 1987) showed by the use of monoclonal antibodies to a nuclear pore complex fraction that 8 different proteins (including one integral membrane protein) shared common epitopes, and all were similarly glycosylated with O-glcNAc at 10-12 sites on mostly serine residues. Further, if the glcNAc residues were removed or masked by galactose, the binding of the monoclonal antibodies was severely reduced, which indicated these residues were part of the antibody binding region.

2) Nuclear Envelope Poly A Polymerase

A 64 kd protein with poly A polymerase activity was isolated from nuclear envelopes of rat liver hepatocytes (Kurl et al. 1988). Poly A polymerase has previously been found to be an enzyme with different isoforms localized either to the cytosol or nucleoplasm of cells (reviewed by Jacob and Rose, 1983). The protein described by Kurl and co-workers, which showed more of a resemblance to the nucleoplasmic enzyme isoform in its physical properties, was found to specifically bind Con A, indicating it to be a glycoprotein. Additionally, this lectin also had an inhibitory effect on the enzyme's activity, suggesting that at least one site of the mannose residue(s) was in the catalytic portion of the molecule. While

it was not stated that the saccharide residues of this protein had a nucleoplasmic orientation, presumably this would be the case because of the saccharide effect on the enzyme function of catalyzing the addition of poly A to mRNA molecules.

E) Proteoglycans and Glycosaminoglycans

The existence of proteoglycans, which carry glycosaminoglycan (GAG) side chains, has also been reported in the nucleoplasm. Kinoshita (1971) reported a prominent uptake of $^{35}SO_4$ into heparin sulfate molecules in sea urchin embryos coinciding with the onset of gastrulation of the urchin embryos. It was also observed that exogenous heparin added to isolated nuclei caused an increase in RNA synthesis. It was concluded that heparin may have an important role *in vivo* in gastrulation by its action on RNA synthesis. Further biochemical studies showed the presence of proteoglyans in the nucleus, where they were preferentially associated with the transcriptionally active chromatin (Kinoshita, 1974). Pulse labeling studies with ^{14}C-glucuronic acid provided evidence that these proteoglycans were synthesized in the cell cytosol and transported to the nucleus, again indicating a potentially important regulatory role for these molecules in RNA transcription. Lastly, when permealized intact embryos were exposed to isolated sea urchin proteoglycans, the proteoglycans were observed to accumulate in the nucleus and bind to chromatin, indicating a possible association *in vivo* between these elements (Kinoshita, 1979).

In other studies, Bhavanandan and Davidson (1975) found an uptake of both ^{3}H-glucosamine and ^{35}S-sulphate into nuclear fractions of cultured mouse melanoma cells. After digestion of the nuclei with DNase and pronase and isolation of the mucopolysaccharide components, the majority of the label was associated with a family of high molecular weight chondroitin sulphate molecules. A small amount of label was also found in heparin sulphate.

Furukawa and Terayama (1977) reported the presence of GAGs in purified nuclei from rat liver consisting mainly of hyaluronic acid with a minor amount of chondroitin sulphate. In followup studies, they reported that the nuclear content of GAGs decreased in regenerating rat liver hepatocytes after partial hepatectomy as compared to normal livers (Furakawa and Terayama, 1979).

In nuclei of rat brain cells, Margolis et al. (1976) reported the presence of GAGs containing 57% chondroitin-4-sulphate, 7% chondroitin-6-sulphate, 29% hyaluronic acid, and 7% heparin sulphate.

Englehardt et al. (1982) found that 25-30 nm granules could be extracted from isolated nuclear matrices by treatment with hyaluronidase enzyme, indicating the presence of hyaluronic acid in this fraction.

After labeling cultured hepatocytes with $^{35}SO_4$, Fedarko and Conrad (1986) described the presence of a pool of unique heparin sulphate molecules in the nucleus; this pool changed

in quantity depending on the growth state of the cells. Followup pulse-labeling studies by Ishihara et al. (1986) showed that these heparin sulphate molecules appeared in the nucleus only 2 hrs after their synthesis, and had a turnover time of approximately 8 hrs.

F) Other Nucleoplasmic Glycoproteins

Margolis et al. (1976) reported that purified nuclei of rat brain cells, stripped of their nuclear envelopes, contained glycoproteins with an average composition of 30% glcNAc, 29% mannose, 19% sialic acid, 15% galactose, 4% galNAc and 3% fucose.

A sulphated and phosphorylated ß-D-galactan was isolated from nuclei of the acellular slime mold P. polycephalum (Farr and Horisberger, 1978).

Kearse and Hart (1989) reported changes in the levels of a nuclear pool of unspecified glycoproteins containing O-linked glcNAc residues in mitogenically activated T-lymphocytes. Shortly after mitogen-induced cellular activation, the levels of O-glcNAc increased rapidly but returned to control levels a few hours later.

Soulard et al. (1991) have described a 43 kd glycoprotein in HeLa cells which is associated with heterogenous ribonucleoprotein (hnRNP) particles. This glycoprotein, which was detected by autoimmune antibodies from sera of dogs developing lupus erythematosus, could be metabolically labeled with ^{14}C-glucosamine and it also bound WGA. These features

indicated the presence of glcNAc residues. This protein did not bind Con A or UEA I, suggesting that mannose and fucose residues were not present.

Codogno and coworkers have also reported the presence of glycosylated proteins related to ribonucleoproteins in HeLa cells. These contained glcNAc residues N-linked to the protein, and in addition did contain mannose and fucose residues (unpublished results cited by Hubert et al., 1989).

Lastly, Meikrantz et al., (1991) have isolated a glycoprotein with phosphotyrosine phosphatase activity from mitotic cells of a human D98/AH-2 cell line. This protein was immunochemically localized to the nucleoplasm in interphase cells, and was shown to be part of the p34 cdc2 histone kinase complex. The protein specifically bound WGA, and this binding could be abrogated by a ß-elimination reaction, indicating the presence of terminal glcNAc residues O-linked to the protein core. Thus, this molecule may be another member of the class of glycoproteins containing single O-linked glcNAc residues described by Torres and Hart (1984).

II) Evidence for Cytosolic Glycoproteins

A) Glycogen

Recent evidence has been provided that cytosolic glycogen is not simply composed of polysaccharide as had long been thought, but is really a proteoglycan composed of the well-known polysaccharide portion attached to a newly-discovered protein, glycogenin (Rodriguez et al., 1985; Aon and Curtino,

1985; Blumfeld et al., 1986). There is also evidence that glycogenin also has glucosyltransferase activity, and it autoglucosylates itself before the enzyme glycogen synthase enzyme adds more glucose residues to complete the synthesis of the molecule (Pitcher et al., 1988a,b). Additionally, it has been reported that another unidentified cytosolic glucosyltransferase must add a first glucose residue onto glycogenin before it autoglucosylates itself (Smythe et al., 1988).

B) Cytosolic Glycoproteins containing O-linked GlcNAc residues

The novel glycoproteins containing O-linked glcNAc residues, first described by Torres and Hart (1984), have been found in the cytosol of a wide variety of eukaryotes (Holt and Hart, 1986; Bennett, 1986; Holt et al., 1987b; Nyame et al., 1987; King and Hounsell, 1989; as well as in the equivalent compartment of viruses (Benko et al., 1988; Calillet-Boudin et al., 1989; Mullis et al., 1990). Such molecules have also been found in the limiting membranes of cellular organelles such as the smooth and rough endoplasmic reticulum where the sugars were found to be cytosolically-oriented (Abejion and Hirshberg, 1988).

Two recent publications have described the presence of N-acetylglucosaminyltransferase activity associated with the cytosolic compartment of rat liver hepatocytes which was capable of adding glcNAc to proteins O-linked to proteins (Haltiwanger et al 1990; Starr and Hanover, 1990). Along the

same lines, a protein of 340 kd with this same activity has now been isolated from rat liver and characterized by Haltiwanger et al. (1992).

C) Cytosolic Glycoproteins containing O-linked Mannose Residues

Srisomsap et al. (1988) have reported the presence of a cytosolic glucosylphosphotransferase, which catalyzes the transfer of α-glucose-1-phosphate from UDP-glucose to a 62 kd cytosolic protein. The protein was found to be contain mannose residues which were O-linked to protein, as indicated by ß-elimination (Srisomsap et al, 1988). Further, the mannosylation of this protein was unaffected by tunicamycin, supporting the concept that N-linked side chains were not involved in the saccharide linkage.

There have also been reports of novel O-linked mannose oligosaccharides which are found attached to the protein core of cytosolic chondroitin sulphate proteoglycan molecules in rat brain cells (Finne et al, 1979; Krusius et al, 1986; Krusius et al, 1987).

D) Other Cytosolic Glycoproteins

A number of early biochemical studies reported evidence for glycoproteins associated with mitochondria (Bosman et al., 1969; Nicolson et al., 1972; Bernard et al., 1971; Glew et al., 1973), ribosomes (Howard et al., 1977; Yoshida et al., 1978), and with the cytosolic surface of rER and Golgi membranes (Bergman and Dallner, 1976), and secretory granules (Feigenson, 1975; Meyer and Burger, 1976).

In more recent studies, Tomek et al. (1987) reported that the small cytoplasmic RNP particles known as prosomes (or proteosomes) (for review see Scherrer, 1990) of Friend erythroleukemia cells bound to Con A and WGA indicating them to have a glycoprotein component with mannose and glcNAc. Schliephacke et al. (1991) also found that prosomes of four species of higher plants had glycoproteins with similar lectin binding affinities to the prosomes stated above.

A recent biochemical study has provided evidence for plasma membrane glycoproteins which contain cytosolically exposed N-glycosidically linked side chains. Pedemonte et al. (1990) reported that the sodium pump α-subunit of dog kidney medullary cells could be labeled by the addition of UDP-^{3}H-galactose and exogenous galactosyltransferase to isolated plasma membrane vesicles, but only if the cytosolic face of the vesicle was exposed to the radioactive sugars. The protein-carbohydrate linkage was sensitive to N-glycosidase F, indicating that the oligosaccharide was N-linked.

Sikorska et al. (1990) found that in several cell lines the M1 subunit of nuclear envelope cytosolically-exposed protein ribonucleotide reductase bound to Con A affinity columns, thus indicating that this molecule is a glycoprotein containing mannose residues.

Kuzmich et al. (1991) have reported that at least one isoform of cytosolic glutathione S-transferase enzyme from rat and human carcinoma cells bound Con A. The binding was Endo H-

sensitive, suggesting that the proteins have high mannose N-linked oligosaccharide side chains.

Lastly, Gonzales-Yanes et al. (1992) provided evidence that a fucoprotein from Dictyostelium slime mold cells is glycosylated in the cytosol by a novel cytosolic fucosyl-transferase. Incorporated ^{3}H-fucose was found to be released after ß-elimination indicating the carbohydrate to be O-linked to the protein core.

B. Evidence from Cytochemical Studies

I) Nucleoplasmic Glycoproteins

A) Lectin Cytochemistry

At the light microscope level, Kurth et al. (1979) reported that fluorescein-conjugated Con A bound specifically to polytene chromosomes of salivary gland cells of Chiromonas thummi, indicating the presence of mannose residues. This binding was found to be preferentially localized to transcriptionally active regions of chromosome IV represented by specific "puff" regions.

Hozier and Furcht (1980) found that fluorescein-conjugated WGA bound to both mitotic chromosomes and the nucleoplasm of interphase nuclei of human lymphocytes, suggesting the presence of glcNAc residues.

Bramwell et al. (1982) found that the nucleoli of Reed-Sternberg cells (diagnostic for Hodgkin's disease) strongly bound fluorescein-conjugated WGA and RCA I lectins, indicating the presence of glcNAc and as well as galactose residues.

Nucleoli of lymphocytes in normal lymph nodes did not show a similar binding.

Similarly, Fatehi et al. (1987) observed that the nuclei of cultured endothelial cells bound fluorescein-conjugated WGA, RCA I, and BSA I lectins, indicating the presence of glcNAc as well as galactose residues.

Recently, Kelly and Hart (1989) showed that Drosophila chromosomes labelled with fluorescein-conjugated WGA. Unlike the study of Kurth et al. (1979), binding was found to be less preferentially localized to transcriptionally active "puff" regions. Chromosomes also became labeled when incubated in the presence of ^{3}H-UDP-galactose and bovine galactosyltransferase, indicating the presence of terminal glcNAc residues. A ß-elimination reaction showed that these were O-linked.

In the study by Tischendorf et al. (1990) (referred to previously) BSA I (galactose) and UEA I (fucose) lectins specifically bound to segments of the Drosophila lampbrush chromosomal loops that were associated with heavy accumulations of ribonucleoprotein, suggesting that the glycoproteins may be ribonucleoproteins.

At the EM level, Horisberger et al. (1981) showed specific binding of RCA I to the chromatin of nuclei of the acellular slime mold P. polycephalum, indicating the presence of galactose residues.

Roth et al. (1983) observed heavy binding of a Con A-HRP-gold complex to condensed chromatin and to the fibrillar part

of the nucleolus in rat liver hepatocytes. The euchromatin, on the other hand, exhibited much fewer binding sites.

Sève et al. (1984) similarly showed specific binding of Con A and WGA lectin-gold complexes to condensed chromatin, euchromatin and nucleolus in the nuclei of lizard ovary cells, indicating the presence of mannose and glcNac residues.

Kan and Pinto da Silva (1986) showed that cross-fractured nuclei of duodenal columnar cells bound both Con A and UEA I, suggesting the presence of mannose and fucose residues. In both cases the labeling was largely restricted to the euchromatin of the nucleoplasm. Kan et al. (1986) have also reported lectin binding in isolated hepatocytes. In normal hepatocytes, UEA I and RCA I specifically bound to euchromatin, and binding was especially heavy at the heterochromatin/euchromatin interface. Nucleoli were also labeled at the edge of the fibrillar cords. When the cells were treated with 5-6-dichloro-1-ß-ribofuranosyl-benzimidazole, large clusters of interchromatin granules were found to be labeled with UEA I and RCA I.

Lucocq et al. (1989) showed that the euchromatin in nuclei of pig and rat hepatocytes specifically labeled with RCA I-gold following the incubation of the tissue sections with UDP-galactose and exogenous galactosyltransferase (GT). This labeling was abolished if glcNAc was included in the incubation with UDP-gal and GT. These results suggested that glcNAc-containing acceptor glycoproteins were present in the

nucleoplasm of these cells. The lectin labeling was not abolished by prior treatment of the sections with Endo F/peptide glycosidase F treatment suggesting that the sugars were O-linked to the protein.

Bolognani Fantin et al. (1989) have reported that nuclei of an embryonic human epithelial line bound LPA, UEA, LTA and WGA lectins indicating the presence of sialic acid, fucose, and glcNAc. The lectin labeling generally decreased when the cells were placed in and became adapted to hyperosmotic medium.

Vannier-Santos et al. (1991) demonstrated binding of a variety of lectins to the nucleoplasm of promastigotes of the parasitic protozoan Leishmania mexicana amazonensis. These included Con A (man), LCH (glc), WGA (glcNAc), RCA I and WHG (gal/galNAc), as well as LPA and LFA (both sialic acid). All of the lectins bound to heterochromatin while Con A, LCH, and WGA also bound to euchromatin, and WGH and LFA bound to nucleoli.

B) Enzyme-gold Cytochemistry

Other cytochemical evidence for nucleoplasmic glycoproteins has been provided by enzyme-gold techniques, in which sugar binding enzymes (e.g. glycosidases) linked to colloidal gold have been used as probes. Using a ß-glucosidase-gold complex, Bendayan and Benhamou (1987) observed ultrastructural labeling of heterochromatin in rat liver, pancreatic acinar, and kidney cell nuclei, providing evidence

for the presence of glucose-containing molecules. In another study, α-mannosidase-gold bound mainly to heterochromatin in nuclei of rat liver, pancreas, kidney and duodenal cells, indicating the presence of mannose residues (Londono and Bendayan, 1987). In contrast, labeling with the lectin Con A-HRP-gold was predominantly localized to the euchromatin.

C) Immunocytochemistry

Welch et al. (1983) showed that a small percentage of nuclei in different cultured cell lines were labeled with an antibody to a 100 kd stress protein. This protein was shown to incorporate ^{3}H-man. Upon heat shock of the cells, the nuclear localization of this protein was found to increase.

Aquino et al. (1984a,b) found nuclear localization of an antibody to chondroitin sulphate proteoglycan in 20-30% of rat brain neurons. In followup studies, Ripellino et al. (1989) found that monoclonal antibodies to specific regions of the polypeptide portion of the same chondroitin sulphate proteoglycan also labeled some nuclei.

In their study of O-linked glcNAc-containing nuclear pore proteins (described earlier), Snow et al. (1987) showed by immunofluorescence that antibodies to these proteins labeled the nuclear periphery in a characteristic punctate fashion. Concurrent immunogold EM studies revealed that the label was localized to the nucleoplasmic face of the nuclear pores. In the study of Davis and Blobel (1987) on nuclear O-glcNAc-containing proteins, one monoclonal antibody (mAb 457) raised

against the p62 nuclear pore protein had a broad specificity for other nuclear O-glcNAc-containing proteins; this antibody was localized by immunofluorescence to all interior regions of the nucleus except nucleoli, and it was interpreted that it might be detecting other nuclear O-glcNAc-containing proteins in the nuclear interior.

Turner et al. (1990) have also used immunofluorescence to detect O-glcNAc-bearing glycoproteins in both the nucleoplasm and cytoplasm in rat cell lines, using monoclonal antibodies which specifically recognise O-glcNAc residues. These antibodies reacted strongly with the nuclear periphery and nucleoplasm and stained the cytoplasm less intensely.

Monoclonal antibodies to blood group-related antigens bearing characteristic terminal saccharides have been used in a number of studies to provide evidence for the presence of nucleoplasmic glycoconjugates. Egami et al. (1990) showed nucleoplasmic labeling with MAb's to blood group A antigens (with galNAc-fucose-galactose trisaccharides) in a pancreatic cancer cell line (PC-1) in Syrian hamsters suggesting the presence of glycoconjugates bearing these sugars in the nucleoplasm of these cells. Such labeling was absent from normal pancreatic cells. Other similar studies include that of Yotsumoto et al. (1990) who showed binding of MAb's against blood group B antigens (galactose-fucose-galactose trisaccharides) to heterochromatin of cells of human eccrine glands as well as Nakajiima et al. (1991) who demonstrated a

similar binding of both blood group A and B antigen-specific MAb's to the heterochromatin of mucous cells of human cervical glands.

Lastly, a number of very controversial reports have provided evidence based on biochemical or cytochemical use of antibodies that some glycoproteins traditionally localized to either the cell surface (with extracellularly-oriented sugars) or the extracellular space can also be localized to the nucleus. The glycoproteins described have included fibronectin (Zardi et al., 1979), the EGF receptor (Radowicz-Szulczynska et al., 1988), human fibroblast IL-1 receptor (Curtis et al., 1990), prolactin receptor (Buckley et al., 1992), and MAC-2 (a macrophage cell surface glycoprotein)(Wang et al., 1992).

The conclusions of studies related to such nuclear localizations have been vigorously challenged (Evans and Bergeron, 1987) based on the possibilities that either the antibodies are nonspecifically binding to nuclear components or that the isolated nuclear fractions are contaminated with extranuclear components (general criticisms of the all the evidence are discussed more fully in a following section).

II) Cytosolic Glycoproteins

In lectin cytochemical studies on rat hepatocytes, Roth et al. (1983) showed labeling of ribosomes and the cytosol between cisternae of the rER with Con A-HRP-gold.

In enzyme-gold cytochemistry, Bendayan (1987) showed labeling of the cytoplasmic face of the rER with ß-glucos-

idase-gold and hyaluronidase-gold.

In immunocytochemical studies, glycoproteins with O-linked glcNAc demonstrated immunogold labeling of the cytosolically-oriented nuclear pore complexes at the EM level (Snow et al., 1987). The cytosolic orientation of some of these glycoproteins was further confirmed in studies carried out by Hanover et al. (1989).

C. Indirect Evidence for Nucleoplasmic and Cytosolic Glycoproteins

I) Evidence for Endogenous Lectins

Indirect evidence for the existence of glycoproteins in the nucleoplasm and/or cytosol has come from studies which have provided evidence for the presence of endogenous lectins in these compartments. Although there is no available proof, it is speculated that these lectins may interact with corresponding glycoprotein acceptors (reviewed in Hubert et al., 1989). Several biochemical studies have described a number of galactose-specific lectins which have a nuclear localization, including carbohydrate binding protein (CBP 35) (Moutsatsos et al., 1986,1987; Jia and Wang, 1988; Liang and Wang, 1988) as well as others (Sevaljavic et al., 1977; Childs et al., 1980; Carding et al., 1985). In a number of cytochemical studies, neoglycoproteins containing glucose, mannose, galactose, and/or fucose residues have been observed to bind to nucleoplasm in sections of undisrupted cells (Hubert et al., 1985; Facy et al., 1990) or isolated nuclei (Seve et al., 1985, 1986, 1988; Bourgeois et al., 1987; Olins

et al., 1988). Some of these lectins were located in the cytosol as well as the nucleoplasm (Childs et al., 1980; Carding et al., 1985; Hubert et al., 1985; Sève et al., 1985; Moutsatos et al., 1986). A predominantly cytoplasmic lectin has been identified in skin cells of Xenopus laevis (Cerra et al., 1984; Bols et al., 1986).

II) Evidence for Cytosolic Glycosidases

Other suggestive evidence the presence of glycoproteins in the cytosol has come from the identification of cytosolic glycosidases, including sialidases (Tulsiani and Carubelli, 1970; Miyagi et al., 1985) and mannosidases (Shoup and Touster, 1976; Bischoff and Kornfeld, 1986; Tulsiani and Touster, 1987).

III) Evidence for Serum DNA-binding Glycoproteins

There have also been a few reports which have described the presence of glycosylated DNA-binding proteins in animal blood serum. These studies are presented as indirect evidence for nucleoplasmic glycoproteins because these proteins have not yet been shown to interact with the nucleus in vivo.

Hoch et al. (1976) purified two major DNA-binding proteins in human serum which were shown to be PAS-positive indicating the presence of carbohydrate. Zardi et al. (1979) showed that fibronectin (mentioned earlier as a cell surface component localized to nuclei) from mouse or human binds with specificity to DNA and that antibodies to chromatin proteins will precipitate this protein from plasma.

III. Criticisms of the Evidence Presented in the Literature for the Existence of Nucleoplasmic and Cytosolic Glycoproteins

It can be seen from the above review of the biochemical and cytochemical literature that a considerable volume of evidence is now available for the existence of what seems to be many different glycoproteins in the nucleoplasm or cytosol. A great majority of this evidence, however, (except for that from some of the most recent studies) has been criticized on various technical grounds.

The evidence from biochemical studies, especially that of the early ones, has been subjected to the major criticism that there is not irrefutable proof that glycoproteins found in the various isolated subcellular fractions representing the nucleoplasm or cytosol are not contaminants from other glycoprotein-rich fractions such as rER, Golgi apparatus, and plasma membrane. The amount of glycoprotein reported in these nucleoplasmic and cytosolic fractions has in most cases been sufficiently small compared to that of the other potentially contaminating compartments, that it could easily be accounted for by even a minute amount of contamination from those other compartments (Jackson, 1976).

A second criticism of these early biochemical studies has been that most of the reported glycoproteins have been only minimally characterized, often only described as PAS-positive bands or Con A binding, etc. However, evidence from some recent studies (most notably those describing glycoproteins

with O-linked glcNAc sugars) have had a much higher level of acceptance. This is due to the improved schemes of fractionation and purification that have been utilized, and the higher degree of biochemical characterization of these molecules (Holt and Hart, 1989).

Cytochemical studies, especially those carried out on sections from undisrupted whole cells, have the advantage over biochemical studies in that the contamination factor is largely eliminated. A major criticism of these studies, however, relates to a possible lack of specificity of the probes detecting the glycoconjugates because of the possibility of false binding of the probe to the section, particularly to cell components such as chromatin in the nucleoplasm (Roth et al., 1987).

Another criticism of the cytochemical studies has been that detailed information about the detected molecules is again lacking, even more so than in biochemical studies. Binding of a certain lectin, for example, tells us only that a certain sugar residue is present, and little else about the molecule, e.g. its size, its protein-carbohydrate linkage, etc. More specificity may be obtained in immunocytochemical studies, but there is the possibility that a given antibody is recognizing a similar epitope from a different molecule, or that the antigen has been redistributed during processing of the tissue for cytochemistry (Jacob et al., 1984; Nigg et al., 1988).

IV. The Contribution of Radioautography to the Detection of Cytosolic and Nucleoplasmic Glycoproteins

The major part of the work of the present thesis can trace its origins to the findings of radioautographic studies carried out over the past three decades in the laboratories of Drs. Leblond and Bennett to investigate the biosynthesis and localization of glycoproteins within various tissues. A variety of tritiated sugars, including ^{3}H-fuc, ^{3}H-gal, ^{3}H-man and ^{3}H-glucosamine (or ^{3}H-glcNAc), were administered to cells *in vivo* and *in vitro*, for various time intervals and then the specialized cytochemical technique of radioautography was used to detect macromolecules which incorporated the label. These studies (as well as similar radioautographic investigations carried out at the time in other laboratories) and other biochemical reports have provided information which led to our traditional concepts of glycosylation of proteins (i.e. glycosylation occurring in the endoplasmic reticulum and Golgi apparatus with subsequent migration of the glycoproteins to other locations in the cell) (reviewed in Bennett, 1991).

On the whole, these radioautographic studies gave no dramatic indications to the investigators at the time that would suggest the presence of cytosolic or nucleoplasmic glycoproteins. In the case of the cytoplasm, the assumption was made from the beginning that observed silver grains would not be attributed to labeled glycoprotein molecules in the cytosol. Limitations in the resolution afforded by the radioautographic technique were such that all such grains

could probably be explained by sources in some nearby cytoplasmic organelle of the central vacuolar system. A notable exception was the labeling attributed to glycogen, but at this time glycogen was not considered to be a glycoprotein. A second exception was the reaction sometimes observed over mitochondria. In some instances a significant proportion of silver grains appeared over these structures after ^{3}H-fucose administration to rat kidney cells (Haddad et al., 1977) and striated duct cells (Hand, 1979), or after ^{3}H-manNAc administration to hepatocytes (Bennett and O'Shaugnessy, 1981), or intestinal columnar and kidney tubule cells (Bennett et al., 1981). In these studies it was accepted that the reaction could represent bona fide metabolic incorporation, since there was independent biochemical evidence for such incorporation.

In the case of the nucleoplasm, most radioautographic studies revealed only a small percentage of silver grains over cell nuclei (less than 10%) after exposure to labeled sugars, and in view of the large proportional area in the cell occupied by the nucleus, the nuclear reaction was usually attributed either to background or to crossfire from radioactive sources in the adjacent cytoplasm (reviewed by Bennett, 1988). If the relative specific activity (% silver grains / % effective area) of labeling of an organelle was much lower than 1.0, the organelle was not considered to contain a significant amount of radioactivity (Ginsel et al.,

1979). Even when larger percentages of grains were occasionally observed over the nucleoplasm, the results were often ignored. Early radioautographic experiments by Bennett and Leblond in the 1960's had also shown appreciable nucleoplasmic labeling of rat duodenal crypt columnar cells exposed to ^{3}H-galactose, but the significance of this was not appreciated at the time (Bennett, personal communication). In other studies, 16% of total grains were observed over the nucleoplasm of rat salivary gland striated duct cells exposed to ^{3}H-fucose (Hand, 1979), and 13 % and 36% were similarly located in plasma cell tumour cells exposed to ^{3}H-galactose and ^{3}H-glcNAc respectively (Zagury et al., 1970). The authors, however, either attributed the reaction to crossfire or declined to speculate on its significance. Bok and Young (1974) similarly noted a substantial reaction over the nucleoplasm of rat photoreceptor cells after exposure to ^{3}H-glucosamine.

Only two early publications suggested that nuclear labeling was actually due to labelled nucleoplasmic glycoproteins. Fromme et al. (1976) reported that in cultured skin fibroblasts exposed for 4 days to ^{35}S-sulphate, a majority of nuclei exhibited substantial reaction in electron microscope radioautographs. A computer-calculated statistical evaluation of the results showed that the silver grains overlying the nuclei originated from ^{35}S radioactivity within the nuclei and not from overall background or crossfire

effects of cytoplasmic radiation sources. Subsequent chemical analyses of the labeled products strongly suggested the label was in sulphated glycosaminoglycans (Fromme et al, 1976).

Reisert (1978) reported relatively high levels of nucleoplasmic labeling (over 20% of total grains) in neurons and glial cells exposed to ^{3}H-manNAc, and suggested that the label might reside in sialic acid residues of glycoconjugates in the nucleoplasm.

The first radioautographic study that revealed dramatic reactions over the nucleoplasm of cells after administration of labeled sugars was carried out by Dr. Bennett and myself at McGill, in collaboration with Dr. Pierre Lavoie at the Université de Montréal. This work formed the basis of my M.Sc thesis (Hemming, 1986) as well as a published article (Bennett et al., 1986). In this study, which had been initiated to study aspects of the axonal flow of ^{3}H-fucose labeled glycoproteins in frog dorsal root ganglion neurons, it was noted that the nuclei of many Schwann and satellite glial cells exhibited heavy nucleoplasmic reactions after exposure to ^{3}H-fucose. Reaction was observed in cells exposed to the labeled precursor for as little as 5 min. Statistical analysis of the silver grain distribution indicated that at least a significant portion of the silver grains overlying the nuclei originated from radioactive sources within the nucleoplasm and not from crossfire from radioactive sources in the nuclear envelope or cytoplasm.

Having established that nuclear reactions appeared over certain cell types in the frog after exposure to labeled fucose, we asked the question as to whether or not other cell types in other species exhibited similar nuclear labeling after exposure to a variety of labeled sugars. While most of the radioautographic studies previously carried out in the laboratory of Dr. Bennett had provided little striking evidence for nucleoplasmic labeling, one early study in which rat duodenal crypt columnar cells were exposed to ^{3}H-galactose had showed prominent nuclear labeling of these cells. As mentioned above, the significance of this finding was not appreciated at the time, and the phenomenon was not further investigated. We therefore repeated this study, and our results provided clear ultrastructural of nucleoplasmic labeling in columnar and goblet cells in both crypts and villi, as well as in fibroblasts of the lamina propria. These results were included in the above mentioned M.Sc thesis and publication (Hemming, 1986; Bennett et al., 1986).

V. Objectives of the Present Work

The work described in the present thesis was undertaken with a number of specific goals. As described above, we had provided radioautographic evidence for nucleoplasmic labeling in one frog cell type and some rat cell types after exposure to ^{3}H-fucose or ^{3}H-galactose. These results were interpreted as evidence for the presence of nucleoplasmic glycoproteins in these cells.

The first objective of the present work was to determine whether radioautographic evidence could be found for the presence of such nucleoplasmic glycoproteins in all animal cells or only in selected cell types. To accomplish this task, a broad group of cell types from tissues exposed to radiolabeled sugars or sulphate have been examined for nucleoplasmic labeling. The results of these investigations, which revealed such labeling in many cell types from several animal species are presented in Chapter 2.

The second objective was to examine the distribution of the label in nuclei. The first step was to determine the extent to which radioautographic reaction observed over the nucleoplasm of cells (exposed to labeled sugars) was actually due to radioactive molecules in the nucleoplasm, as opposed to molecules in the surrounding cytoplasm or nuclear envelope. The experimental approach used to resolve this question involved quantitating the nucleoplasmic labeling observed in undisrupted cells and then comparing the general distribution of this label with that observed over nuclei isolated away from their cytoplasm and nuclear membranes. The best candidates in terms of an appropriate cell model for these fractionation experiments appeared to be any of a number of cultured cell types. However, it first had to be determined if such cells exhibited nuclear labeling. Thus, the cells were exposed to a variety of labeled sugars and processed for radioautography. The results of these experiments, in which

prominent reactions were observed over the nucleoplasm of interphase cells as well as over the chromosomes of dividing cells, are presented in Chapter 3. This chapter also includes the results of experiments in which the turnover time of labeled molecules in the nucleoplasm was investigated using a pulse-chase approach.

The results of the aforementioned nuclear isolation experiments are described in chapter 4. Radioautographic and biochemical assessment of incorporated label in nuclei isolated from myeloma cells indicated that most of the radioautographic reaction observed over the nucleoplasm of whole cells was due to radioactive molecules in the nucleoplasm, as opposed to molecules in the surrounding cytoplasm or nuclear envelope.

The final goal of these studies was to partially characterize the molecules responsible for the nucleoplasmic labeling in these cultured cells. To this end, some biochemical analysis was carried out on the labeled molecules in nuclear fractions from cells exposed to ^{3}H-sugars. Lectin blotting experiments were also carried out on the nuclear fractions containing the labeled molecules to provide additional evidence for the presence of glycoproteins and help determine the sugars present. The results of these studies are presented in Chapter 4.

I. Introduction

As mentioned in the general introduction, the first goal of the present work was to investigate the tissue distribution of cells exhibiting nucleoplasmic labeling (as an indicator of putative nucleoplasmic glycoproteins) in many different animal cell types after exposure to radiolabeled sugars or sulphate to establish whether or not such labeling was a ubiquitous occurrence in all animal cells. Though it was acknowledged that most earlier EM radioautographic studies had not described nuclear labeling, it must be taken into account that many of these studies tended to ignore radioautographic reactions associated with the nucleus. Therefore, the lack of reported radioautographical evidence in this regard could be due more to the authors' bias than to an actual absence of reaction over nuclei.

To accomplish the task of examining the labeling of a comprehensive list of cell types, LM radioautographs from some previous studies in the laboratory of Dr. Bennett have been reexamined, and where evidence existed for nuclear labeling, tissues were processed for EM radioautography. In addition, new experiments utilizing various ^{3}H-sugars or ^{35}S-sulphate were also carried out. In this chapter, the results of observations in which nucleoplasmic labeling was seen in many cell types in the frog, mouse, and rat are presented and discussed. The distribution of label within the nucleoplasm has also been examined qualitatively at the EM level to try to

identify the labeled subcompartments.

II. Materials and Methods

A. Biological Materials and Labeled Precursors

Young (40 gm) Sherman rats as well as young (15 gm) and adult Swiss albino mice were obtained from the animal colony of the McGill Anatomy Department. Young and adult frogs (Rana catesbeiana) were obtained from the laboratory of Dr. Pierre Lavoie at the Universitie de Montréal.

The following labeled precursors: L-[5,6-^{3}H]-fucose (spec. act. 60 Ci/m mole), D-[1-^{3}H(N)]-galactose (spec. act. 4.0, 14.2, or 55 Ci/mmole), N-[mannosamine-6-^{3}H(N)]-acetyl-mannosamine (spec. act. 2.2 Ci/m mole) (for sialic acid residues), D-[2-^{3}H(N)]-mannose (spec. act. 30 Ci/m mole), N-[glucosamine-1,6-^{3}H-(N)]-acetylglucosamine (spec. act. 30-60 Ci/m mole), D-glucosamine-6-^{3}H HCl (spec. act. 3.7 Ci/mmole), and [^{35}S]-sodium sulfate (spec. act. 1200 mCi/m mole) were obtained from New England Nuclear, Boston.

B. Radioautographic Experiments with Intact Tissues

In experiments using whole animals, the isotopes were dissolved in saline and injected intravenously in rats and mice, and intracardially in frogs under anesthesia. In some experiments on eye tissues, the isotopes were administered intravitreously (also under anesthesia). The animals were sacrificed after various time intervals by intracardiac perfusion with 2.5% glutaraldehyde in either Sorensen's phosphate buffer or 0.1 M sodium cacodylate buffer, preceded

by a 30 second pre-wash with lactated Ringer's solution. In experiments using D-glucosamine-6-^{3}H, the glutaraldehyde was replaced by 3% paraformaldehyde, and 1% cold glucosamine-HCl was added to the buffer wash.

In experiments using isolated organs, other approaches were used: 1) ^{3}H-galactose was injected into the lumen of the gall bladder of adult mice in situ, and after 5 min the organ was removed and fixed by immersion in 2.5% glutaraldehyde. 2) ^{3}H-mannose was administered to jejunal explants of adult mice in organ culture, and after 2 hours the tissue was fixed by immersion in 2.5% glutaraldehyde. 3) Finally, ^{3}H-fucose, ^{3}H-galactose or ^{3}H-manNAc were administered to adult frog dorsal root ganglia in an oxygenated media, and after various time intervals the tissues were fixed by immersion in 2.5% glutaraldehyde.

C. Processing of Tissues for LM and EM Radioautography

In all of the above experiments, the tissues were washed in buffer after the primary aldehyde fixation, postfixed in 1% potassium ferrocyanide-reduced osmium and dehydrated in ethanol. The ethanol was then replaced with propylene oxide, and the specimens were infiltrated with Epon 812 - propylene oxide mixtures and embedded in pure Epon 812. For LM microscope radioautography, sections of 0.5 or 1.0 μm thickness were stained with iron hematoxylin, coated with Kodak NTB2 emulsion, and later developed in Kodak D-170 after various exposure time periods. For EM radioautography, thin

sections (silver to pale gold in interference color) were carbon coated, then coated with Ilford L4 emulsion and exposed for various time periods. The radioautographs were developed either in Kodak D19B developer (filamentous grain development) or by the solution physical fine grain development procedure. The sections were then stained with uranyl acetate and lead citrate, and examined using a Phillips 400 electron microscope.

III. Results

A. Radioautographic Experiments on Intact Tissues

I) Cell Types Exhibiting Nuclear Labeling

The radioautographic findings of the present study are summarized in Table 1 (at the end of Discussion), which lists those cell types exhibiting nuclear radioautographic reactions after exposure to the following labeled precursors:

A) ^{3}H-Fucose Experiments

A number of frog cell types routinely exhibited heavy nuclear labeling. Reaction was first observed in Schwann and satellite cells of dorsal root ganglia exposed *in vitro* at as little as 5 minutes (Bennett et al., 1986). A typical reaction observed at short exposure intervals is shown in Fig 1. When the incubations with labeled ^{3}H-fucose were followed by chase periods in unlabeled medium, some labeling of nuclei was observed in the same cells even after a 17 hr chase, the longest time interval examined (Figs. 2-3). When whole animals were injected intracardially with ^{3}H-fucose and sacrificed 30 or 60 minutes later, nuclear labeling was again seen in Schwann and satellite cells (Fig. 4) as well as in many other cell types, including spinal cord capillary endothelial cells (Fig. 5), neurons (Fig. 6), duodenal columnar cells (Fig. 7), pancreatic acinar cells (Fig. 8), and hepatocytes (Fig. 9).

Most rat cell types exhibited only very modest nuclear labeling. Some reaction was observed over nuclei of many cell types, especially at their periphery, including intestinal

epithelial cells (Fig. 10), hepatocytes (Fig. 11), and kidney proximal convoluted tubule cells. Certain rat cell types exhibited more prominent nuclear reactions; these included neurons and capillary endothelial cells in the retina Fig. 13), retinal pigment epithelial cells (Fig. 14), ciliary epithelial cells (Bennett and Haddad, 1986), and lens epithelial cells (Haddad and Bennett, 1988).

In the mouse, only selected tissues were examined, but substantial nuclear reactions were observed in duodenal columnar cells (Fig. 15) at short exposure times. Interestingly, in these cells the nuclear reaction was found to be vastly reduced 4 hrs after injection of the radiolabeled fucose. Other cells which showed prominent labeling were hepatocytes, and kidney proximal tubule cells (Fig. 16).

B) ^{3}H-Galactose Experiments

In the frog, electron microscope radioautographic studies were carried out only on dorsal root ganglia incubated for 1 hour with ^{3}H-galactose, and these studies revealed nuclear labeling which was easily noticeable in neurons (Fig. 17) and quite intense in Schwann and satellite cells (Fig. 18). Light microscope radioautographic studies of tissues from frogs killed one hour after intracardiac injection of ^{3}H-galactose showed prominent nuclear reactions in several other cell types, but this was accompanied by comparatively heavier cytoplasmic reactions. In the rat, prominent reactions were observed in a number of duodenal cell types, including

columnar and goblet cells of the villi (Fig. 19) and crypts (Fig. 20), as well as capillary endothelial cells (Fig. 21), lymphocytes (Fig. 22), and fibrocytes in the lamina propria (Fig.23). Reaction was also seen over the chromosomes of dividing crypt cells at all stages of mitosis (Figs. 24-26). Liver bile ductule cells displayed intense nuclear reactions (Fig. 27), while lighter but significant labeling occurred in kidney tubule cells, pancreatic acinar and islet cells, and adrenal cortex cells.

In the mouse, only the gall bladder was examined, and here the epithelial cells displayed substantial labeling over their nuclei (Fig. 28).

C) ^{3}H-Glucosamine and ^{3}H-GlcNAc Experiments

Frog and mouse tissues were not examined with either of these glcNAc precursors. In rat tissues, EM radioautographs showed prominent nuclear reactions in duodenal columnar cells (Fig. 29), hepatocytes (Fig. 30), hepatic duct cells (Fig. 31), and pancreatic duct and islet cells (Figs. 32-33). LM radioautographs revealed nuclear labeling in many other cell types, including kidney tubule cells, stratified epithelial cells of the skin, esophagus, and stomach, adrenal cortex cells, choroid plexus cells, and ciliary epithelial cells.

D) ^{3}H-Mannose Experiments

Frog tissues were not examined. In the rat, radioautography was carried out only on duodenal tissue, and significant nuclear reaction was observed in villous columnar

cells (Figs. 35) and enteroendocrine cells (Fig. 36). LM radioautographs revealed moderate nuclear reactions in several other cell types, including pancreatic islet cells, kidney tubule cells, neurons, choroid plexus cells, ciliary epithelial cells, and some lymphocytes.

Only one experiment was done in the mouse, in which explants of jejunal tissue were incubated with ^{3}H-mannose for 2 hours and examined by LM radioautography. Significant nuclear labeling of the villous columnar cells was observed (Fig. 34).

E) ^{3}H-N-Acetylmannosamine Experiments

The only frog tissue examined with this precursor was the dorsal root ganglion, where LM radioautographs revealed nuclear reactions over neurons, Schwann and satellite cells.

In the rat, some cell types, eg. kidney distal convoluted tubule cells (Fig. 37), exhibited light but consistent labeling of their nuclei, while most other cells exhibited only very modest nuclear reaction.

In tissues of the mouse (examined only at 10 min after administration of the precursor), significant nuclear reactions appeared over a number of cell types. In the colonic epithelium, LM and EM radioautographs showed that nuclear reaction was prominent in upper crypt cells (Figs. 38-39) but almost absent in surface cells (Figs. 38,40). Other cells exhibiting nuclear reactions at the EM level included duodenal hepatocytes (Fig. 41), kidney proximal tubule cells (Fig. 42),

and villous columnar cells (Fig. 43).

F) ^{35}S-Sulphate Experiments

Frog tissues were not examined. In the mouse, EM radioautography showed prominent nuclear reactions in hepatocytes (Fig. 44), duodenal villous columnar cells (Fig. 45) and colonic goblet and columnar cells (Fig. 46), and kidney proximal convoluted tubule, while LM radioautography revealed nuclear reactions in many other cell types.

No EM radioautographic studies were carried out in the rat, but studies at the LM level revealed nuclear reactions in an array of cell types similar to that seen in the mouse (Table 1).

B. Distribution of Radioautographic Grains

The distribution of silver grains overlying nuclei varied with different cell types and different labeled precursors. In the case of frog Schwann and CNS capillary endothelial cells, which have abundant peripheral chromatin, many grains lay over the peripheral heterochromatin or over the euchromatin/heterochromatin interface after exposure to ^{3}H-fucose (Figs. 1,5).

In other nuclei with less peripheral chromatin, more of the silver grains were centrally located over euchromatin, e.g. frog neurons (Fig. 6), mouse duodenal columnar cells (Fig. 15) and frog hepatocytes (Fig. 9) exposed to ^{3}H-fucose; mouse bile ductule (Fig. 27) and gall bladder cells (Fig. 28) exposed to ^{3}H-galactose; mouse hepatocytes (Fig. 41) and

duodenal columnar cells (Fig. 43) exposed to ^{3}H-N-acetyl-mannosamine; rat duodenal columnar cells (Fig. 29), hepatocytes (Fig. 30) and islet cells (Fig. 33) exposed to ^{3}H-N-acetylglucosamine; and mouse hepatocytes (Fig. 44) and duodenal intestinal cells (Fig. 45) exposed to ^{35}S-sulfate. In such instances, a smaller percentage of grains were localized over the euchromatin/ heterochromatin interface.

In many cell types, a portion of the silver grains were associated with nucleoli, e.g. frog neurons (Fig. 6) and duodenal columnar cells (Fig. 7) after exposure to ^{3}H-fucose; rat crypt and villus columnar cells after exposure to ^{3}H-galactose (Figs. 19,20) and mouse intestinal columnar cells after exposure to ^{35}S-sulphate (Fig. 45).

It should be noted that after exposure to ^{3}H-mannose, some cell types exhibited nuclear reactions in which most of the grains appeared to be near the nuclear periphery, e.g. rat duodenal enteroendocrine cells (Fig. 36), and duodenal columnar cells. In other cell types a higher proportion of centrally located grains was observed e.g. lymphocytes (Figure not shown).

In some cells of the frog dorsal root ganglia where the label was chased for 17 hrs with unlabeled media, a striking change was noted in the distribution of the nuclear label. Whereas many silver grains were observed over interior regions of the nucleoplasm at short labeling intervals, after a 17 hr chase in unlabeled medium the silver grains were almost

exclusively over the periphery, either over the peripheral heterochromatin or over the nuclear envelope. A reaction over the lumen of the nuclear envelope was very evident in some cells in these latter experiments where there was an increased separation between the outer and inner nuclear membranes observed which created an greatly expanded lumen (Fig. 3).

IV. Discussion

A. Rationale for using Radioautography to Indicate the Presence of Nucleoplasmic Glycoproteins

As with other cytochemical techniques using intact tissues, the major advantage of using radioautography in the present study was that it allowed us to study the incidence and distribution of particular molecules (in this case putative labeled nucleoplasmic glycoproteins) without having to disrupt the cells. This circumvented one of the biggest problems of biochemical studies, i.e. the potentially incorrect assignment of molecules (such as glycoproteins) to particular cellular compartments (such as nuclei) due to the contamination of subcellular fractions with similar types of molecules from other fractions.

The radioautographic method in fact offered additional advantages over other forms of cytochemistry. Unlike immunocytochemistry, lectin cytochemistry, or other cytochemical staining procedures using exogenous probes to identify molecules, radioautography is not subject to the frequently encountered problem of limited accessibility to the molecular sites trying to be detected, leading to a "false negative" reaction. This is because the molecules being detected are intrinsically labeled by having incorporated the radioactive precursors, and the embedding medium forms no significant barrier to the detection process of this label. Radioautography is also less subject to nonspecific "false positive" reactions than other cytochemical techniques such as

lectin cytochemistry or immunocytochemistry. With these techniques, for example, nonspecific binding of lectins or antibodies to chemically fixed nuclei has frequently been observed. This makes interpretation of positive results sometimes difficult. Though the background silver grains in a radioautographic constitute a form of "false positive" reaction, this tends to be to more easily recognized, as this background is usually uniform throughout the tissue section.

A final advantage of the radioautographic technique as used at the light microscope level in our hands was that we could examine large histological paraffin sections in which were assembled virtually all body tissues of an experimental animal which had received a labeled sugar. This provided us the opportunity to sample and compare the distribution of nucleoplasmic labeling in a wide variety of cells <u>in vivo</u>. Where detected, such nucleoplasmic labeling could then be confirmed at the ultrastructural level with reasonably good resolution of the label.

B. <u>Pattern of Cell types exhibiting Nucleoplasmic Reactions</u>

Almost all cells exhibited at least occasional silver grains over their nuclei, but only certain cell types tended to exhibit prominent nuclear reactions after exposure to various labeled precursors. As seen in Table 1, moderate to heavy nucleoplasmic labeling was often observed in intestinal epithelial cells, hepatocytes, kidney tubule cells, glandular duct cells, neurons, and lymphocytes. Nonetheless, each of

these cell types sometimes exhibited only modest nuclear labeling (depending on the species or the precursor used). In addition, certain cell types exhibited prominent nuclear labeling only in certain species or only after exposure to certain precursors (e.g. rat bile ductule cells after ^{3}H-galactose). An example to illustrate this point is that heavy nuclear labeling was observed in epithelial cells of the rat intestine after exposure to ^{3}H-galactose while in the same cells exposed to radiolabeled fucose, only a small reaction was observed. In epithelial cells of mouse intestine exposed to ^{3}H-fucose, however, a much more prominent reaction was observed.

The cells which did exhibit prominent reaction did not tend to fit into any single category of cell type, encompassing cells as diverse as epithelial cells, connective tissue cells, lymphatic tissue cells, and neurons. Epithelial cells, however, tended to consistently show more nuclear labeling than cells of the other groups.

Nucleoplasmic labeling also did not appear to be restricted to cells having a high replication frequency. Thus, radioautographic reactions occurred over the nuclei of rapidly renewing cells (intestinal epithelium), as well as slowly renewing cells (hepatocytes and kidney tubule cells) and non-renewing cells (neurons). In some instances, a cell lost most of its nuclear labeling as it matured (e.g. when colonic crypt cells became surface columnar cells). In other instances,

labeling was observed in cells as old as the animal (e.g. neurons).

While no firm conclusions can be drawn from all these observations, the differential patterns of labeling in cells would seem to indicate that there is a heterogeny in the types and/or numbers of labeled nucleoplasmic molecules in different cells, reflecting some tissue and species specificity. If it is given that some or all of the label resides in glycoproteins within the nucleus, this is not really a surprising result when it is considered how many different nucleoplasmic glycoproteins are described in the scientific literature. While some of the reports may not correspond to true nuclear glycoproteins (as mentioned in the general introduction under "Criticisms..."), at least some of these reports are no doubt valid and there really might be a wide variety of bonafide different nucleoplasmic glycoproteins.

It must be also remembered that the radioautographic reaction observed over a cell is not a measure of the total existing cellular pool of those species of molecules incorporating the administered label. Rather, it is only the newly synthesized molecules of these species (i.e. those which incorporated the label during their synthesis), that are responsible for the radioautographic reaction. Thus the labeling observed in these studies is not necessarily an accurate indicator of the total amounts of glycoprotein contained in the nucleus of any particular cell type. For

example, a nucleus with small amount of glycoproteins which are being rapidly renewed might show considerable radioautographic reaction. On the other hand, a nucleus containing many glycoproteins which are being only slowly renewed would show little or no radioautographic reaction. Thus it cannot be concluded in the present study that unlabeled nuclei do not contain glycoproteins.

C. Distribution of Label within Nuclei

The patterns of the distribution of label observed within nuclei varied in different cell types after exposure to different labeled precursors. In the present chapter the results are presented in a qualitative manner, since many cell types were surveyed and our primary interest was more to determine which cell types exhibited nuclear labeling than to examine the precise distribution of the label. Such an examination has been carried out in cultured cell types in Chapter 3.

In cells exposed to ^{3}H-mannose, the majority of the silver grains in many of the cell types tended to be localized near the nuclear periphery. In cells exposed to all of the other labeled precursors in this study (as well as some cell types exposed to ^{3}H-mannose) the observed nuclear reactions included many silver grains located over central regions of the nucleus as well as grains over the periphery. In the nucleoplasm, all compartments appeared to be labeled (heterochromatin, euchromatin, nucleolus), but the highest percentage of silver

grains tended to be over euchromatin. In cells with large amounts of heterochromatin, the majority of the silver grains tended to be localized over the euchromatin/heterochromatin interface. This finding is interesting in that it has been reported that this is the site an active site of RNA transcription (Fakan and Nobis, 1978). At least in the case of some cells exposed to ^{3}H-fucose (i.e. frog Schwann and satellite cells, Fig 3.), some of the peripheral nucleoplasmic labeling appeared to be derived from sources within the lumen of the nuclear envelope, a finding which is somewhat surprising in that fucose is a terminal sugar and fucosyltransferase is thought to be only in the Golgi apparatus. However, labeling of the rER has been described in a few studies after the cells were exposed to ^{3}H-fucose (Pelletier et al., 1973; Magner et al., 1992) and it is known that the lumen of the nuclear envelope is in continuity with the lumen of the rER.

The above descriptions were based on a direct scoring approach to radioautographic analysis, i.e. the assumption that the radioactive source of a silver grain lies directly below that grain. It is acknowledged that with the spacial limitations of the radioautographic method this is not necessarily the case, and this problem is discussed more fully in Chapter 3.

D. Temporal Patterns of Nucleoplasmic Labeling

In terms of distribution of nucleoplasmic label after

different intervals of exposure to the radiolabeled precursors, nuclei in some cell types were found to be labeled as early as 5 minutes after exposure to ^{3}H-sugars. Since the synthesis of the polypeptide chains of nucleoplasmic proteins is thought to occur only in the cytoplasm, the present results suggest either that the proteins are glycosylated in the cytoplasm and rapidly transported to the nucleus or that the glycosylation of these molecules is occurring in the nucleoplasm itself.

Our radioautographic results indicate that the turnover rates of nucleoplasmic glycoproteins may vary. On the one hand, the disappearance of nucleoplasmic labeling in the nuclei of mouse duodenal cells by 4 hours after exposure to ^{3}H-fucose suggests that some nucleoplasmic proteins may be rapidly turning over. On the other hand, the persistence of some nucleoplasmic labeling in Schwann cells of frog dorsal root ganglia at 17 hours after exposure to ^{3}H-fucose indicates that at least some nucleoplasmic glycoproteins turn over very slowly. As described in our results, the distribution of nucleoplasmic labeling in these cells seemed to change with time from being both internal and peripheral to being exclusively peripheral. This might be explained by the presence of different nucleoplasmic glycoproteins with different turnover rates. Thus, labeled proteins in the internal regions of the nuclei might turnover more rapidly and thus disappear faster than labeled proteins located at the

periphery.

E. Summary of Chapter 2

In summary, radioautographic reaction over nuclei was observed in many different cell types in 3 animal species after exposure to 5 tritiated sugars or radiolabeled sulphate. These results suggest that nuclear labeling after the administration of such precursors is common if not ubiquitous in animal cells and, by extrapolation, nucleoplasmic glycoproteins are present to some degree in all animal cells. Furthermore, differences in the extent, distribution, and turnover time of the nuclear labeling, depending on cell type and radiolabeled precursor, would suggest that these glycoproteins are heterogenous in nature, possibly indicative of tissue and/or species specificity. It must be pointed out, however, certain limitations in the radioautographic method did not allow for unequivocal conclusions in this study and so further investigations remained to be carried out to provide further evidence to support the above postulates.

Table 1- Cell Types Exhibiting Nuclear Reactions After Administration of ^{3}H-Sugars or ^{35}S-Labeled Sulphate

Cell Type	Fuc	Gal	GlcNAc	Man	ManNAc	SO_4
Rat						
duodenal cells						
columnar c.	+	+++	++	++	+/-	+++
goblet c.	+	+++	++	ne	-	+++
enteroendocrine c.	ne	ne	ne	++	ne	ne
lamina propria endo cell	ne	++	ne	ne	ne	ne
pancreatic cells						
acinar c.	+	++	++	+	ne	ne
islet c.	+	++	+++	++	↓	ne
duct c.	+	+++	++	+		ne
colonic columnar c.	+	++	++	+		+++
hepatocyte	+	++	+++	++		+++
bile ductule c.	ne	+++	+++	++		ne
kidney prox. conv. tubl. c.	+	++	++	++		++
adrenal cortex c.	+	++	ne	++		ne
chorold plexus c.	+	ne	++	++		↓
ciliary epithelial c.	+	ne	++	++		
stomach stratified epith. c.	+	ne	++	ne		
lymphocyte	+	+++	ne	++		
fibrocyte	+	+++	++	+		
C.N.S. neuron	ne	ne	ne	++		
retina cap. endo. c.	++	ne	ne	ne		
retina RPE cell	++	ne	ne	ne		
lens capsule cell	++	ne	ne	ne	↓	↓
Mouse						
duodenal cells						
columnar c.	++	ne	ne	++	++	+++
goblet c.	++	↓	↓	++	++	+++
jejunal columnar c.	ne			ne	++	+++
colonic cells				↓		
surface columnar c.	++				+/-	+++
crypt columnar c.	++				++	+++
goblet c.	++				+	++
pancreas acinar c.	++				++	++
hepatocyte	++				++	++
kidney prox con tub c	++	↓			++	ne
gall bladder epithelial c.	ne	++			ne	ne
thyroid follicular c.	ne	ne			+	++
spermatogonia	ne	↓			++	++
lymphocyte	ne				ne	++
adrenal medulla	ne		↓	↓	ne	ne
Frog						
neuron c.	++	++	ne	ne	++	ne
Schwann c.	+++	+++	↓	↓	++	↓
satellite c.	+++	+++			++	
cap. endothelial c.	+++	ne			ne	
duodenal columnar c.	++	++			↓	
hepatocyte	++	++				
kidney tubule c.	++	++				
pancreatic acinar c.	++	++				
gallbladder epithelial c.	++	ne	↓	↓	↓	↓
ciliary epithelial c.	++	ne				

+++ =very strong + =small
++ = medium +/- = little or none
ne= not examined

Fig. 1 EM radioautograph of a Schwann cell from a frog dorsal root ganglia incubated for 15 min in vitro with ^{3}H-fucose.

A heavy radioautographic reaction is seen over the Schwann cell nucleus (N). Many of the grains are associated with peripheral heterochromatin and the nuclear envelope, while others are located over more internal patches of heterochromatin as well as euchromatin. In the cytoplasm a heavy cluster of grains overlies the Golgi apparatus (G). (My: Myelin; Ax: Axoplasm)

Exposure: 12 mo (Phys. dev.) x 21,000


G
My
N
1

Figs. 2-3 EM radioautograph of Schwann cells from a frog dorsal root ganglion incubated for 1 hr in vitro with ^{3}H-fucose, followed by a 17 hr post-incubation in unlabeled medium.

Fig. 2 A Normal Schwann Cell

Nuclear labeling is found mostly over the periphery of the nucleus (N). There is also labeling over the Golgi apparatus (G) as well as the plasma membrane (pm). (n: nucleolus; My: myelin; Ax: axoplasm)

Exposure: 4 mo (Phys. dev.) x 21,000

Fig. 3 A Schwann cell undergoing early apoptosis

As above, reaction which is localized over the nucleus (N) is confined to mostly peripheral areas. In some locations, as seen at (A), the perinuclear space between the outer nuclear membrane (ONM) and the inner nuclear membrane has become greatly enlarged. At these enlargements, reaction appears over the perinuclear space, as well as over the inner and outer nuclear membranes. This space remains in continuity with the remainder of the perinuclear space (arrow). Also observed are heavily-labeled ribosome-studded profiles of rER (B) in the cytoplasm; these resemble the nuclear envelope enlargement seen at A and may be in continuity with such enlargements. (My:myelin; ax:Axoplasm)

Exposure: 4 mo (phys. dev.) x 21,000

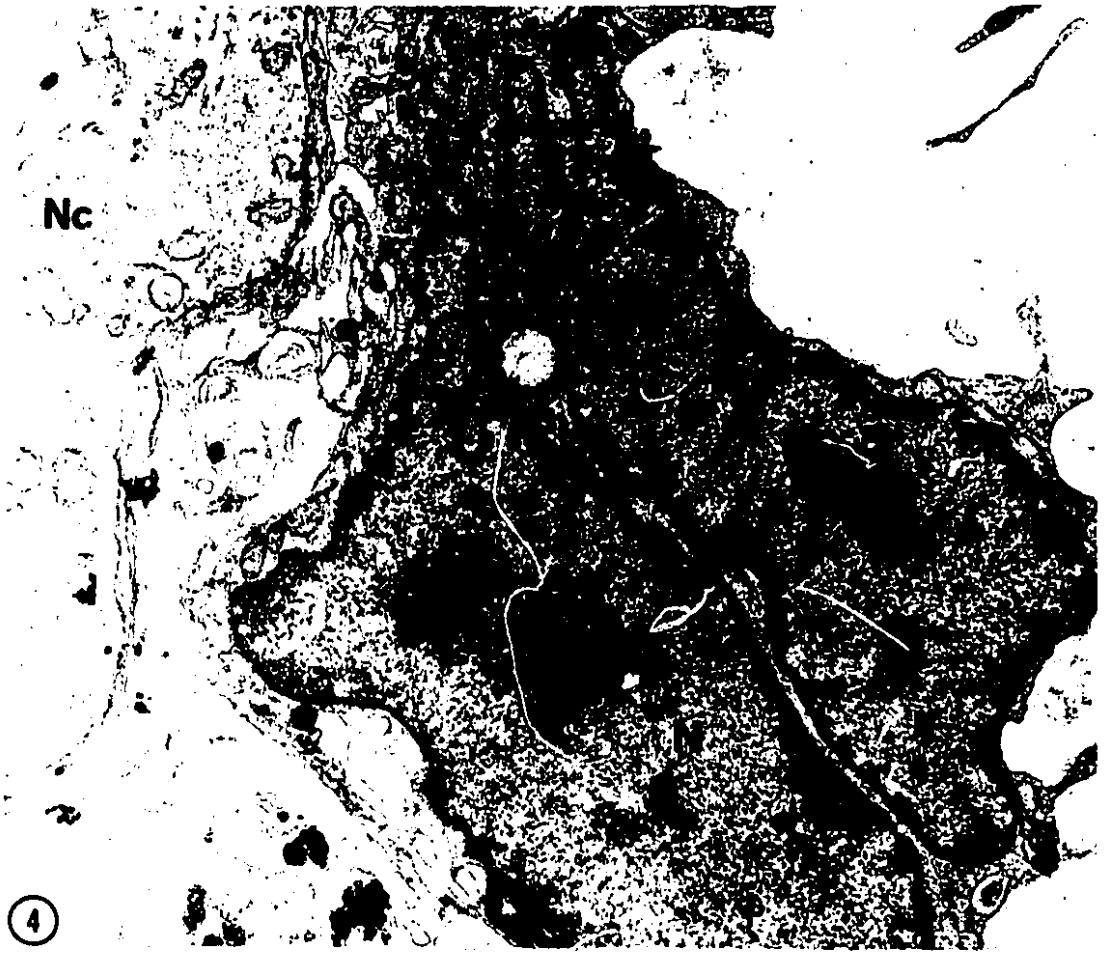

pm
n
My
Ax
2


My
N
B
3

Fig. 4 EM radioautograph of a satellite cell of a dorsal root ganglion from a frog killed 30 min after an intracardiac injection of ^{3}H-fucose.

Reaction is primarily localized over peripheral and central regions of the nucleus (N). Occasional grains are associated with nucleoli (n). (Nc: cytoplasm of a neuron cell body)

Exposure: 5 mo (Chem. dev.) x 11,500



④

Fig. 5 EM radioautograph of a capillary endothelial cell from the spinal cord of a frog killed 1 hr after an intracardiac injection of ^{3}H-fucose.

A heavy reaction covers the nucleus (N), in which many silver grains are over the periphery but some are more centrally located. Of these, some are associated with the nucleolus (n). A light reaction is observed over the cytoplasm. In the capillary lumen, a red blood cell (RBC) shows no reaction. (npl: neuropil)

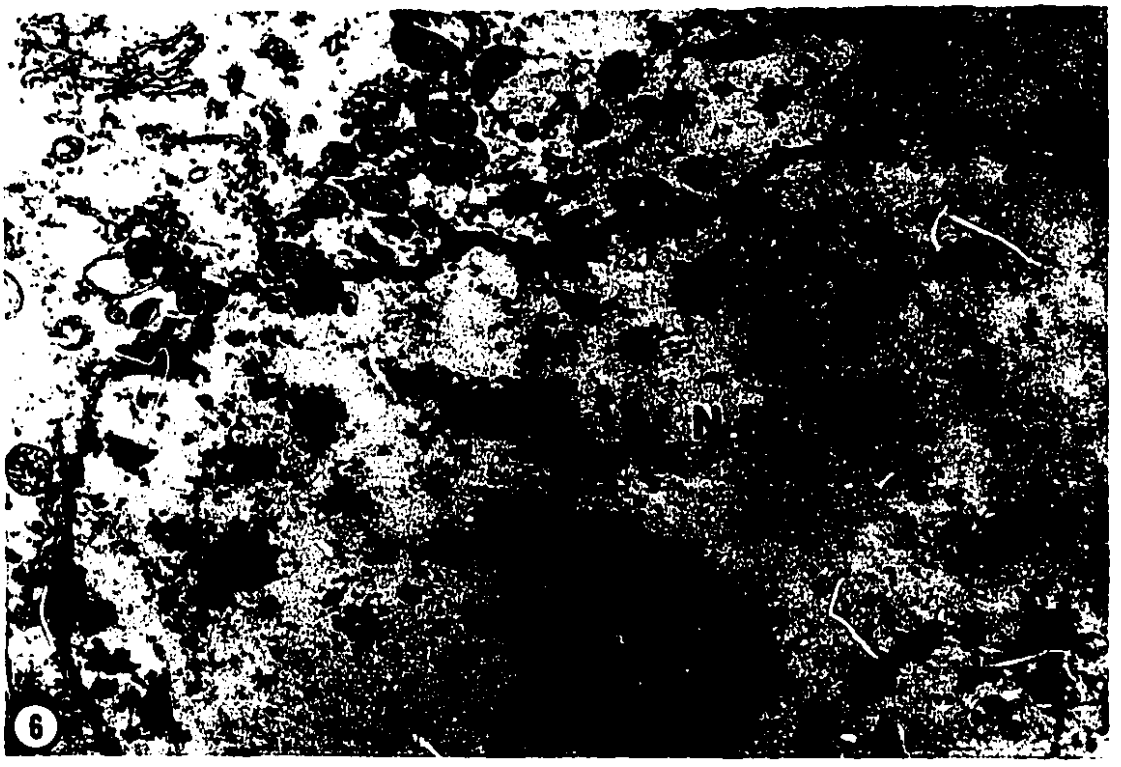
Exposure: 6 mo (Chem. dev.) x 18,000

Fig. 6 EM radioautograph of a neuron from the spinal cord of a frog killed 1 hr after an intracardiac injection of ^{3}H-fucose.

Many silver grains are observed over the nucleus (N); of these most occur over the central regions of the nucleoplasm, and some are assiciated with the nucleolus (n). Some grains are also seen scattered over the cytoplasm.

Exposure: 6 mo (Chem. dev.) x 11,000

5



6

Fig. 7 EM radioautograph of duodenal columnar cells from a frog killed 1 hr after an intracardiac injection of ^{3}H-fucose.

A substantial reaction is seen over the nuclei (N), where many of the grains overly the interior regions. Some grains lie near nucleoli (n). Over the cytoplasm, an intense reaction covers the Golgi complexes (G), and some grains occur over the lateral plasma membrane (lpm). (mv: microvilli)

Exposure: 6 mo (Chem. dev.) x 10,500

Fig. 8 EM radioautograph of pancreatic acinar cells from a frog killed 1 hr after an intracardiac injection of ^{3}H-fucose.

Many grains overlie the nuclei (N), both over the peripheral and central regions. Some grains are associated with nucleoli (n). In the cytoplasm, heavy reaction is observed over the Golgi complexes (G) and a lighter reaction occurs over the secretory granules (sg).

Exposure: 6 mo (Chem dev) x 10,500

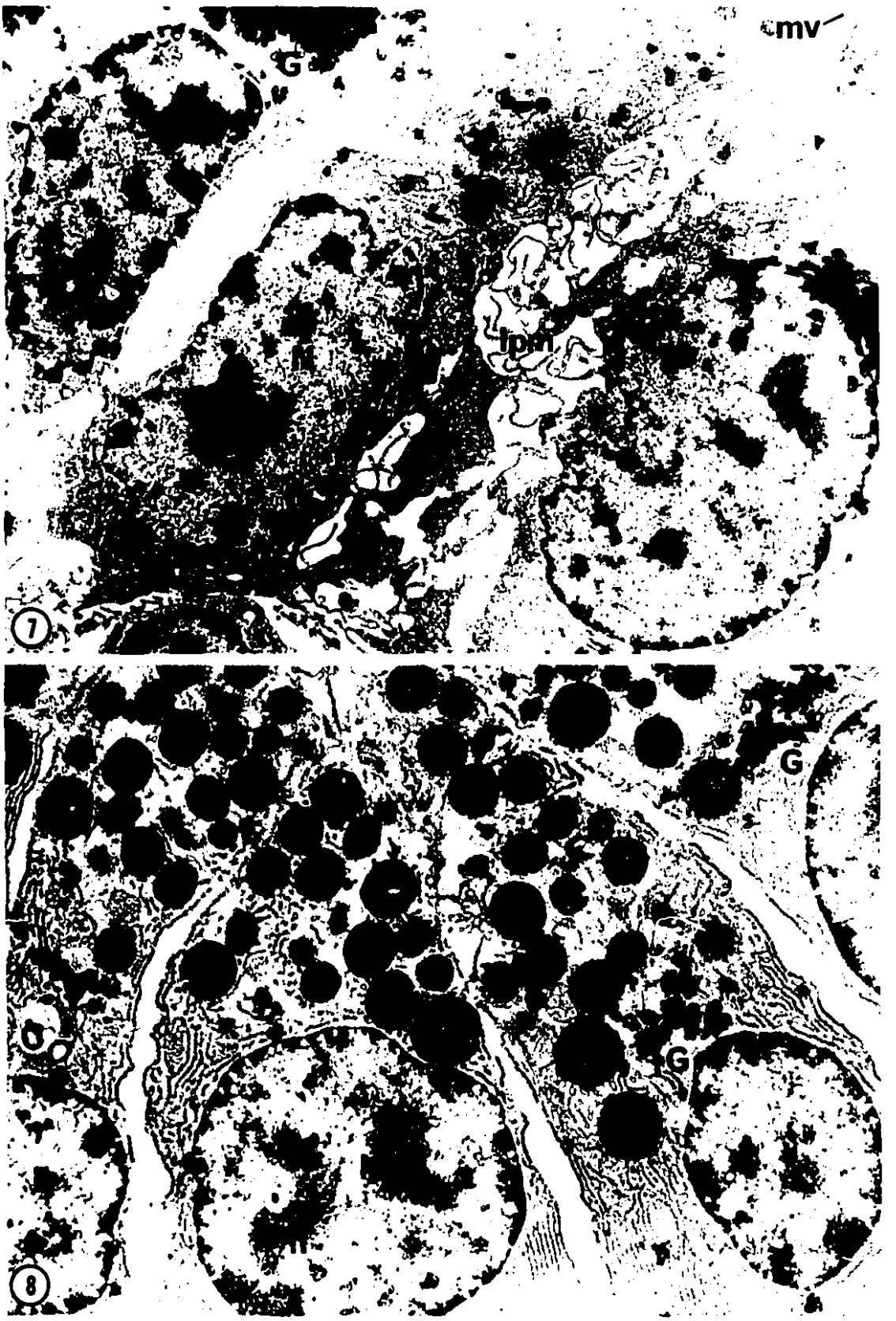

mv
G
lpm
7
G
G
8

Fig. 9 EM radioautograph of hepatocytes from a frog killed 1 hr after an intracardiac injection of ^{3}H-fucose.

A fairly heavy reaction occurs over the nuclei (N), in which most of the grains are centrally located, and some are associated with nucleoli (n). In the cytoplasm, a very heavy reaction covers a Golgi complex (G). Occasional grains are seen over the lateral plasma membrane (lpm). (BC: bile canaliculus)

Exposure: 6 mo (Chem. dev.) x 12,000

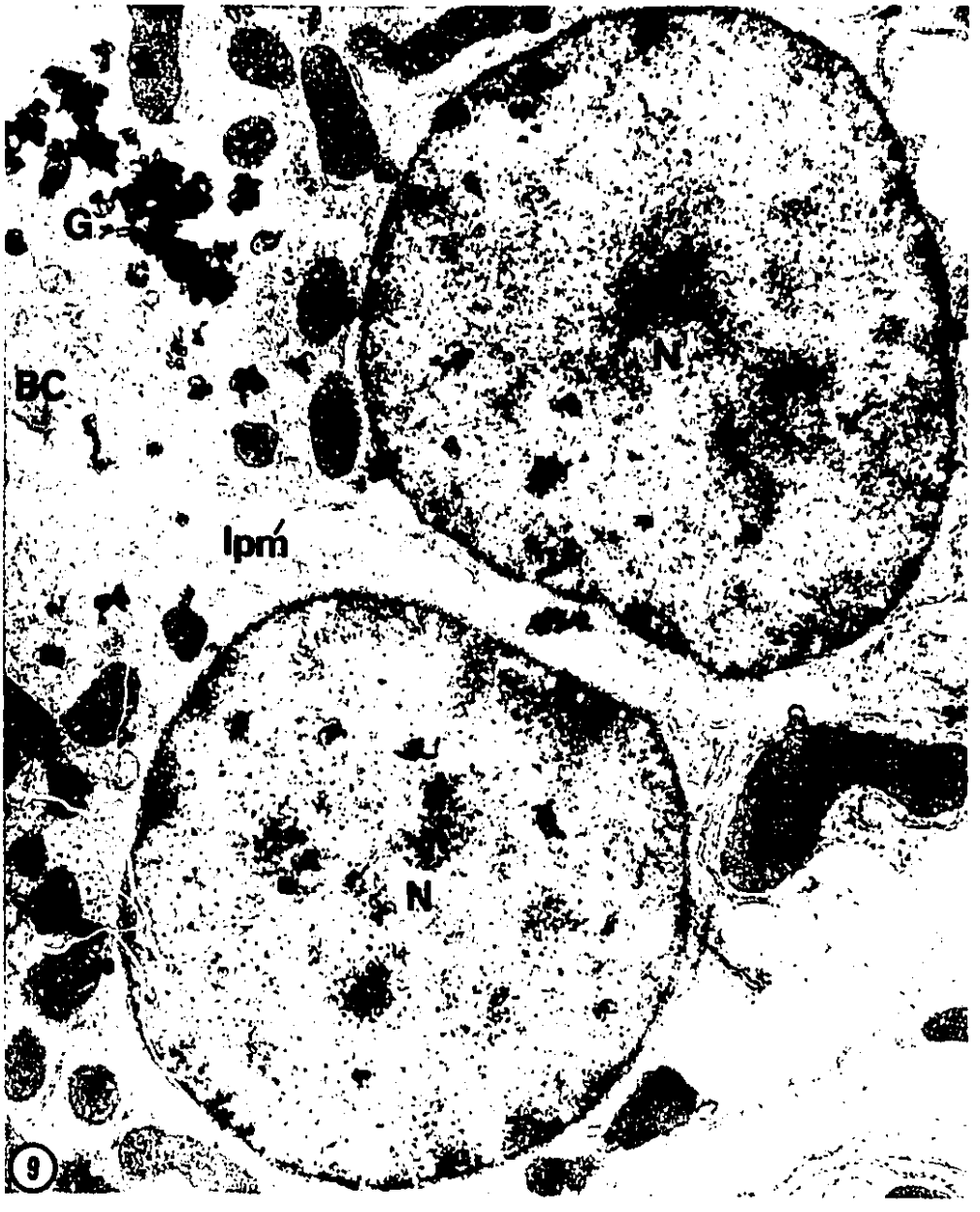

G
BC
lpm
N
N
9

Fig. 10 EM radioautograph of duodenal villus cells from a rat killed 30 min after an intravenous injection of ^{3}H-fucose.

These cells exhibit only a light reaction over their nuclei (N), combined with a heavier reaction over the cytoplasm. The nuclear reaction is almost entirely peripheral, with few grains over the interior regions. In the cytoplasm, grains are localized over the Golgi apparatus (G), lysosomes (L), microvilli (mv), and the lateral plasma membrane (lpm).

Exposure: 2 mo (Chem. dev.) x 8,700


10

Fig. 11 EM radioautograph of hepatocytes from a rat killed 1 hr after an intravenous injection of ^{3}H-fucose.

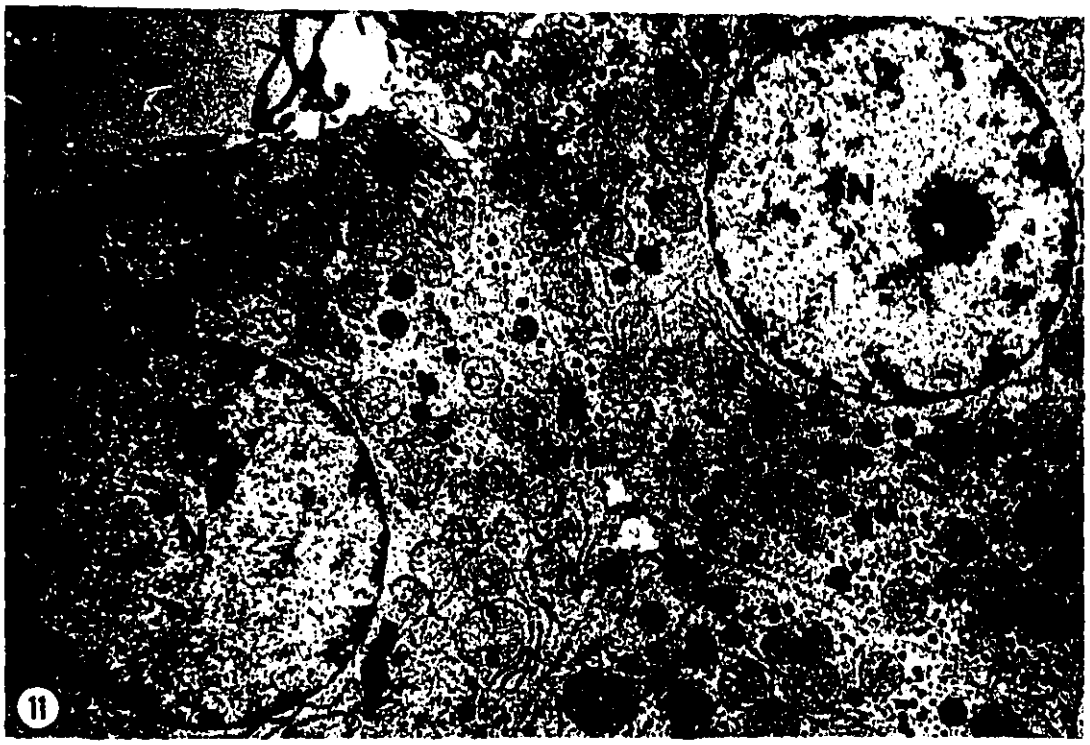
The overall radioautographic reaction in these cells is light but it is evident that a sihnificant number of grains are associated with the nuclei (N). These are mainly observed over the rim of heterochromatin inside the nuclear envelope. Of the interiorly located grains, one appears to be associated with a nucleolus (n). In the cytoplasm, silver grains tend to be localized over lysosomes (L) and the plasma membrane (pm). (BC: bile canaliculus)

Exposure: 4 mo (Chem. dev.) x 9,000

Fig. 12 EM radioautograph of a kidney proximal convoluted tubule cell from a rat killed 20 min after an intravenous injection of ^{3}H-fucose.

The nucleus is observed to be lightly labeled; three silver grains appear over the euchromatin, while one is associated with the peripheral heterochromatin. In the cytoplasm, lysosomes (l) and microvilli (mv) are among the structures labeled. (BM: Basement membrane)

Exposure: 3 mo (Chem. dev.) x 12,000


N
11

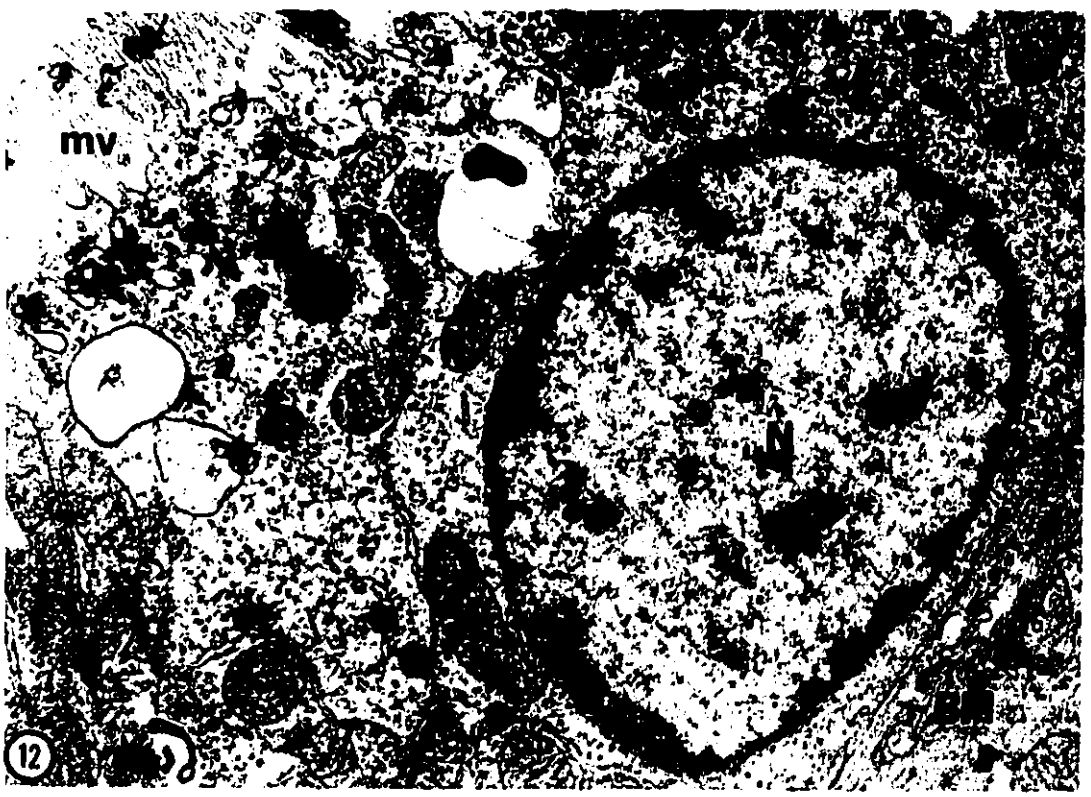

mv
N
12

Fig. 13 EM radioautograph of cells of the inner nuclear layer of the retina from a rat killed 1 hr after intravitreal injection of ^{3}H-fucose.

A significant reaction occurs over the nuclei (N) of neurons, as well as that of a capillary endothelial cell (N'). In the neuronal nuclei, a large proportion of the grains overlie the central regions, and some are associated with nucleoli (n). A heavy reaction is also seen over the Golgi complex (G) of these cells, and many other grains are associated with the plasma membrane. (lu: capillary lumen)

Exposure: 6 mo (Phys. dev.) x 12,000

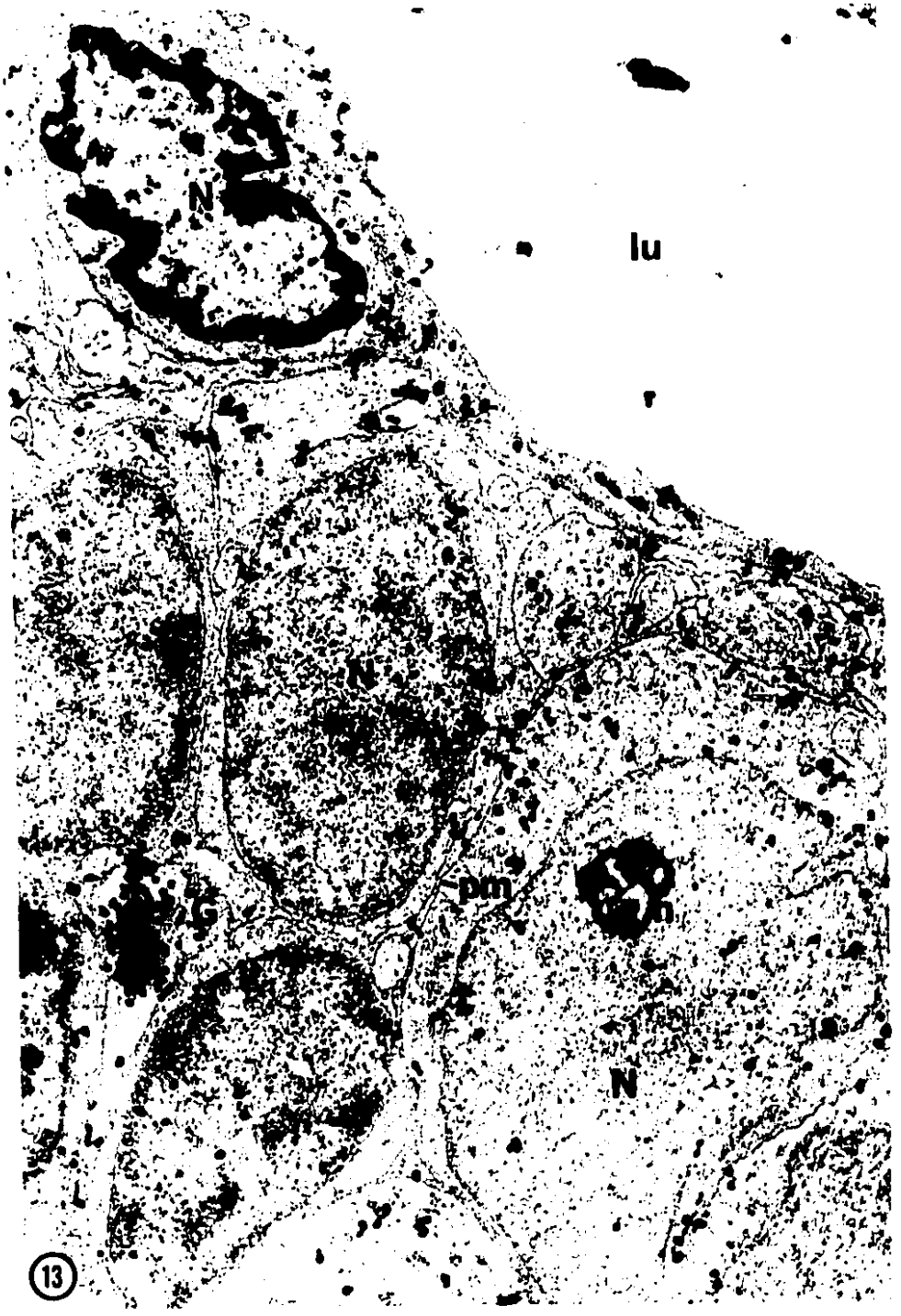

N
lu
N
pm
G
N
L
13

Fig. 14 EM radioautograph of retinal pigment epithelial cells from a rat killed 1 hr after an intravitreal injection of ^{3}H-fucose.

A large uptake of label is observed in the retinal pigment epithelial cells. The reaction is largely cytoplasmic but there is also a substantial reaction in the nucleus (N), with silver grains associated with the peripheral heterochromatin, and a few grains seen more interiorly over the euchromatin. (Ch: choroid; bpm: basal plasma membrane; OS: photoreceptor outer segment)

Exposure: 6 mo (Phys. dev.) x 12,000


Ch
L
14

Fig. 15 EM radioautograph of duodenal columnar cells from a mouse killed 20 min after an intravenous injection of ^{3}H-fucose.

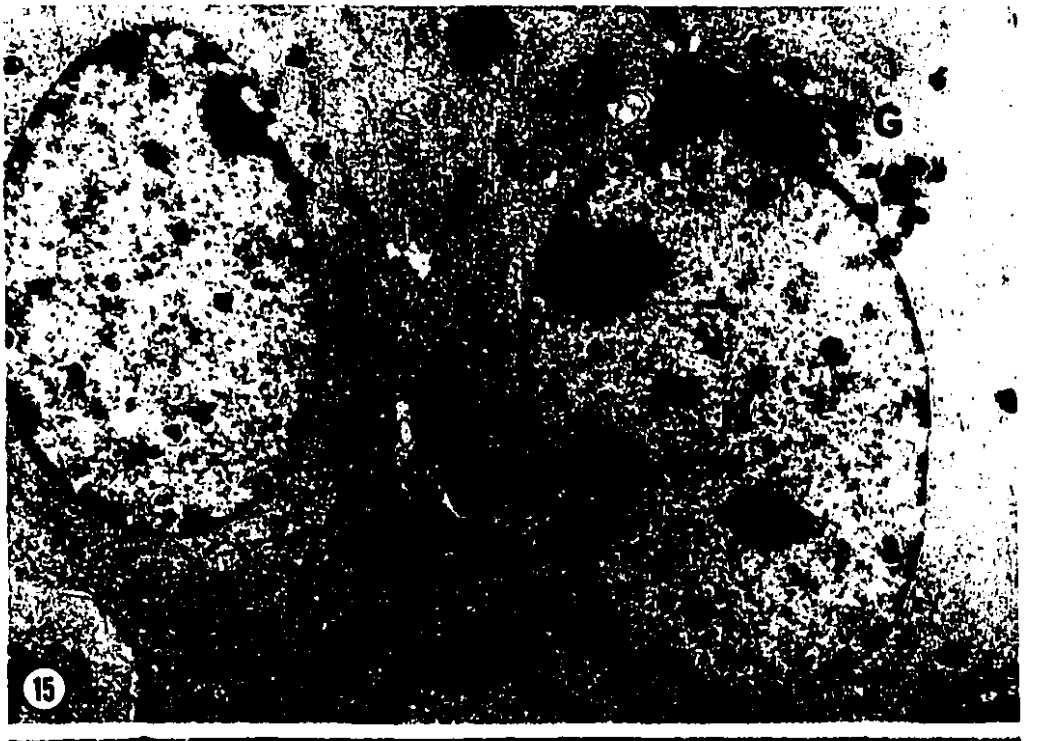
Many grains are seen over the nuclei (N), and of these most are centrally located, some being associated with nucleoli (n). In the cytoplasm, a very heavy reaction occurs over the Golgi apparatus (G), and a few grains occur over the lateral plasma membrane (lpm).

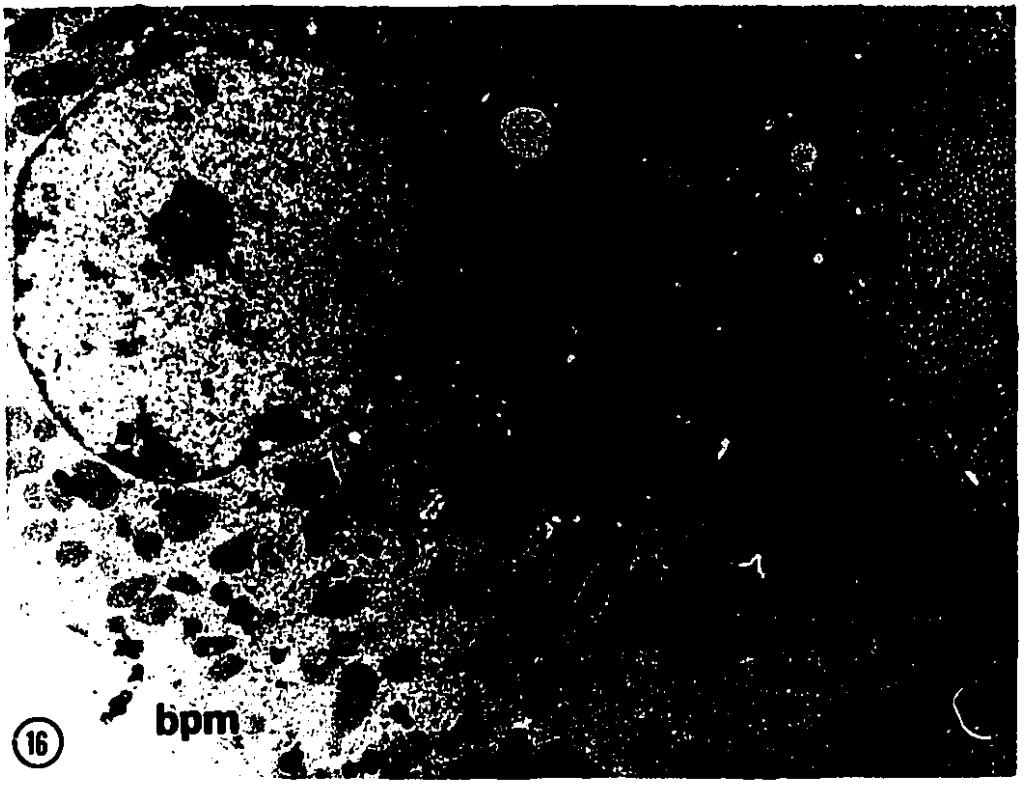
Exposure: 5 mo (Chem. dev.) x 12,500

Fig. 16 EM radioautograph of kidney proximal convoluted tubule cells from a mouse killed 30 min after an intravenous injection of ^{3}H-fucose.

These cells exhibit a fairly heavy reaction over both their cytoplasm and a lighter but significant reaction over their nuclei (N). In the latter, many grains are centrally located, and some are associated with nucleoli (n). The reaction over the cytoplasm is diffuse, and includes silver grains overlying the Golgi apparatus (G) and the microvilli (mv). (bpm: basal plasma membrane).

Exposure: 4 mo (Chem. dev.) x 13,000


G
15


bpm
16

Figs. 17-18 EM radioautographs of cells from a frog dorsal root ganglion incubated for 1 hr with ^{3}H-galactose.

Fig. 17 Neuron

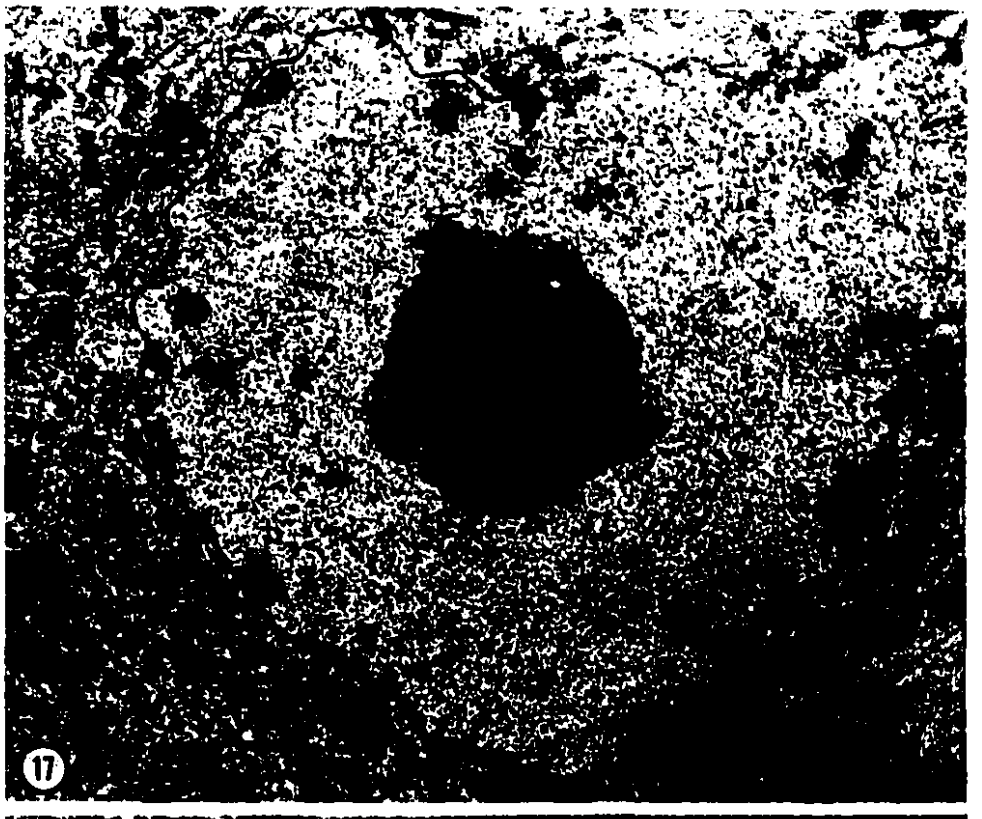
A nuclear reaction is observed where the nucleolus (n) is heavily labeled. Most of the nucleolar label is seen over its periphery near its interface with other nuclear elements such as the nucleolar-associated heterochromatin or the euchromatin. A few grains overly the euchromatin as well.

Exposure: 5.5 mo (Chem. dev.) x 12,000

Fig. 18 Schwann cell

A very heavy reaction overlies the nucleus (N), in which many of the grains are centrally located. A few grains are seen over the adjacent thin rim of cytoplasm. The surrounding collagen (Col) and the adjacent axon (Ax) are unlabeled.

Exposure: 5.5 mo (Chem. dev.) x 12,000



17

18

Fig. 19 EM radioautograph of duodenal villus epithelial cells from a rat killed 1 hr after intravenous injection of ^{3}H-galactose.

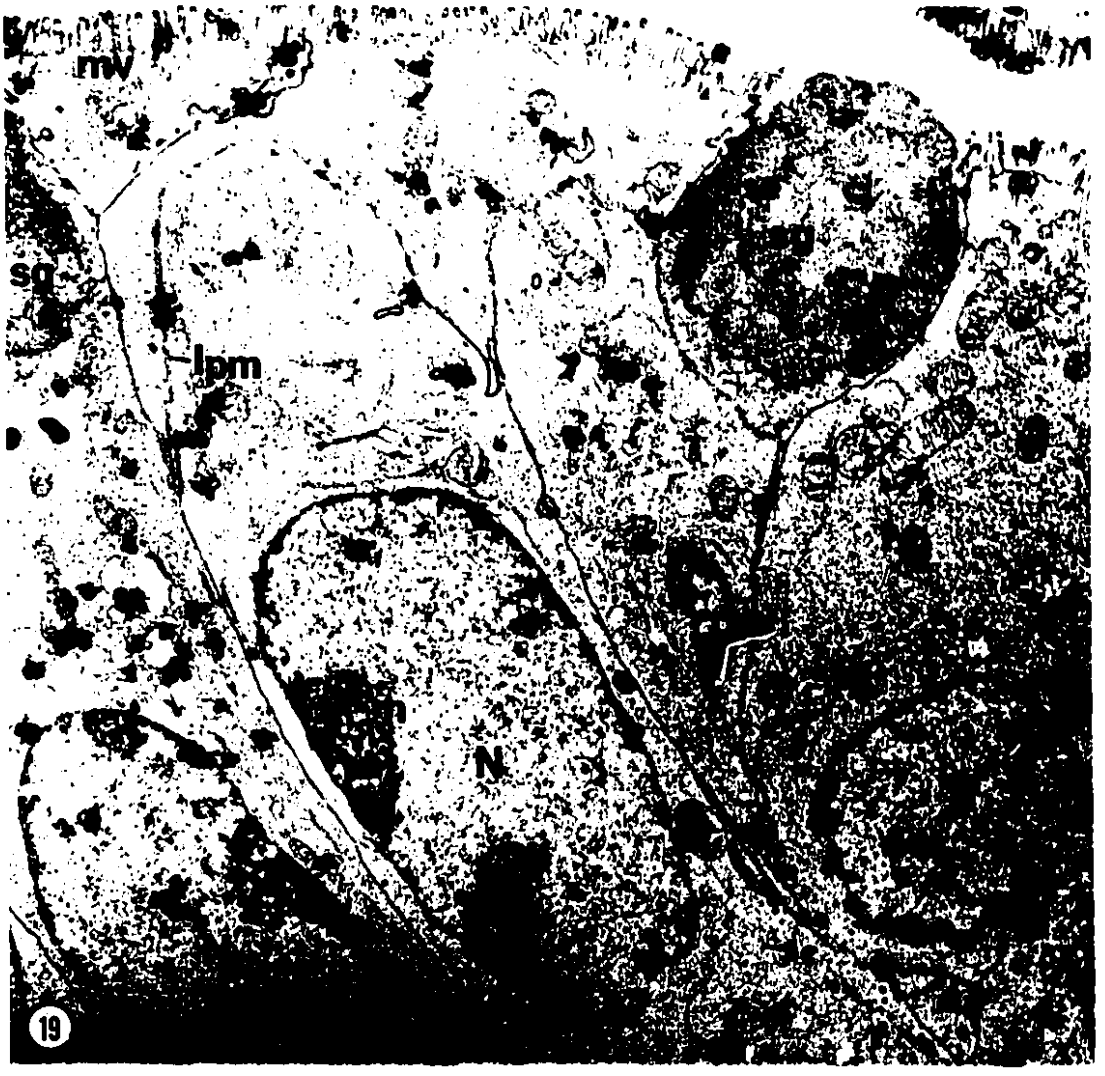
The nuclei of both the villus columnar cells (N) and the goblet cells (N') exhibit labeling. In these reactions, grains are seen over both the periphery and interior regions, and the nucleoli (n) are also labeled. In the cytoplasm, the reaction is localized over the Golgi apparatus (G) of the goblet cell, the microvilli (mv), the lateral plasma membranes (lpm), and the secretory granules (sg) of the goblet cells.

Exposure: 9 mo (Chem. dev.) x 11,000

Fig. 20 EM radioautograph of duodenal crypt columnar cells from a rat killed 15 min after an intravenous injection with ^{3}H-galactose.

A strong reaction is observed over the nuclei (N). Again,the silver grains are scattered throughout the nuclei, with some associated with the nucleolus (n). Reaction over the surrounding cytoplasm is comparatively light. (bpm: basal plasma membrane)

Exposure: 9 mo (Chem. dev.) x 10,000


mv
sg
lpm
N
19

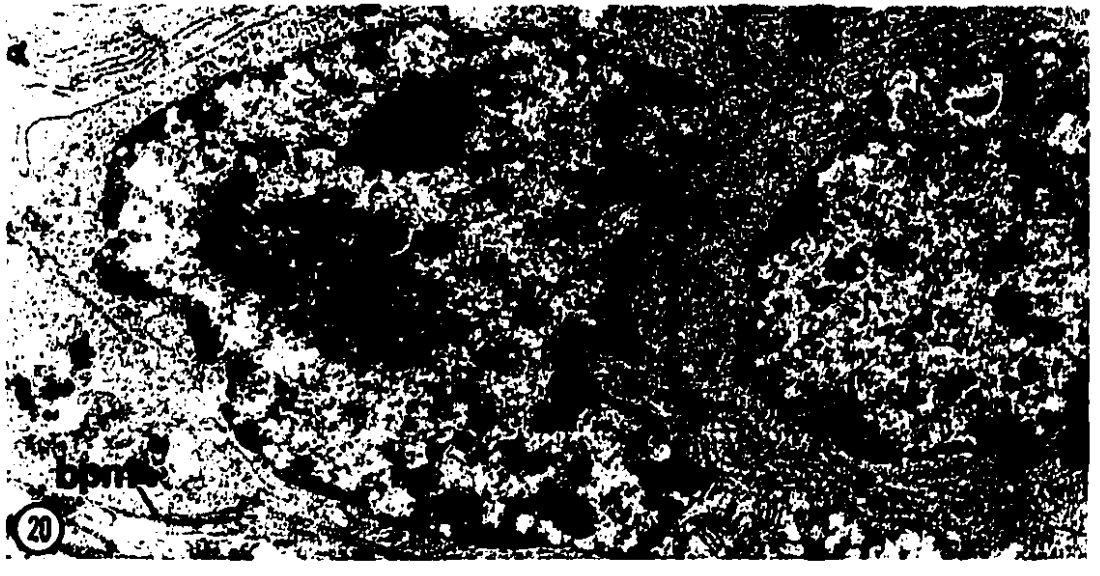

bpm
20

Fig. 21 EM radioautograph of capillary endothelial cell in the duodenal lamina propria of a rat killed 5 hrs after intravenous injection of ^{3}H-galactose.

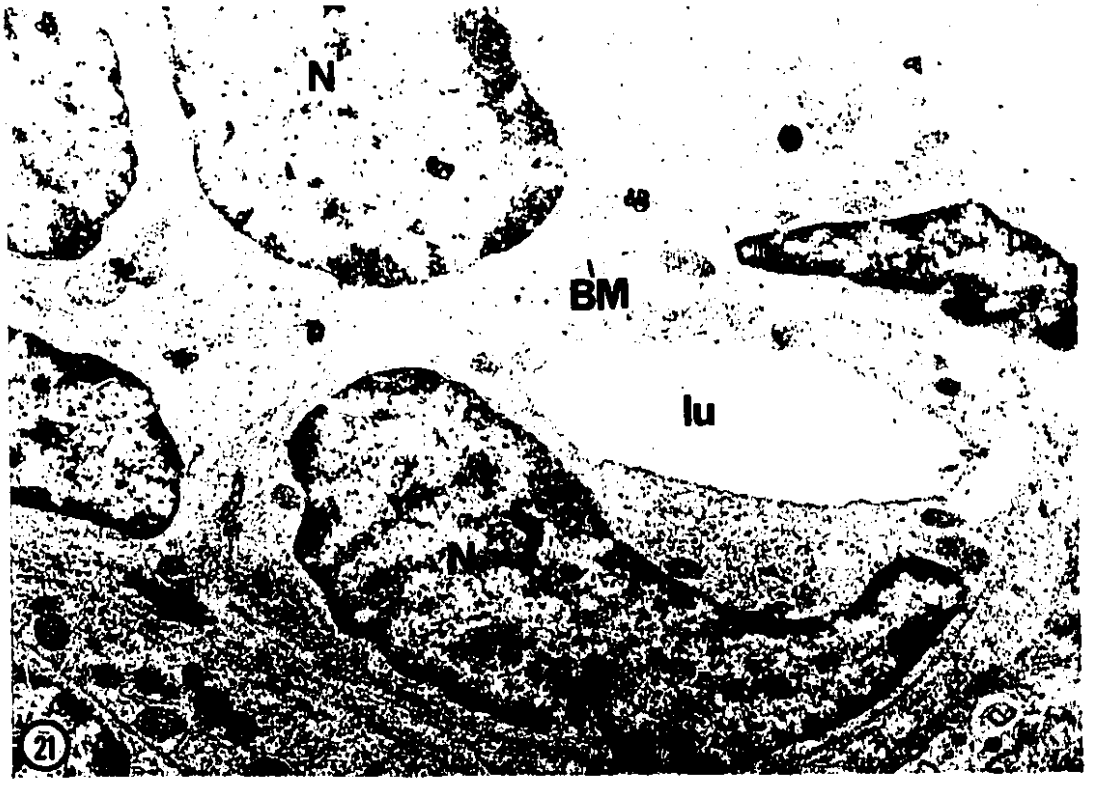
A light reaction is observed over the nucleus of the endothelial cell (N). Grains are also seen over the nuclei of adjacent crypt columnar epithelial cells (N'). (lu: capillary lumen; BM: basement membrane)

Exposure: 9 mo (Chem. dev.) x 10,000

Fig. 22 EM radioautograph of a migrating in the duodenal mucosa of a rat killed 1 hr after intravenous injection of ^{3}H-galactose.

The nucleus of the lymphocyte shows significant labeling, with most of the grains at the nuclear periphery. Note that part of the nuclear envelope (NE) appears internally here, due to the plane of section. (ICS: intercellular space; lpm: lateral plasma membane of columnar epithelial cells; BM: basement membrane; LP: lamina propria)

Exposure: 9 mo (Chem. dev.) x 12,000


N
BM
lu
N
21


ICS
lpm
M
LP
22

Fig. 23 EM radioautograph of fibrocytes in the duodenal lamina propria of a rat killed 1 hr after an intravenous injection of ^{3}H-galactose.

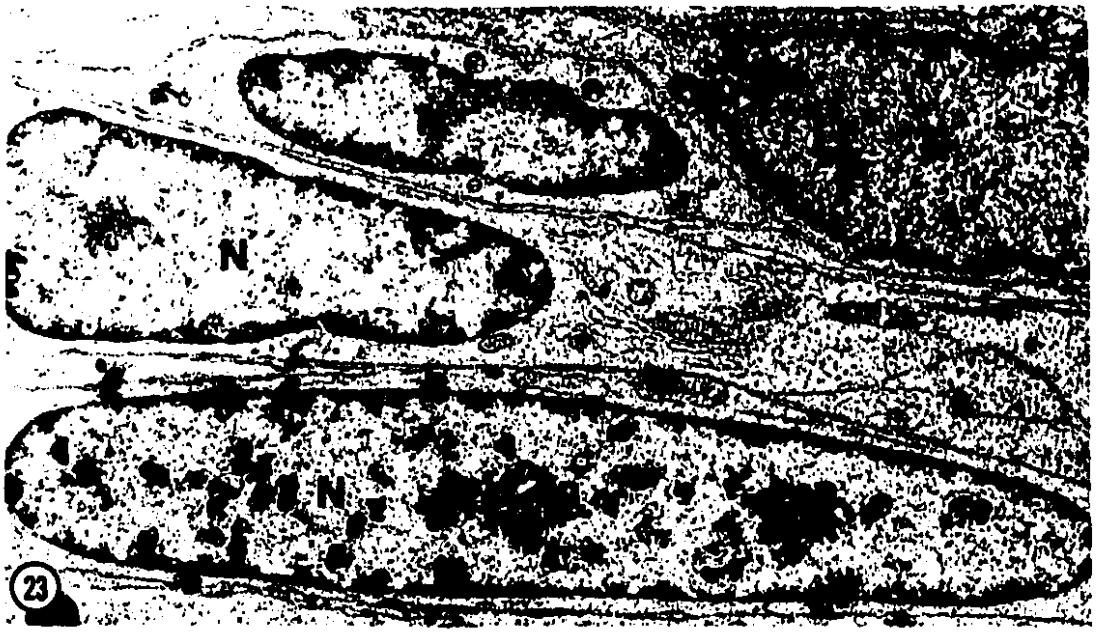
Some fibrocytes show very heavy labeling of their nuclei (N). The silver grains are distributed throughout the nucleus, and the nucleolus (n) is labeled. Other fibrocytes in this figure show less reaction over their nuclei (N').

Exposure: 9 mo (Chem dev.) x 12,000

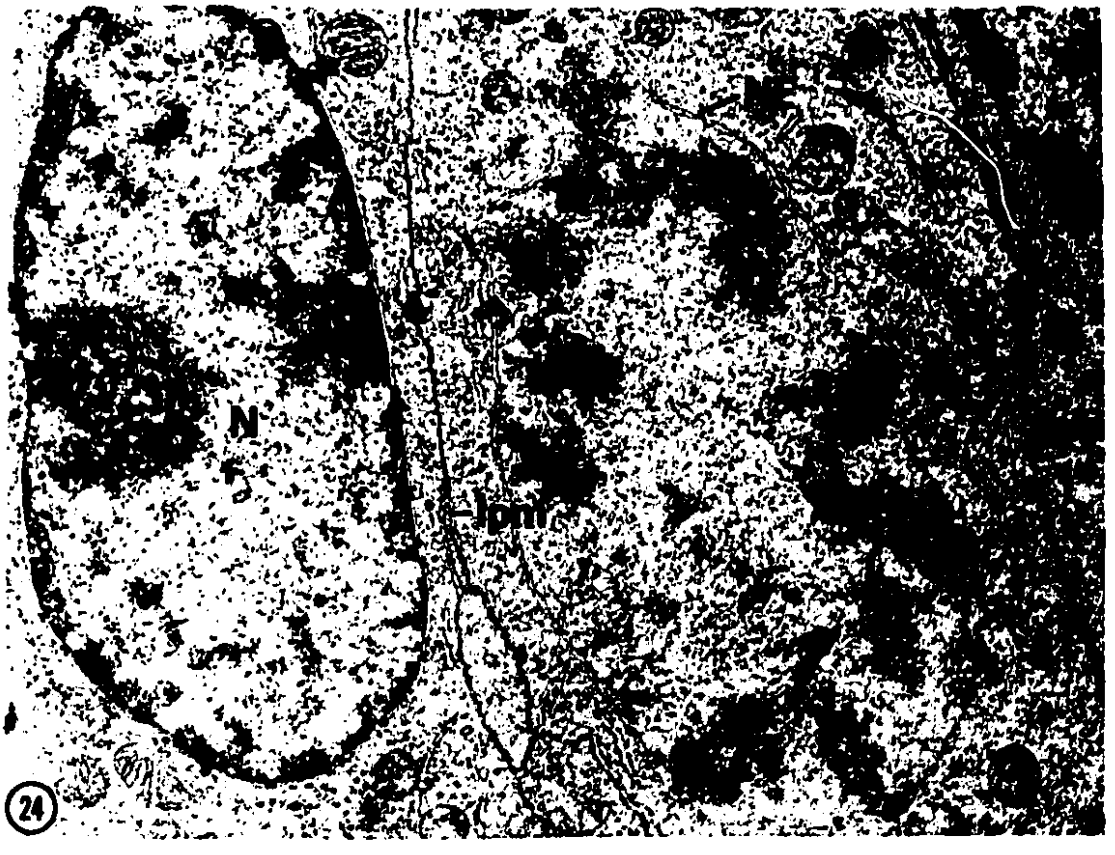
Fig. 24 EM radioautograph of a duodenal crypt columnar cell undergoing mitosis from a rat killed 5 hrs after intravenous injection of ^{3}H-galactose.

In this figure, the mitotic cell is in late prophase, and the forming chromosomes (cc) have separated from the nucleus envelope (NE). In some locations, the latter has begun to disappear (arrowhead). Most of the grains are localized over the condensed chromosomes, but two may be over the nuclear envelope. At the left and far right of the figure are interphase columnar cells which exhibit reactions over their nuclei (N). The grains are mostly located peripherally, but two (one very small) are observed over the nucleolus (n). Two grains overlie a lateral plasma membranes (lpm).

Exposure: 9 mo (Chem dev.) x 12,000



23

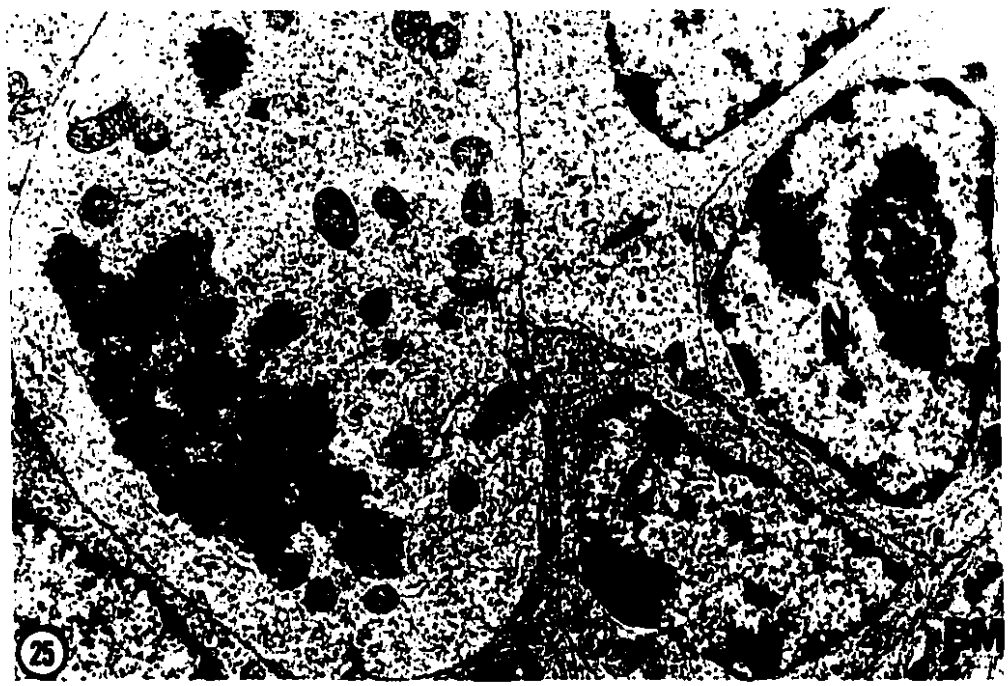


24

Figs. 25-26 EM radioautographs of sectons of duodenal crypt columnar cells undergoing mitosis from a rat killed 5 hrs after intravenous injection with ^{3}H-galactose.

The mitotic cell in Fig. 25 is in anaphase, and no nuclear envelope is present. The cell in Fig. 26 is in telophase, and the nuclear envelope has reformed. In both cells, the radioautographic grains are localized over the condensed chromosomal material (cc). In each figure, the surrounding interphase crypt cells exhibit reaction over their nuclei (N) and nucleoli (n).

Exposure: 9 mo (Chem. dev.) x 10,000


25
PM

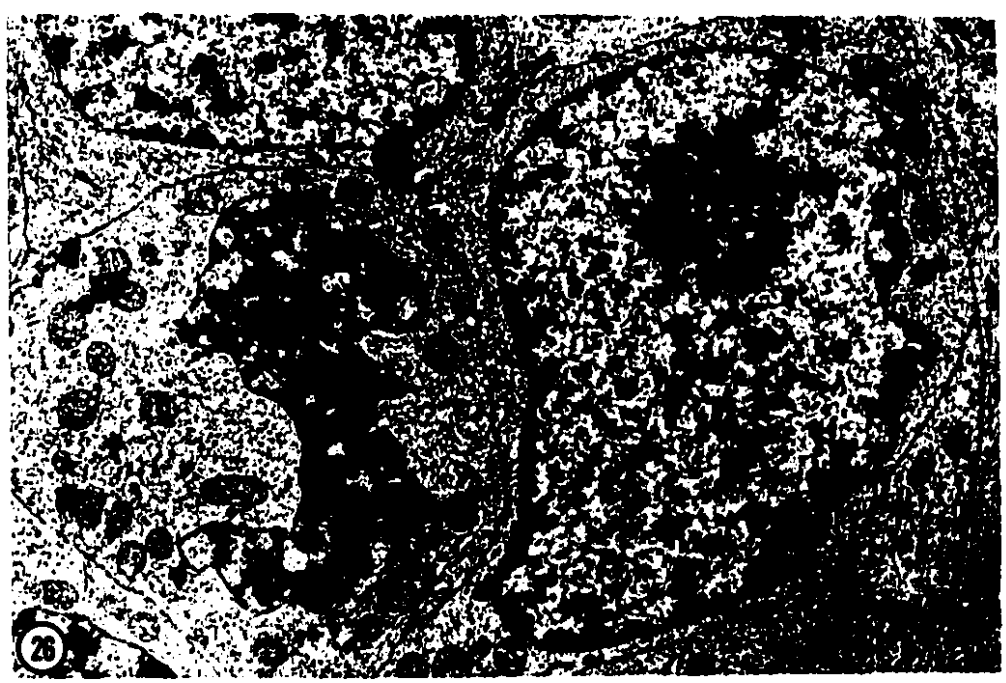

26

Fig. 27 EM radioautograph of a bile ductule cell from a liver segment incubated in vitro for 5 min with ^{3}H-galactose.

An intense reaction covers the nucleus (N), with grains distributed over both the periphery and the central regions. The cytoplasm exhibits a diffuse reaction of moderate intensity, and some grains are associated with the microvillous plasma membrane bordering a bile ductule (BD).

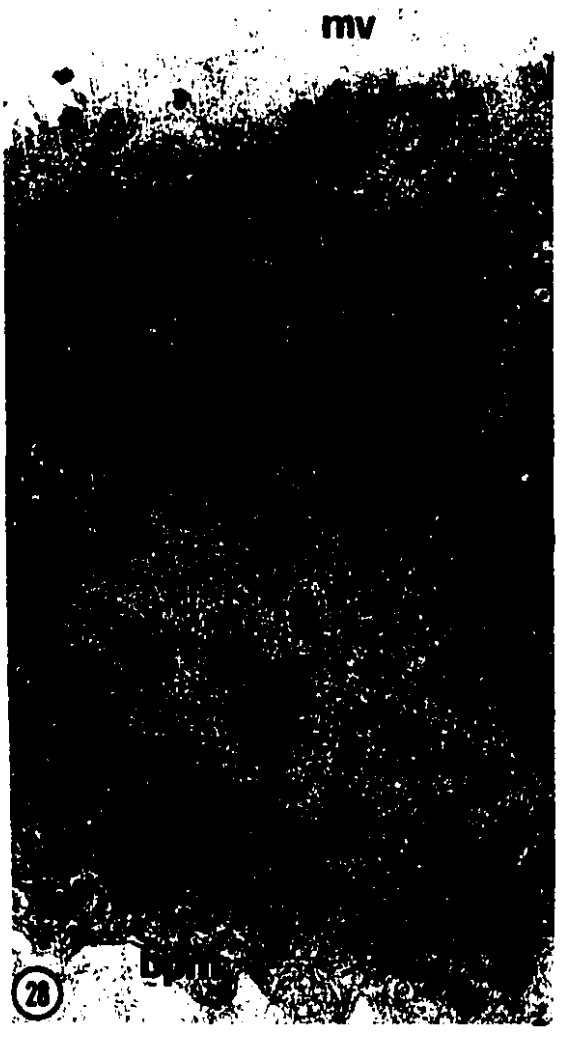
Exposure: 3 mo (Chem. dev.) x 17,000

Fig. 28 EM radioautograph of a mouse gall bladder epithelial cell removed from the animal 5 min after intraluminal injection of ^{3}H-galactose.

A moderate reaction is observed over the nucleus (N), with many grains occurring over central regions. In the cytoplasm, several grains are associated with the Golgi apparatus (G), while others are randomly distributed. Occasional grains are associated with the apical microvilli (mv) and the basal plasma membrane (bpm).

Exposure: 3 mo (Chem. dev.) x 13,000

27



28

Fig. 29 EM radioautograph of duodenal columnar cells from a rat killed 5 min after an intravenous injection of ^{3}H-glucosamine.

Substantial reaction is observed over the nuclei (N), occurring both peripherallly and internally. In the cytoplasm, many grains are associated with Golgi stacks (G) and the lateral plasma membrane (lpm).

Exposure: 1 mo (Chem. dev.) x 12,500


G
N
29

Fig. 30 EM radioautograph of a hepatocyte from a rat killed 5 min after an intravenous injection of ^{3}H-glucosamine.

The nucleus exhibits a fairly heavy reaction, with many grains over its central euchromatin regions (E). Some grains are associated with the nucleolus (n). In the cytoplasm there is a diffuse reaction in which may grains are over rough endoplasmic reticulum (rER) or mitochondria (M). A bile canaliculus (BC) is unlabeled.

Exposure: 1 mo (Chem. dev.) x 12,500

Fig. 31 EM radioautograph of a bile ductule cell from a rat killed 5 min after an intravenous injection of ^{3}H-glucosamine.

Heavy reaction is observed over the nucleus of the bile ductule cell (N), with grains over both the periphery and the internal regions. In the adjacent hepatocytes, heavy reaction covers mitochomdria (M) and/or rough endoplasmic reticulum (rER). Some grains overlie the plasma membrane bordering a bile ductule (BD).

Exposure: 3 mo (Chem. dev.) x 12,000

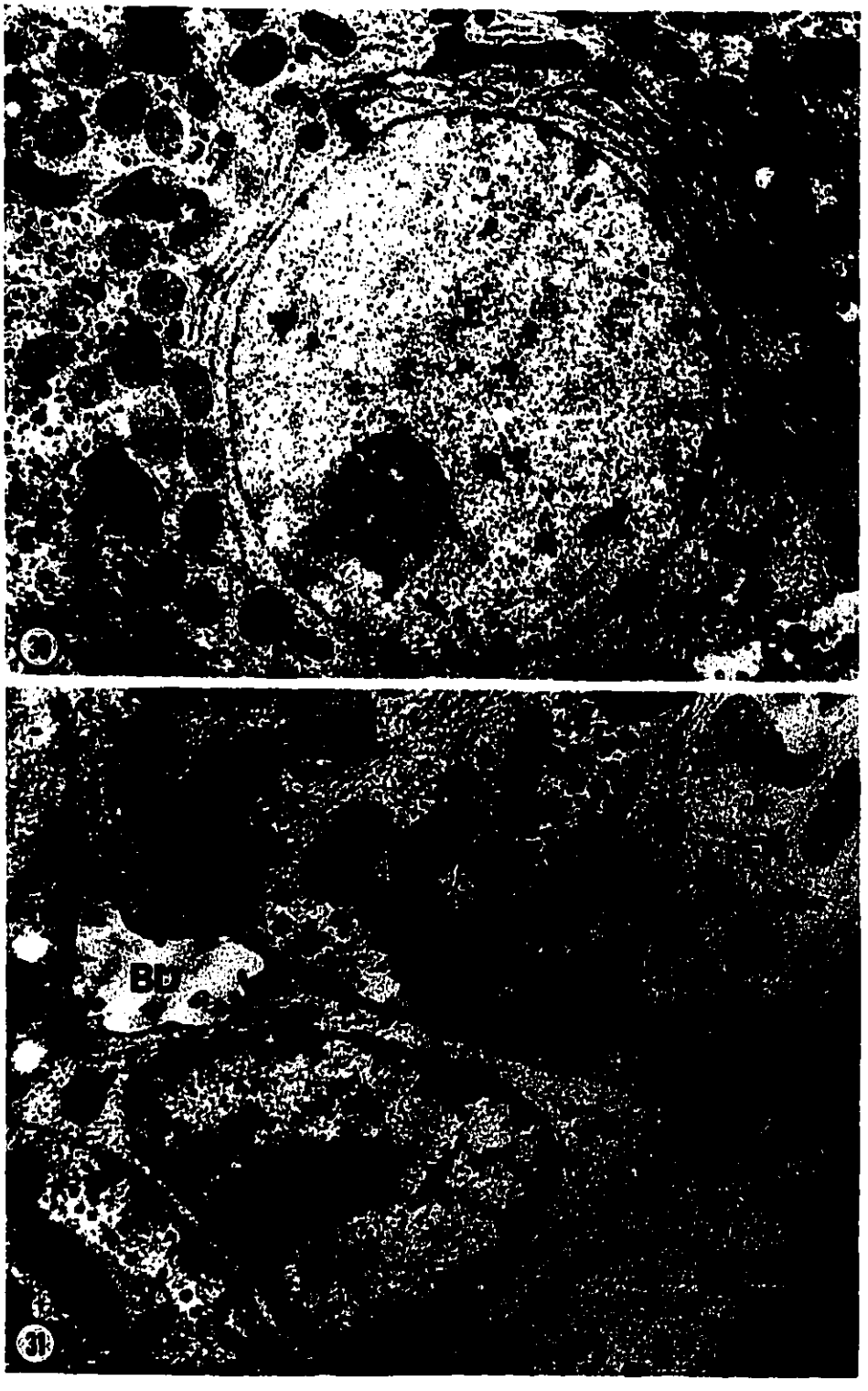

BB

Fig. 32 EM radioautograph of pancreatic duct cells from a rat killed 5 min after an intravenous injection of ^{3}H-glucosamine.

Reactions of moderate intensity are seen over the nuclei (N), in which many of the grains are centrally located. The cytoplasm is only lightly labeled, but occasional grains are observed over the lateral plasma membrane (lpm) and the apical plasma membrane facing the lumen (lu).

Exposure: 2.5 mo (Chem. dev.) x 11,000

Fig. 33 Electron microscope radioautograph of pancreatic Islet of Langerhans cell from a rat killed 5 min after an intravenous injection of ^{3}H-N-acetylglucosamine.

A fairly heavy reaction covers the nucleus (N), in which most of the grains are centrally located. In the cytoplasm, grains are associated with Golgi complexes (G) or secretory granules (sg). Occasional grains occur over or near the lateral plasma membrane (lpm) or the plasma membrane facing the sinusoid (sin).

Exposure: 2.5 mo (Chem. dev.) x 11,000


Ju
32

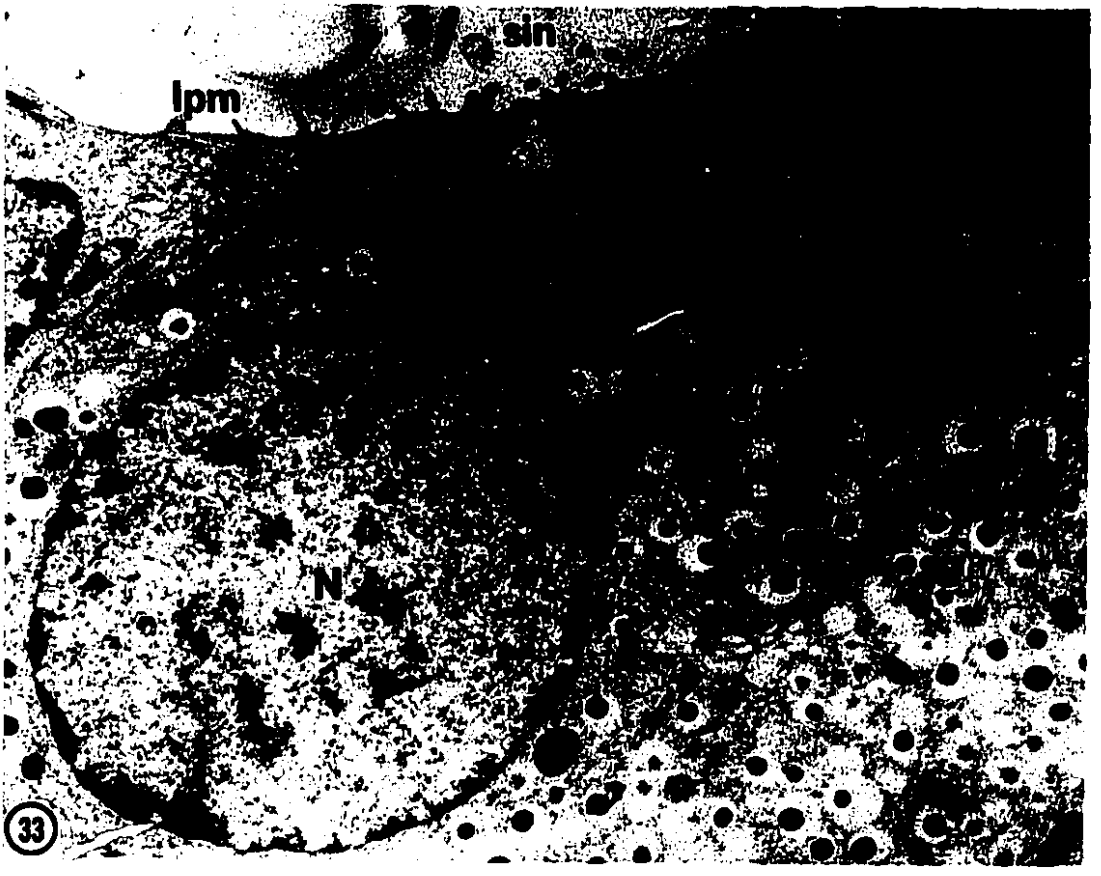

sin
lpm
N
33

Fig. 34 LM radioautograph of villous columnar cells of a mouse jejunal explant incubated with ^{3}H-mannose for 2 hours.

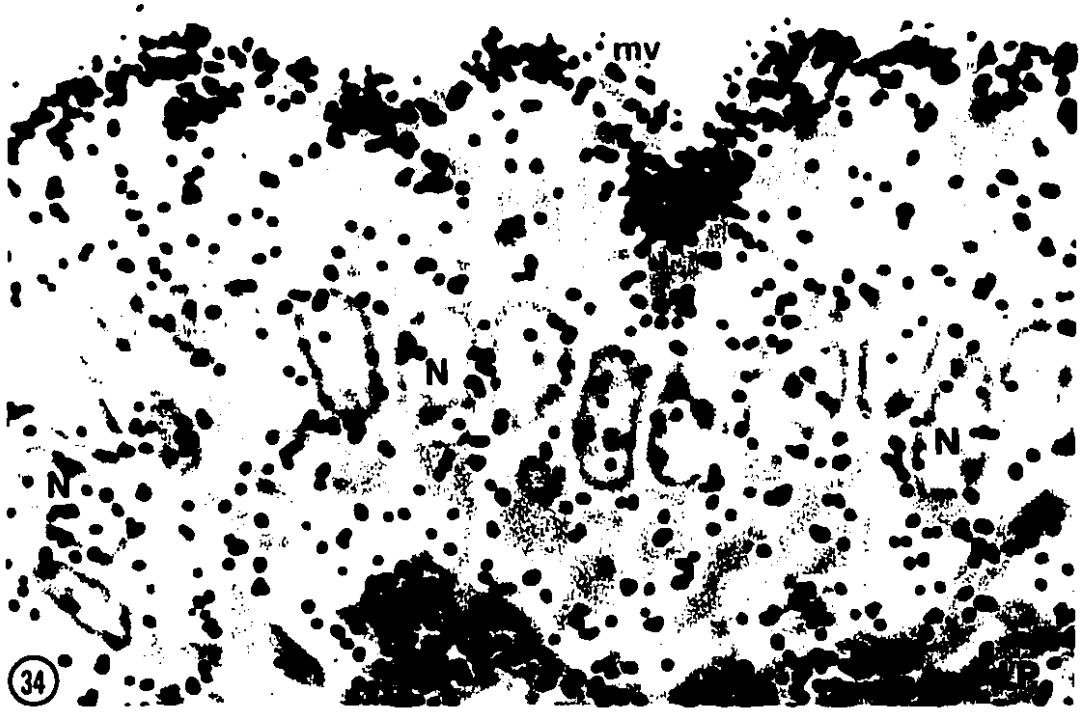
These cells exhibit substantial reaction over their nuclei (N). In addition, many grains are observed over the cytoplasm and especially over the apical microvillous border (mv). (LP: lamina propria)

Exposure: 3 mo x 1,600

Fig. 35 EM radioautograph of a cross-section of duodenal villous columnar cells from a rat killed 1 hr after an injection of ^{3}H-mannose.

Although the cellular reaction is quite light in general, a fair number of radioautographic grains are seen over the nuclei (N). Many of these grains are peripherally located, but some are internal. In the cytoplasm, some grains are over the lateral plasma membranes (lpm).

Exposure: 6 mo (Chem. dev.) x 12,000


mv
N
N
N
34

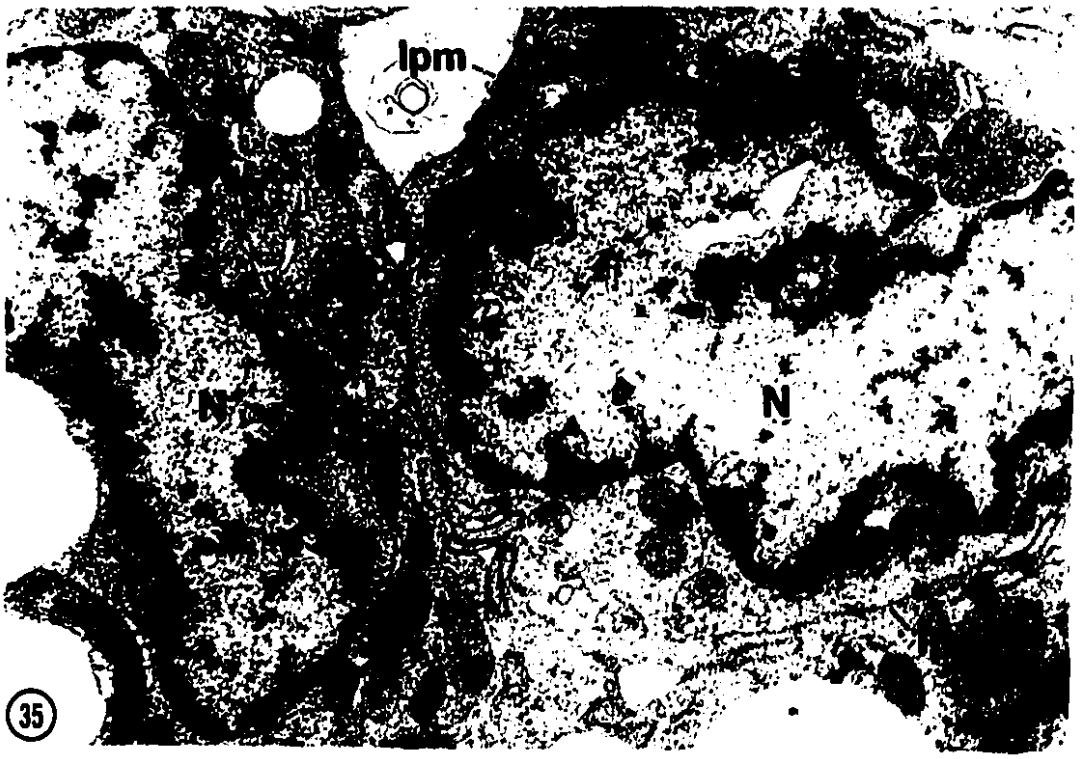

lpm
N
N
35

Fig. 36 EM radioautograph of a duodenal enteroendocrine cell from a rat killed 10 min after an intravenous injection of ^{3}H-mannose.

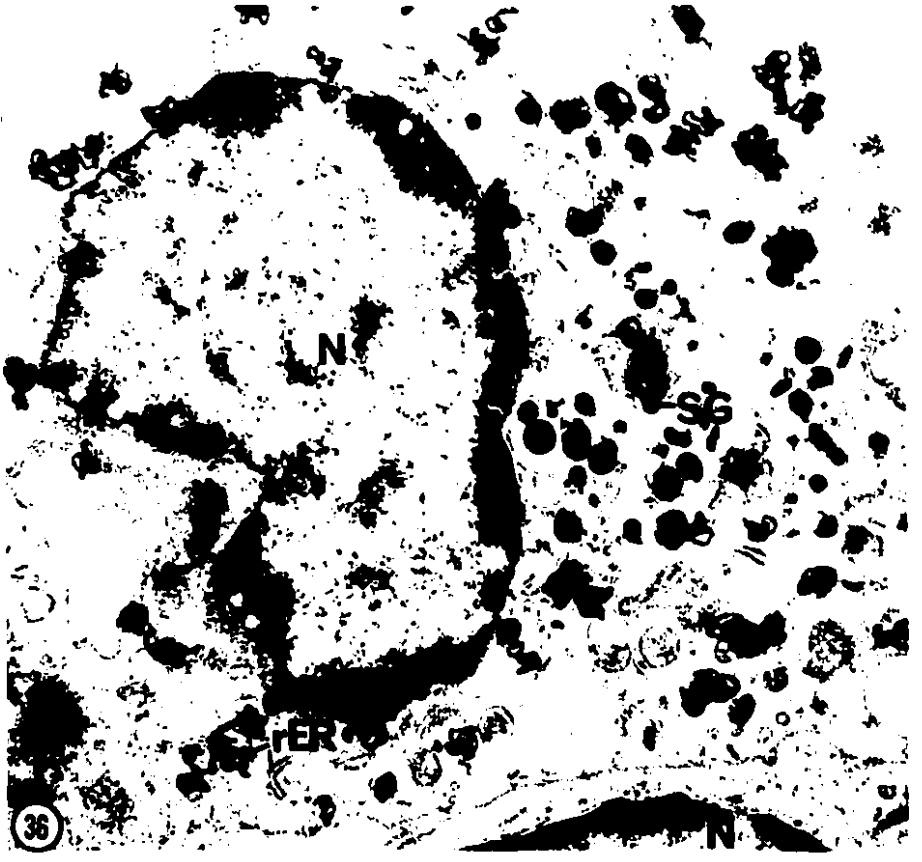
Several grains are observed over the periphery of the nucleus (N), although none are seen over the internal regions. In the cytoplasm, grains appear to be associated with rough endoplasmic reticulum (rER) and secretory granules (SG).

Exposure: 7 mo (Chem. dev.) x 12,000

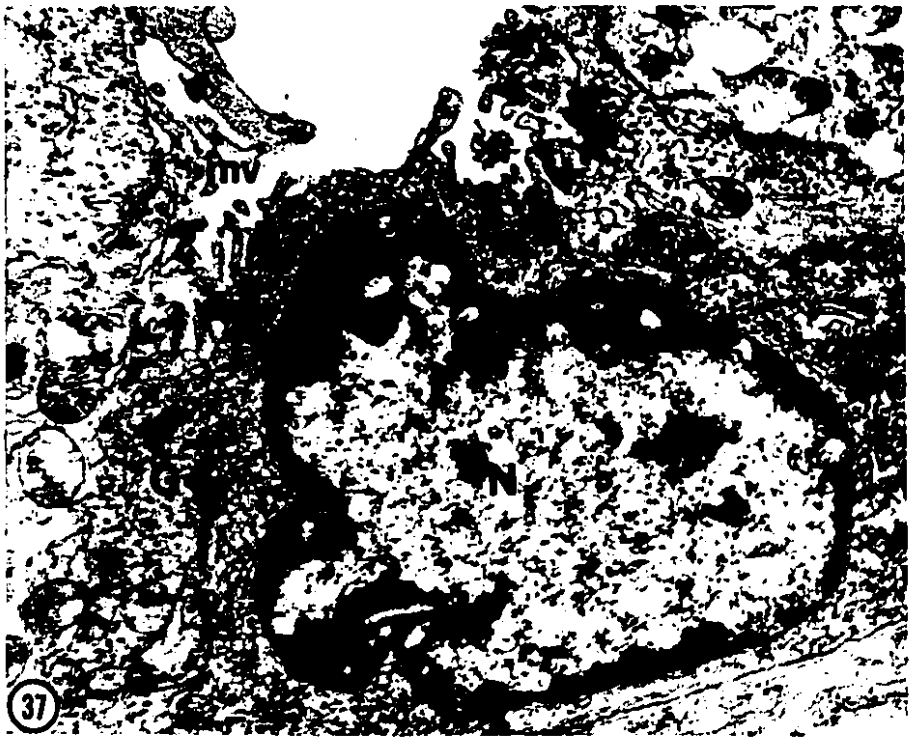
Fig. 37 EM radioautograph of a kidney distal convoluted tubule cell from a rat killed 20 min after an intravenous injection of ^{3}H-N-acetylmannosamine.

The nucleus (N) exhibits a light reaction, with three peripheral grains and one internal grain. In the cytoplasm, grains are localized over the Golgi apparatus (G) and the apical microvilli (mv). (bpm: basal plasma membrane).

Exposure: 4 mo (Chem. dev.) x 11,000



36



37

Fig. 38 LM radioautograph of colonic mucosa from a mouse killed 10 min after an intravenous injection of ^{3}H-N-acetylmannosamine.

At the right of the figure, surface columnar cells exhibit a localized supranuclear Golgi reaction (G), and only occasional grains occur over the nuclei (N'). At the left of the figure, upper crypt cells also exhibit a Golgi reaction, but there is also substantial reaction over the remainder of the cytoplasm, and many grains appear over the nuclei (N).

Exposure: 4 mo x 1,200

Fig. 39 EM radioautograph of upper crypt columnar cells from the colonic mucosa shown in Fig. 38.

The cells exhibit a strong Golgi reaction (G) and also a substantial reaction over their nuclei (N). Several grains are associated with the nucleolus.

Exposure: 6 mo (Chem. dev.) x 6,000

Fig. 40 Electron microscope radioautograph of surface columnar cells from the colonic mucosa shown in Fig. 38.

Heavy reaction covers the Golgi complexes (G), but almost no grains are over the nuclei (N'), even in those areas adjacent to the heavy Golgi reactions. Two grains are seen over the periphery of the nucleus at lower right.

Exposure: 6 mo (Chem. dev.) x 6,000

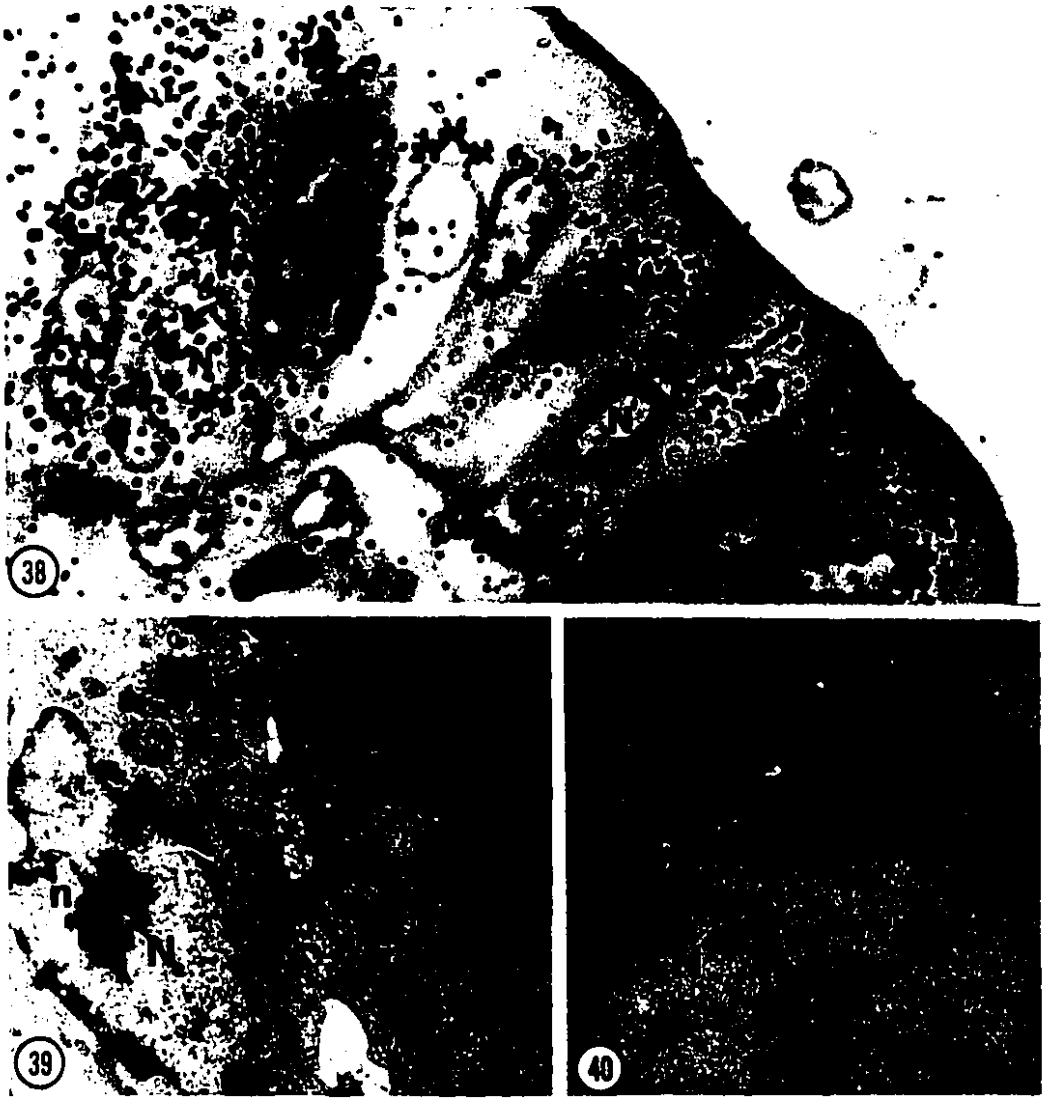

38
N
39
N
40

Fig. 41 Electron microscope radioautograph of hepatocytes from a mouse killed 5 min after an intravenous injection of ^{3}H-N-acetylmannosamine.

Several grains occur over the nuclei (N), and most of these are distributed over the central regions. One grain is at the edge of a nucleolus (n). In the cytoplasm, most reaction is associated with pale staining lysosomes (L). Some grains occur over or near the lateral plasma membranes (lpm) and bile canaliculi (BC).

Exposure: 11 mo (Chem. dev.) x 13,000

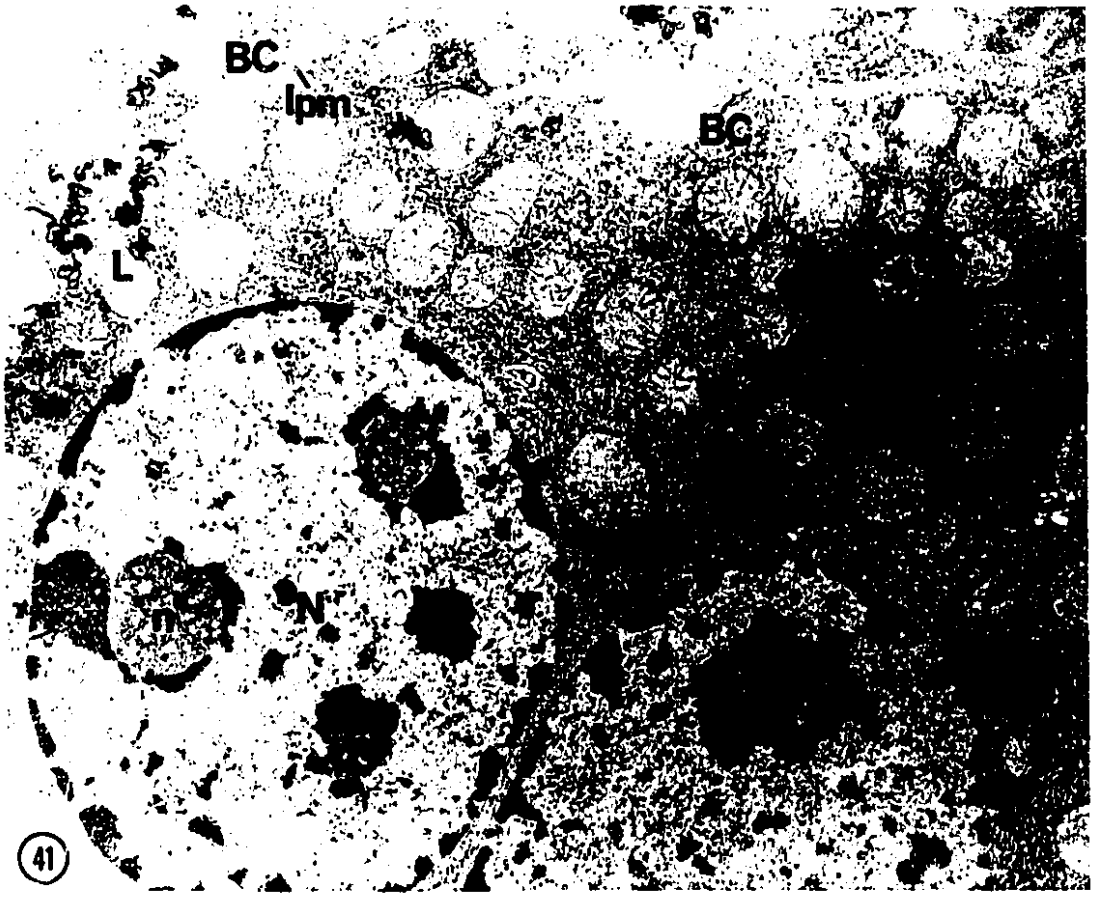

BC
lpm
BC
L
n
N
41

Fig. 42 Electron microscope radioautograph of a kidney proximal tubule cell from a mouse killed 5 min after an intravenous injection of ^{3}H-N-acetylmannosamine.

A fairly heavy reaction occurs over the nucleus (N). Over twelve grains are distributed over the nuclear periphery and internal regions. One lies at the edge of the nucleolus (n). The cytoplasm exhibits a light diffuse reaction.

Exposure: 11 mo (Chem. dev.) x 13,000

Fig. 43 Electron microscope radioautograph of a duodenal columnar cell from a mouse killed 5 min after an intravenous injection of ^{3}H-N-acetylmannosamine.

A fairly heavy reaction is observed over the Golgi complex (G), but several silver grains also occur over the nucleus (N), where most are centrally located. No reaction is seen at this time interval over the apical microvillous border (mv).

Exposure: 11 mo (Chem. dev.) x12,000

42

43

Fig. 44 EM radioautograph of hepatocytes from a mouse killed 4 hr after an intravenous injection of ^{35}S-sulfate.

Several grains over observed over the nuclei (N) of this binucleate hepatocyte, and many of these are centrally located. In the cytoplasm, many grains are located over mitochondria. Some are over or near the lateral plasma membrane (lpm). (BC: bile canaliculi).

Exposure: 6 mo (Chem. dev.) x 13,000

Fig. 45 EM radioautograph of duodenal villous columnar cells from a mouse killed 4 hr after an intravenous injection of ^{35}H-sulfate.

Very heavy reaction covers the nuclei (N), where many grains are centrally located, and some are associated with nucleoli (n). In the cytoplasm, many grains are associated with Golgi stacks (G) and the lateral plasma membranes (lpm).

Exposure: 6 mo (Chem. dev.) x 13,500


N
44

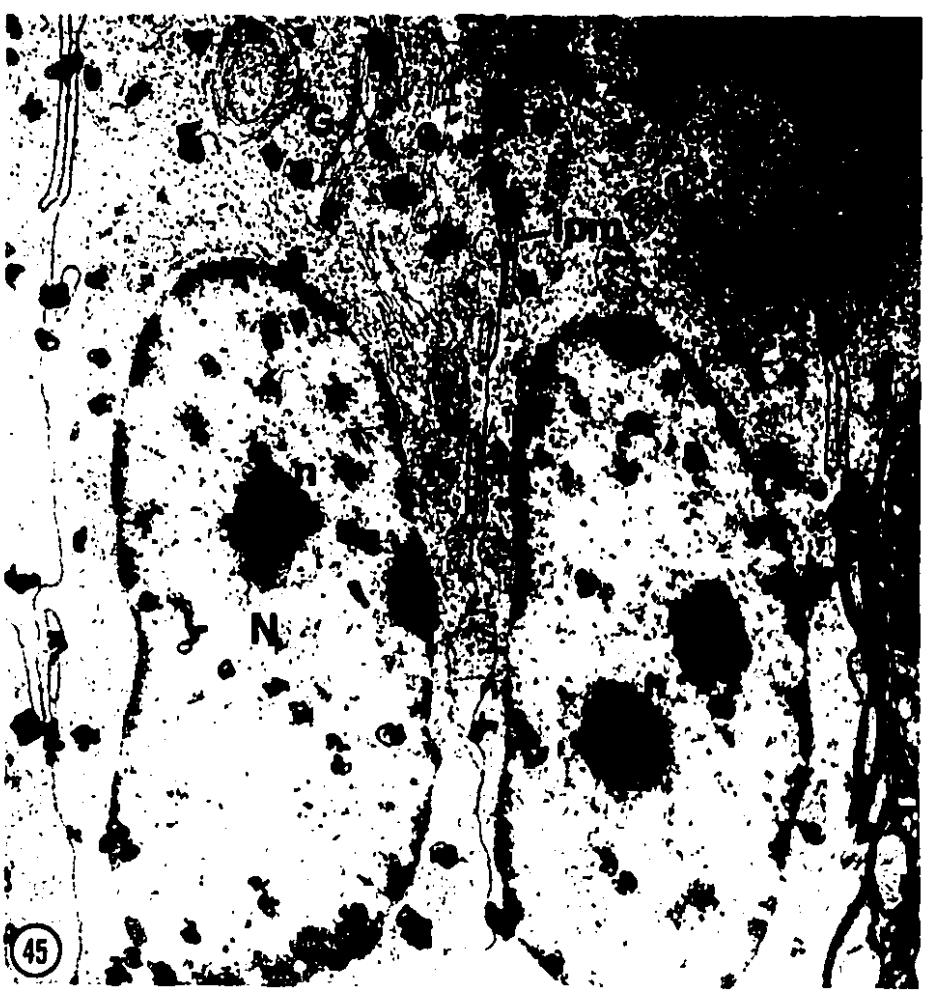

G
pm
N
45

Fig. 46 Electron microscope radioautograph of colonic goblet and columnar cells from a mouse killed 4 hr after an intravenous injection of ^{35}H-sulfate.

Substantial reaction is observed over the nuclei of both the goblet cell (N) and the columnar cells (N'). Many grains are centrally located, and some are associated with nucleoli (n). In the cytoplasm, a heavy reaction occurs over the Golgi apparatus (G) of the goblet cell, and intense reactions is seen over the secretory granules (SG). Some grains occur over the lateral plasma membranes (lpm).

Exposure: 6 mo (Chem. dev.) x 13,500

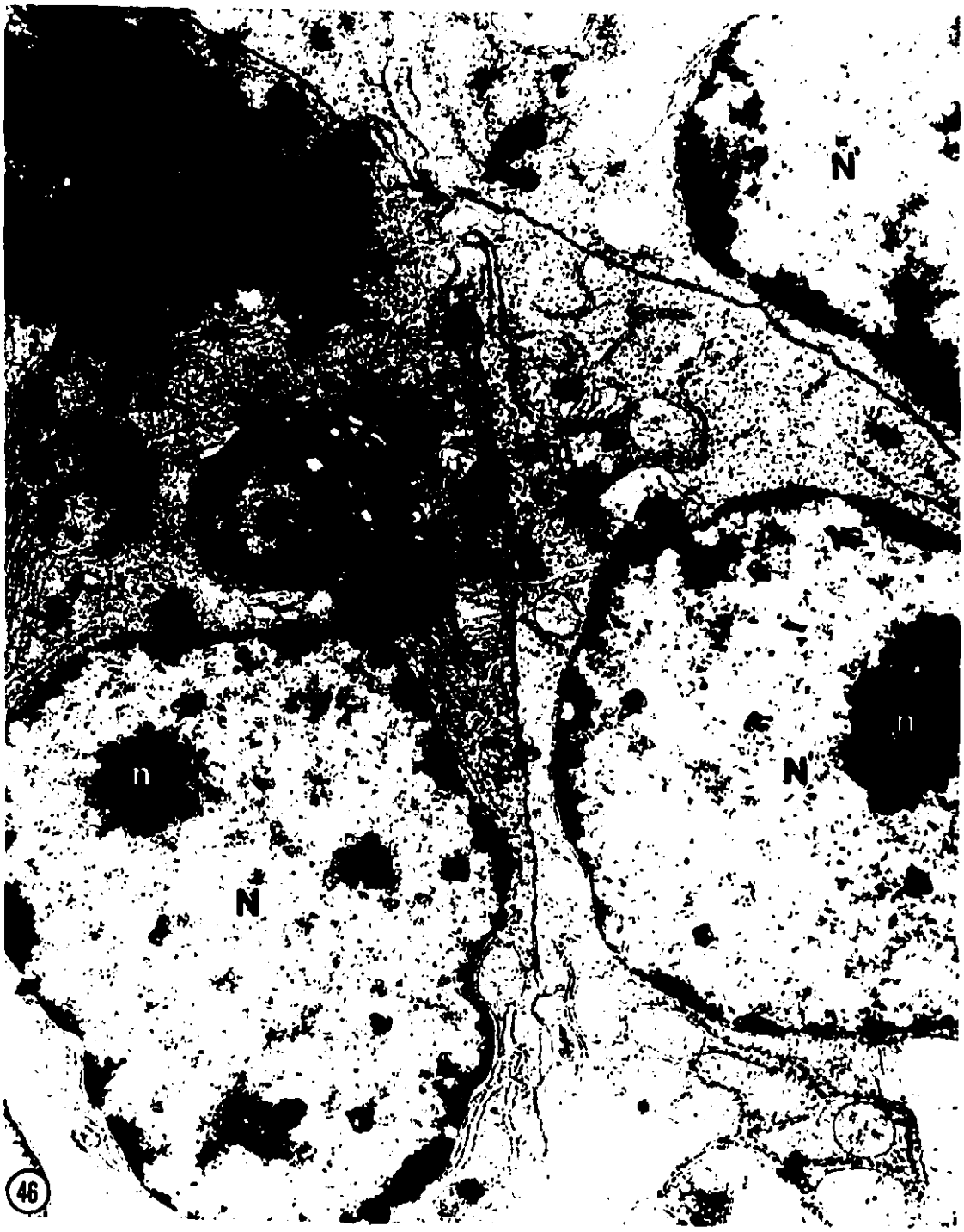

N'
n
N
n
N'
46

Table 1- Cell Types Exhibiting Nuclear Reactions After Administration of ^{3}H-Sugars or ^{35}S-Labeled Sulphate

Cell Type	Fuc	Gal	GlcNAc	Man	ManNAc	SO$_4$
Rat						
duodenal cells						
columnar c.	+	+++	++	++	+/-	+++
goblet c.	+	+++	++	ne	-	+++
enteroendocrine c.	ne	ne	ne	++	ne	ne
lamina propria endo cell	ne	++	ne	ne	ne	ne
pancreatic cells						
acinar c.	+	++	++	+	ne	ne
islet c.	+	++	+++	++		ne
duct c.	+	+++	++	+		ne
colonic columnar c.	+	++	++	+		+++
hepatocyte	+	++	+++	++		+++
bile ductule c.	ne	+++	+++	++		ne
kidney prox. conv. tubl. c.	+	++	++	++		++
adrenal cortex c.	+	++	ne	++		ne
choroid plexus c.	+	ne	++	++		
ciliary epithelial c.	+	ne	++	++		
stomach stratified epith. c.	+	ne	++	ne		
lymphocyte	+	+++	ne	++		
fibrocyte	+	+++	++	+		
C.N.S. neuron	ne	ne	ne	++		
retina cap. endo. c.	++	ne	ne	ne		
retina RPE cell	++	ne	ne	ne		
lens capsule cell	++	ne	ne	ne		
Mouse						
duodenal cells						
columnar c.	++	ne	ne	++	++	+++
goblet c.	++			++	++	+++
jejunal columnar c.	ne			ne	++	+++
colonic cells						
surface columnar c.	++				+/-	+++
crypt columnar c.	++				++	+++
goblet c.	++				+	++
pancreas acinar c.	++				++	++
hepatocyte	++				++	++
kidney prox con tub c	++				++	ne
gall bladder epithelial c.	ne	++			ne	ne
thyroid follicular c.	ne	ne			+	++
spermatogonia	ne				++	++
lymphocyte	ne				ne	++
adrenal medulla	ne				ne	ne
Frog						
neuron c.	++	++	ne	ne	++	ne
Schwann c.	+++	+++			++	
satellite c.	+++	+++			++	
cap. endothelial c.	+++	ne			ne	
duodenal columnar c.	++	++				
hepatocyte	++	++				
kidney tubule c.	++	++				
pancreatic acinar c.	++	++				
gallbladder epithelial c.	++	ne				
ciliary epithelial c.	++	ne				

+++ =very strong + =small
++ = medium +/- = little or none
ne= not examined

Chapter 3 - Radioautographic detection and localization of nucleoplasmic glycoproteins in interphase and dividing cultured cells

I. Introduction

The radioautographic studies described in Chapter 2 suggested the existence of nucleoplasmic glycoproteins in a great variety of cell types in different animal species after exposure to ^{3}H-sugars or ^{35}S-sulfate. However, due to limitations of the radioautographic technique two questions remained in part or completely unanswered. The first question arises due to the known limitation in the spatial resolution of label afforded by the radioautographic method. These limitations are such that it is known that the radioactive source for an observed silver grain does not always lie in the structure immediately below that grain and may in fact be located at some distance away from it. With this the case, when silver grains are observed over a nucleus, it might be asked to what extent these grains are actually due to emissions from molecules located in the nucleoplasm. Or to rephrase the question, what is the relative contribution of extra-nucleoplasmic radioactive sources (e.g. nuclear envelope and adjacent cytosol) to the observed nucleoplasmic label?

In the case of the more centrally located grains, there is little doubt that the radioactive sources must lie within the nucleoplasm. In previous analysis of the distribution pattern of radioautographic grains over the nuclei of frog Schwann cells, the question was asked as to whether or not the nuclear labeling distribution could be explained by radioactive sources residing in the nuclear envelope (or adjacent

cytosol). The nuclear distribution was compared with a hypothetical distribution pattern expected if the nuclear envelope was the sole source of label and it was found that this hypothetical distribution pattern could not explain the more interiorly located nucleoplasmic grains. It was therefore concluded that at least the more interiorly located silver grains must be derived from nucleoplasmic sources (Bennett et al., 1986).

For grains located at the nuclear periphery, it was more difficult to draw firm conclusions as to the true source of label, since at this site a number of potentially labeled compartments, e.g. peripheral euchromatin, peripheral heterochromatin, nuclear envelope, or adjacent cytosol could be possible sources of the label. To determine more definitively the source of this peripheral nuclear labeling, it was desirable to utilize cells in which the cell nucleus could be separated from the cytoplasm and even the nuclear envelope by biochemical fractionation procedures. Such cells would be exposed to a labeled precursor and subsequently fractionated, and radioautography would then be used to compare labeling of the isolated nuclei with those of undisrupted cells.

It was obviously necessary that such a model cell type exhibit substantial nucleoplasmic labeling. In our <u>in vivo</u> survey studies, the heaviest examples of nucleoplasmic labeling occurred in certain cell types, e.g. rat duodenal

crypt columnar cells, and frog Schwann cells. Attempts to obtain uncontaminated nuclei from duodenal cells proved to be very difficult due to their extensive cytoskeleton, and the amount of tissue required for isolations of Schwann cell nuclei was considered prohibitive. Successful isolations of liver cell nuclei were achieved, but the level of nucleoplasmic labeling in these cells was insufficient. Therefore an alternative cell type was needed which could be obtained in adequate quantities, show at least reasonable nucleoplasmic labeling after exposure to ^{3}H-sugars, and be fractionated to yield isolated nuclei almost completely free from cytoplasmic contamination.

The idea of turning to cultured cells for our experiments seemed to be appealing for a number of reasons. Firstly, at this time a collaboration with Dr. Yves Raymond made possible the use of a cultured myeloma cell line which could yield an adequate amount of starting material for joint radioautographic and biochemical studies on nuclear fractions. Heavy nucleoplasmic labeling of lymphocytic cells in the spleen and bone marrow had been observed in our previous studies making such a cell line a feasible starting candidate. Subsequent morphological observation of initial isolated nuclear fractions from these cells showed them be minimally contaminated with recognizeable cytoplasmic elements which satisfied the second of the three aforementioned desired criteria. What remained to be determined was whether the cells

would show appreciable nucleoplasmic labeling. Other cultured cell types, i.e. YAC T-cell lymphoma cells and Friend erythroleukemia cells, were also made available to us at this time by Dr. Sandra Miller of our Anatomy Department.

A further advantage of using these cultured cells was that many could be seen in mitosis, providing an opportunity to observe radioautographic labeling of nuclear components both during interphase and mitosis.

The present chapter presents the radioautographic findings observed in whole cultured myeloma cells exposed to labeled sugars for different time intervals. The results of labeling studies on YAC lymphoma and Friend Erythroleukemia cells are also described. Our findings, which indicated the presence of substantial nucleoplasmic labeling in myeloma cells (as well as in the YAC and Friend cells) confirmed these cultured cells to be a legitimate choice as a model for fractionation studies to examine more fully the source(s) of the observed nucleoplasmic labeling. (Those studies are described in Chapter 4). Quantitative studies of the distribution of silver grains over cytoplasmic and nuclear compartments in cultured cells exposed to the above precursors are also presented which expand on the qualitative observations of Chapter 1 pertaining to the areas of localization of labeled molecules (or putative glycoproteins) within the nuclei.

Also presented in this chapter are observations on the labeling patterns of nuclei of occasional myeloma cells, which

appeared to be in different stages of apoptosis with drastic changes in their nuclear morphology.

Lastly, light microscope results of two sets of different metabolic labeling experiments on cultured cells are presented. In the first set of experiments, nucleoplasmic labeling is examined in myeloma cells in pulse-chase experiments after exposure to some radiolabeled sugars. In the second set, the effect of tunicamycin administration on nucleoplasmic labeling of YAC lymphoma and myeloma cells exposed to radiolabeled sugars has also been examined.

II. Materials and Methods

A. Radioautographic Experiments on Cultured Cells

I) Biological Materials and Labeled Precursors

Suspension cultures of mouse myeloma cells (P3X63AG8.653 non-antibody secreting) were provided as a generous gift from Dr. Yves Raymond of the Institut du Cancer de Montréal. YAC T-cell lymphoma cells and Friend Eythroleukemia cells were provided as a generous gift from Dr. Sandra Miller of the McGill Anatomy Department. The following labeled precursors: L-[5,6-^{3}H]- fucose (spec. act. 60 Ci/m mole), D-[1-^{3}H(N)]-galactose (spec. act. 55 Ci/mmole), N-[mannosamine-6-^{3}H(N)]-acetylmannosamine (spec. act. 30.0 Ci/m mole), D-[2-^{3}H(N)]-mannose (spec. act. 30 Ci/m mole), N-[glucosamine-1,6-^{3}H-(N)]-acetylglucosamine (spec. act. 32.4 Ci/m mole) and [^{35}S]-sodium sulfate (spec. act. 1200 mCi/m mole) were obtained from New England Nuclear, Boston.

II) Experimental Procedures

The mouse myeloma cells were grown in RPMI 1640 media buffered with 10 mM HEPES (pH 7.2) and supplemented with 20% fetal calf serum, 1.1% sodium pyruvate, 2.92% glutamine, with penicillin/streptomycin (pen/strep) and kanamycin as antibiotics (Gibco Laboratories). YAC T-cell lymphoma cells were grown in RPMI 1640 media buffered with 25 mM HEPES (pH 7.2) and supplemented with 10% fetal calf serum with pen/step as antibiotics. Friend Eythroleukemia cells were grown in modified Basal Eagle media supplemented with 15% fetal calf serum, sodium bicarbonate, and 2% essential and nonessential amino acids.

The tritiated sugars ^{3}H-mannose, ^{3}H-N-glcNAc, ^{3}H-galactose, ^{3}H-fucose, or ^{3}H-N-acetylmannosamine (New England Nuclear) were evaporated under sterile conditions with a stream of dry N_2 and reconstituted in an appropriate small volume of sterile PBS. These sugars were then administered to the above cultures in log phase for various time intervals.

A) 18 Hr Incubations

Myeloma cells were incubated for 18 hrs with 50 μCi/ml of ^{3}H-mannose, ^{3}H-N-acetylglucosamine, ^{3}H-galactose, ^{3}H-fucose or ^{3}H-N-acetylmannosamine or 2 μCi/ml ^{35}S-sulphate. YAC lymphoma and Friend Erythroleukemia cells were incubated with 5 μCi/ml of ^{3}H-galactose or ^{3}H-fucose. At the end of the labeling period, the cells were pelleted by centrifugation at 400 x g for 10 min. The cells were washed twice with 5 mM PBS (pH

7.2), and were then fixed at 4°C with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2 hrs. The fixed cells were transferred to 1.5 ml plastic Eppendorf microfuge tubes, and centrifuged for 4 min. These were washed with 0.1 M cacodylate buffer (pH 7.4) containing 4% sucrose, and then post-fixed in 1% potassium ferrocyanide-reduced osmium for 1 hr. The pellets were then processed for radioautography as described for the blocks of animal tissue, and examined using both light and electron microscopy. Three categories of cells were analyzed within the cultured cell population, namely cells in interphase, cells in various phases of mitotic division, and finally, some cells undergoing apoptotic cell death.

B) 2-60 Min Incubations

For some radioautographic studies, much shorter radiolabeling intervals were used. In some experiments, myeloma cells were incubated for 10 min or 60 min with 10 μCi/ml ^{3}H-mannose, ^{3}H-glucosamine, and ^{3}H-galactose. At the end of the labeling period, the cells were washed with PBS and fixed and processed as above. In other experiments, myeloma cells were incubated for 2 min with 50 μCi/ml ^{3}H-N-glcNAc. These were pelleted at 4°C, and immediately fixed and processed as with the other experiments.

C) Pulse-Chase Incubations

In pulse-chase experiments, myeloma cells were labeled for 4 hrs with 10 μCi/ml ^{3}H-fucose and then chased for 0, 6, or 18

hrs in medium containing a 100 fold excess of cold fucose. In a second experiment, the cells were labeled for 4 hrs with 10 μCi/ml ^{3}H-galactose and then chased for 0 or 18 hrs in medium containing a 10 fold excess of cold galactose. The cells were then pelleted, washed, fixed, and processed for radioautography as described above.

D) Incubation in the Presence of Tunicamycin

To assess the effect of the N-linked glycosylation inhibitor tunicamycin on biosynthesis of nuclear glycoproteins, myeloma cells were pre-incubated for 4 hr with this drug as follows: A 10 mg/ml stock solution of tunicamycin dissolved in dimethylsulfoxide (DMSO) was added to the media to a final concentration of 10 μg/ml. At this concentration, the medium contained 0.5 μl/ml DMSO. Control cells were pre-incubated in medium also containing DMSO but lacking tunicamycin. Both experimental and control cells were then radiolabeled with 10 μCi/ml of ^{3}H-gal for an additional 4 hr. The cells were then pelleted, washed, fixed, and processed for radioautography as described above.

III) Quantitative Analysis of EM Radioautographs

For analytical purposes, each silver grain (or a portion thereof) was assigned to one of the following cell compartments of interphase cells:

I) Cytoplasm (C)
II) Nuclear envelope (NE)
III) Heterochromatin (H)
IV) Euchromatin (E)
V) Nucleolus (n)

In addition, a sixth separately tabulated category entitled "heterochromatin/euchromatin interface" was included consisting of the line formed by contiguous points of apposition between the condensed heterochromatin and the dispersed euchromatin.

In radioautographs developed by the chemical development procedure, the possible source of a silver grain was designated as any of the above compartments lying within an "assignment distance" of 230 nm from the centre of that grain. This distance is based on the calculation by Nadler that a circle with this radius, drawn around the center of a silver grain, would have a 95% probability of containing the radioactive source which gave rise to the grain (Nadler, 1971). When two or more compartments lay within 230 nm of the center of one silver grain, that grain was considered to be shared, and its value was divided equally by the sharing compartments.

With solution physical (fine grain) development, the resolution obtainable is approximately twice that of the chemical development procedure (Kopriwa et al., 1984). Thus an "assignment distance" of 115 nm was used in analyzing radioautographs developed with this procedure. In these radioautographs, a value of "one grain" was assigned both to isolated single silver particles and to compact clusters of particles, on the assumption that both the former and the latter were probably derived from one silver bromide crystal during development.

III. Results

A. Radioautographic Studies with Intact Cultured Cells

I) ^{3}H-Galactose Experiments

A) Mouse Myeloma Cells-18 hr Incubations

1) Interphase Cells

EM radioautographs of cells incubated for 18 hrs with ^{3}H-galactose showed heavy nuclear reactions with both chemical (Fig.47) and physical development (Fig. 48). Quantitation of silver grains (Table 2) showed that approximately 22.3% of the grains were localized over the nucleus in the former case and 19.5% with the latter. Within the nucleus 1.3-2.1% of the grains were assigned to the nuclear envelope (using values derived from assessments made with both grain development procedures), 4.7-5.1% to the heterochromatin, 9.1-11.6% to the euchromatin, and 4.0% to the nucleolus, depending on the type of grain development used (Table 2). 5.7-9.1% of the silver grains lay within one assignment distance of the heterochromatin/euchromatin interface.

The distribution of grains outside the nucleus was not quantified but there appeared to be prominent labeling of the plasma membrane, lysosomes, Golgi apparatus, and surprisingly, the rER and the cytosol.

2) Dividing Cells

Many silver grains remained associated with the chromosomes at all stages of mitosis (Figs. 49-51). In Fig. 49, for example, a cell in metaphase is seen which shows

prominent labeling of its chromosomes as well as the nucleolar remnant. The grains were seen mostly at the periphery of this latter structure. At anaphase (Fig. 50), the chromosomes still were strikingly labeled. Some of these grains were at the periphery, where the forming nuclear envelope was observed. At telophase (Fig. 51), many grains were scattered over both peripheral and central regions of the reforming nucleus, and occasional grains were observed at the periphery of the nucleolus.

In the cytoplasm, at all stages of mitosis there was labeling of the plasma membrane and lysosomes which was accompanied by a diffuse labeling of the cytosol.

B) Mouse YAC Lymphoma Cells - 18 hr Incubations

1) Interphase Cells

Radioautographs developed with solution physical development showed a very heavy reaction over both the nucleus as well as the cytoplasm, as seen after 3 months of exposure (Fig. 52). Quantitation of silver grains (Table 3) showed that approximately 28.3% of the grains were localized over the nucleus. Within the nucleus 2.4% of the grains were assigned to the nuclear envelope, 8.3% to the heterochromatin, 11.3% to the euchromatin, and 6.2 to the nucleolus. At the heterochromatin/euchromatin interface, 9.5% of the total cellular grains were observed.

In the rest of the cell, the labeling pattern was similar to the myeloma cells. Grains were again observed over the

plasma membrane, lysosomes, Golgi apparatus, and to a small extent, the cytosol.

2) Dividing Cells

In mitotic cells, there was a striking localization of silver grains to the chromosomes, both over their peripheral and internal regions. A fairly light reaction appeared over the cytoplasm and plasma membrane (Fig. 53).

C) Mouse Friend Erythroleukemia Cells - 18 hr Incubations

1) Interphase Cells

Radioautographs developed with solution physical development showed a moderately heavy reaction over the nucleus along with heavy labeling of the cytoplasm (Fig. 54). Quantitation of silver grains (Table 4) showed that approximately 21.3% of the grains were localized over the nucleus. Within the nucleus 2.1% of the grains were assigned to the nuclear envelope, 5.9% to the heterochromatin, 10.2% to the euchromatin, and 3.1% to the nucleolus. 8.9% of the total cellular grains appeared over the heterochromatin/ euchromatin interface.

In the cytoplasm, the Golgi apparatus was heavily labeled. The plasma membrane was also labeled as was were lysosomes, the rER and the cytosol.

2) Dividing Cells

In mitotic cells in late prophase, there were radioautographic silver grains over the lacy chromosomes, localized mostly to their periphery (Fig. 55). Some grains

appeared at the edge of nucleolar remnants. Only occasional grains appeared over the nuclear envelope, which had pulled away from the forming chromosomes.

In the cytoplasm, there was a diffuse reaction of moderate intensity with some grains overlying the cell surface.

D) Myeloma Cells - Short Incubation Times

Light microscope radioautographs of myeloma cells after a 4 hr incubation showed fairly heavy cytoplasmic reactions, with many grains over the cell surface. Several grains were also observed over many individual nuclei, however, especially in association with the nucleoli (Fig.56).

Light microscope radioautographs of myeloma cells after a 1 hr incubation were similar to the above but also exhibited heavy grain clusters probably overlying Golgi regions. Again grains were observed over nuclei, especially in association with the nucleoli (Fig. 57).

After an incubation for 10 min, the cells exhibited a lighter overall reaction but the distribution was similar to that seen at 1 hr, with a number of grains over still observed over nuclei. Again, many of these grains were associated with nucleoli (Fig. 58).

E) Myeloma Cells - Pulse-Chase Experiments

In myeloma cells incubated for 4 hr with ^{3}H-galactose and then incubated for a further 18 hr in medium containing only cold galactose, LM radioautographs showed a substantial decrease in cytoplasmic and cell surface reaction (Fig. 59),

as compared to cells incubated for 4 hr with ^{3}H-galactose and not chased (Fig. 58). Reaction over the nuclei, on the other hand, decreased only moderately.

F) YAC and Myeloma Cells - Tunicamycin Experiments

In YAC cells incubated for 4 hr with ^{3}H-galactose in the absence of tunicamycin, LM radioautographs showed an intense cytoplasmic reaction with much cell surface labeling (Fig. 60). A lighter but consistent reaction was observed over the nucleus, with some grain localization to the nucleoli. In cells incubated under the same conditions but in the presence of tunicamycin, the cytoplasmic reaction and cell surface labeling was dramatically decreased. The nuclear reaction, in contrast, was not significantly diminished by this tunicamycin treatment (Fig. 61).

Myeloma cells incubated for 4 hr with ^{3}H-galactose in the presence of tunicamycin, exhibited a decrease in cytoplasmic labeling compared to control cells, but the decrease was less marked than that observed in the above YAC cells. As in the case of YAC cells, the nuclear reaction was unaffected by tunicamycin treatment (figure not shown).

II) ^{3}H-Fucose Experiments

A) Myeloma cells - 18 hr Incubations

1) Interphase Cells

EM radioautographs showed moderate nuclear reactions over these cells, accompanied by substantial cytoplasmic reaction (Fig. 62). Quantitation of the distribution of silver grains

developed by either the chemical or physical fine grain development procedures showed that approximately 17.7% of the grains were localized over the nucleus when chemical development was used while 14.1% were observed when the latter procedure was used (Table 2). Within the nucleus, 2.0-2.4% of the grains were assigned to the nuclear envelope, 3.9-4.7% to the heterochromatin, 7.1-8.9% to the euchromatin, and >1.0-2.4% to the nucleolus. There was a wide variance in grains assigned to the heterochromatin/euchromatin interface depending on the method of grain development with 9.3% assigned to this interface with grains derived from chemical development while that figure dropped to only 1.7% with physical fine grain development.

2) Dividing Cells

In mitotic cells (Fig. 63), some silver grains remained associated with the chromosomes, mostly at their periphery. Other grains were observed at the periphery of nucleolar remnants.

In the cytoplasm substantial reaction overlay lysosomes and the cell surface.

B) YAC Lymphoma Cells - 18 hr Incubations

1) Interphase Cells

EM radioautographs showed moderate nuclear reactions over these cells, accompanied by substantial cytoplasmic reaction (Fig. 64). Quantitation of silver grains showed that approximately 16.6% of the grains were localized over the

nucleus. Within the nucleus, 3.4% of the grains were assigned to the nuclear envelope, 5.7% to the heterochromatin, 5.8% to the euchromatin, and 1.7% to the nucleolus. Surprisingly, 11.2% of the total cellular grains could be assigned to the heterochromatin/euchromatin interface (Table 3).

2) Dividing Cells

In dividing cells, the overall cellular reaction was light but occasional grains were consistently observed at the edges of condensed chromosomes (Figure not shown).

C) Friend Erythroleukemia Cells - 18 hr Incubations

1) Interphase Cells

EM radioautographs showed light nuclear reactions over these cells, accompanied by moderate cytoplasmic reaction (Fig. 65). Quantitation of silver grains showed that approximately 19.7% of the grains were localized over the nucleus. Within the nucleus, 3.0% of the grains were assigned to the nuclear envelope, 6.3% to the heterochromatin, 6.2% to the euchromatin, and 4.2% to the nucleolus. 9.0% of the cellular grains appeared to overly the heterochromatin/euchromatin interface (Table 4).

2) Dividing Cells

In mitotic cells, a few silver grains remained associated with the chromosomes and seemed to be located mostly at their periphery. The nucleolar remnants also exhibited some labeling. In cells in late prophase (Fig. 66), only a few grains were associated with the nuclear envelope.

In the cytoplasm, heavy reaction persisted over lysosomes, the Golgi apparatus and the cell surface.

D) Myeloma Cells - Pulse-Chase Experiments

When myeloma cells were incubated with ^{3}H-fucose for 4 hr, LM radioautographs showed a heavy reaction over the cytoplasm and cell surface. In addition, the nuclei exhibited substantial reaction, especially in association with the nucleoli (Fig. 67).

When myeloma cells were incubated with ^{3}H-fucose for 4 hr, followed by a 6 hr chase in medium containing cold fucose (Fig. 68), the cells exhibited essentially the same radioautographic reaction as that seen after a 4 hr incubation with no chase.

When the myeloma cells were incubated with ^{3}H-fucose for 4 hr, followed by a 18 hr chase in medium containing cold fucose (Fig. 69), the heavy reaction over the cytoplasm and cell surface was greatly reduced but the nuclei continued to exhibit an observable reaction. It could now be seen that, in addition to the internal reaction, the nuclei exhibited many grains near their periphery.

III) ^{3}H-GlcNAc Experiments

A) Myeloma Cells - 18 hr Incubations

1) Interphase Cells

EM radioautographs of myeloma cells showed a relatively light overall reaction compared to that seen after ^{3}H-galactose or ^{3}H-fucose, but the nuclei in these cells were

prominently labeled (Fig. 70). Quantitation of silver grains produced by chemical development showed that approximately 48.9% of the grains were localized over the nucleus. Within the nucleus, 4.2% of the grains were assigned to the nuclear envelope, 12.1% to the heterochromatin, 21.7% to the euchromatin, and 11% to the nucleolus (Table 2). 24.3% of the silver grains lay within one assignment distance of the heterochromatin/euchromatin interface.

2) Dividing Cells

In dividing myeloma cells, silver grains were dramatically localized to the chromosomes at all stages of mitosis (Figs. 71-73). Fig. 71 shows a cell in late prophase where the chromosomes were labeled over both their peripheral and internal regions. Some grains were associated with the nuclear envelope which had pulled away from the chromosomes. At anaphase (Fig. 72) the nuclear envelope was absent, but heavy reaction remained over the peripheral and internal regions of chromosomes. At telophase (Fig. 73), the nuclear envelope had reformed against many chromosomes, and some grains appeared to directly overly this structure.

B) Short Incubation Times (1 Hr, 10 min, 2 min)

Light microscope radioautographs of myeloma cells after a 1 hr incubation revealed a strong reaction over the nuclei (Fig. 74), while only a light reaction was observed over the cytoplasm. Many of the nuclear grains were associated with nucleoli.

After incubations for 10 minutes and even 2 minutes, the nuclei exhibited lighter but still definite reactions which again accounted for the majority of silver grains over the cells (Figs. 75-76).

IV) ^{3}H-Mannose Experiments

A) Myeloma Cells - 18 Hr Incubations

1) Interphase Cells

Radioautographs of myeloma cells developed with the physical development procedure showed a moderate reaction over the cytoplasm and a light reaction over the nucleus (Fig.77). Quantitation of the grains showed 13.9% to be over the nucleus. This compared with 15.4% of grains over the nucleus if chemical grain development was used. 3.1-3.2% were assigned to the nuclear envelope, 3.6-4.9% to the heterochromatin, 6.0-6.4 to the euchromatin, and 0.9-1.4 to the nucleolus (Table 2). Only 2.5% of the silver grains lay within one assignment distance of the heterochromatin/euchromatin interface in radioautographs developed with the physical fine grain procedure while 10.5% of the grains were assigned to the interface when chemical grain development was used.

2) Dividing Cells

Only a very light reaction remained over the chromosomes and nucleolar remnants of dividing cell. At late prophase (Fig. 78), substantial reaction remained over the nuclear envelope which had pulled away from the forming chromosomes. This reaction matched that over nearby cisternae of rER.

B) Short Incubation Times (1 hr, 10 min)

Light microscope radioautographs of myeloma cells after a 1 hr incubation showed a reaction of moderate intensity in which a significant number of grains were often observed over the nucleus (Fig. 79). At 10 minutes, the cells exhibited a much lighter overall reaction, grains only occasionally appeared over nuclei (Fig. 80).

V) ^{3}H-ManNAc Experiments

A) Myeloma Cells

1) Interphase Cells

Radioautographs of myeloma cells developed with chemical development showed a light reaction over both the cytoplasm and over the nucleus (Fig. 81). Quantitation of grains (Table 2) showed 16.3% over the nucleus. Of these, 1.9% were over the nuclear envelope, 4.7% were over the heterochromatin, 5.7% were over the euchromatin, and 4.1% were over the nucleolus. 10.3% of the silver grains lay within one assignment distance of the heterochromatin/euchromatin interface.

2) Dividing Cells

Radioautographs of myeloma cells in mitosis (Figs. 82-83) showed a very light overall reaction. Of the grains present, however, a significant number were associated with the chromosomes. No grains were seen over nucleolar remnants.

VI) ^{35}S-Sulphate Experiments

A) Myeloma Cells - 18 hr Incubations

1) Interphase Cells

EM radioautographs of interphase myeloma cells exposed to ^{35}S-sulphate showed a small cellular reaction but many nuclei appeared to be labeled. No quantitation of the distribution of the silver grains was carried out, but as with the ^{3}H-sugars, grains were observed over both the periphery and internal regions of the nucleus (Fig 83a).

2) Dividing Cells

EM radioautographs of mitotic cells exposed to ^{35}S-sulphate was also lightly labeled. However, some labeling at the periphery of chromosomes could be seen (Fig 83b).

VII) Observations on Apoptotic Cells

A small proportion of cells in the myeloma and Friend erythroleukemia cell culture populations exhibited changes which indicated that they were undergoing apoptotic cell death at the time of incubation with the different ^{3}H-sugars (see Discussion).

In myeloma cell cultures incubated with ^{3}H-fucose, radioautographs of cells in the early stages of apoptosis (Fig. 84) showed a light but discrete reaction over the nucleus. Within this compartment, grains were found over the patches of marginated heterochromatin, over the internal euchromatin, and over masses of nucleolar remnant material.

At later stage of apoptosis (Fig. 85), a discrete reaction was again seen over the nucleus, in which silver grains were observed over marginated heterochromatin and euchromatin. Several grains were associated with the nuclear envelope. In

some regions, the nuclear envelope dilated, and in these regions some grains could be assigned exclusively to the perinuclear space (which is equivalent to the rER lumen). In the cytoplasm, many silver grains occurred over expanded profiles of endoplasmic reticulum.

At the latest stage of apoptosis (Fig. 86), the nucleus had broken up and isolated circular masses of heterochromatin, which exhibited some labeling, were scattered in the cytoplasm. These were associated with fragments of expanded nuclear envelope. As at earlier times, these had several grains over their lumen and limiting membrane. In the remainder of the cytoplasm, there were many expanded profiles of rER which showed identical reaction.

In myeloma cells cultures incubated with ^{3}H-galactose, cells at an early stage of apoptosis exhibited substantial reaction over patches of marginated heterochromatin, as well as over nucleolar remnant material (Fig. 87). In the cytoplasm, intense reaction was observed over lysosomes and the cell surface.

Finally, in Friend erythroleukemia cell cultures incubated with ^{3}H-galactose, some cells appeared to be in an apoptotic state. Fig. 88 shows a nucleus which may have been in the process of being extruded from the cell. In these cells, a significant number of silver grains were located over the marginated heterochromatin, with somewhat fewer over the euchromatin.

IV. Discussion

The present radioautographic studies revealed that after the administration of ^{3}H-sugars the interphase cells of three lines of cultured cells exhibited nucleoplasmic reaction which was at least as intense and often more intense than that observed in many cell types of intact tissues. The method of analysis of the radioautographic distribution of label in these cells is discussed below, followed by a discussion of the distribution of label in cells exposed to different ^{3}H-sugars.

A. Analysis of Electron Microscope Radioautographs

As mentioned in the introduction to this chapter, due to limitations in the spacial resolution of the radioautographic method, a silver grain may not necessarily overly the radioactive source. It can only be stated with some degree of certainty that the true source of label will lie within a known assignment distance from the centre of any observed grain. In the present study, using tritium as the radiolabel, the assignment distance (for a probability of 95%) has been calculated to be 230 nm using chemical grain development (Nadler, 1971). Using solution physical development, the assignment distance is 115 nm, since this procedure has twice the resolution (Kopriwa et al, 1984). These assignment distances were therefore used in our analysis of radioautographic labeling of cultured cells. Using this method, when more than one compartment lay within this

assignment distance of a particular silver grain, each compartment was assigned a partial grain count.

The use of assignment distances and the sharing of grains made it difficult to draw firm conclusions as to the true source of label for grains in some instances. In grains located at the nuclear periphery, a number of potentially labeled compartments, including peripheral euchromatin, peripheral heterochromatin, nuclear envelope, adjacent cytosol, often lay within one assigment distance of the grain. Consequently, all such grains were shared by various compartments, and we frequently assigned one-half or even one-quarter of a grain in relevant cases to appropriate compartments as a way of correcting for the limitations in the spatial resolution.

A similar problem arose in determining the relative labeling of the various intranuclear subcompartments. The heterochromatin usually appeared as small dense patches in the more internal parts of the nucleoplasm or as thin rims of area lining the nuclear envelope or around the nucleolus. Under these circumstances, there was some difficulty in determining with a high degree of certainty which compartment (nucleolus, heterochromatin, euchromatin, or nuclear envelope) contained the actual label. It is possible that with this approach some of the smaller but extended compartments such as the heterochromatin and nuclear envelope might have ended up undercredited in terms of relative labeling because grains

over these areas were almost always shared. On the other hand, the euchromatin may have been overcredited because, in addition to many unshared grains, it was the recipient of many partial grains because of its close proximity to all the other compartments.

In cases where grain counts were carried out on radioautographs of the same cell developed with the chemical and the solution physical procedures, there were some differences in the assignment of grains to different compartments, perhaps based on the smaller assignment distances used in the latter procedure. Thus, in myeloma cells exposed to ^{3}H-galactose for 18 hr, 22.3% of the grains were attributed to the nucleus with chemical development but only 19.5% with solution physical development. This is perhaps because all cytoplasmic grains whose centers were within 230 nm of the nuclear envelope were shared between cytoplasm and nucleus in the chemical development procedure, while only those within 115 nm were shared in the solution physical development procedure. Similarly, using chemical development, 9.3% of the grains were within one assignment distance (230 nm) of the heterochromatin/euchromatin interface in myeloma cells exposed to ^{3}H-fucose. This included many grains over the nuclear periphery. In contrast, using physical development, only 1.7% of the grains lay within one assignment distance (115 nm) of the heterochromatin:euchromatin interface where it was noted that very few of the peripheral grains were now

assigned to the interface. As a general principle, in dealing with compartments which are essentially two dimensional and have almost no volume, e.g. membranes, H/E interface, decreasing the assignment distance will result in fewer grains being attributed to that compartment. Using the direct scoring method (with a zero assignment distance), only a very small proportion of the grains would be assigned to such compartments.

B. Distribution of Silver Grains in Radioautographs

I) 18 Hr Incubations

A) Interphase cells

1) Galactose

Myeloma cells exposed to ^{3}H-galactose exhibited heavy radioautographic reactions, in which the nuclei accounted for a substantial portion (~20%) of the total cell label (Table 1). Most of this nuclear labeling was nucleoplasmic with less than 10% of the nuclear labeling accounted for by the nuclear envelope. The euchromatin was the most labeled nucleoplasmic compartment but the heterochromatin and nucleolar compartments were significantly labeled as well. The heterochromatin/ euchromatin interface compartment was moderately labeled. This suggests that galactose incorporation might be involved in some aspect of the transcription process, since this interface has been shown to be a site of active transcription (Fakan and Nobis, 1978).

In the rest of the cell, reaction was localized, as

expected from previous studies, over the Golgi apparatus, plasma membrane and lysosomes. Labeling of the rER was less expected, but has been reported in occasional cell types (Nelson et al., 1978). If galactose is indeed added to transmembrane glycoprotein molecules in the rER in our cells, such incorporation into transmembrane molecules of the nuclear envelope (which in fact is a specialized part of the rER) could account in part for the reaction observed over the nuclear periphery.

The fairly heavy reaction scattered over the rest of the cytoplasm is also interesting. In most previous radioautographic studies, grains seen over this compartment tended to be attributed to whatever organelle of the central vacuolar system that lay closest. However, in light of recent evidence for cytosolic glycoproteins (reviewed in Hart et al., 1989) it is possible that the label actually does reside in the cytosol itself.

YAC lymphoma cells and Friend Erythroleukemia cells exhibited radioautographic reactions somewhat similar to those in myeloma cells although in comparing the distribution of label using values derived with fine grain development, much more nuclear label was assigned to the H/E interface of the YAC and Friend than to the myeloma cell. This again may represent the participation of the labeled molecules (maybe glycoproteins such as the HMGs proteins in transcriptional events). Also interesting was the finding that the

nucleoplasmic label over the YAC lymphoma cells was found to be the highest (~28% of total) of the three cell types after exposure to ^{3}H-galactose. Myeloma cells were chosen as the model cell type for biochemical studies, however, because the cells yielded cleaner nuclei as assessed morphologically when fractionated.

2) Fucose

Myeloma cells exposed to ^{3}H-fucose exhibited radioautographic reactions which were somewhat lighter than those observed after ^{3}H-galactose, and their nuclei accounted for slightly less (~17%) of the total cell label (Table 1). The grains were distributed over both the periphery and the interior of the nucleus with a slightly higher percentage attributed to the nuclear envelope than with galactose. Many grains were associated with the heterochromatin/euchromatin interface with chemical development, suggesting, as with galactose, that fucose incorporation might be involved in some aspect of transcription process. The much lower value of grain percentage given the H/E interface with fine grain development is slightly misleading in that it was our observation that the difference between the two values (ie. that from chemical development vs. that from physical development) seemed to be largely due to a dramatic decrease in the peripheral label assigned to the peripheral H/E interface just inside the nuclear envelope. The internal H/E sites seemed to be much more labeled than the overall average of 1.7%. This may

reflect a heterogeny in ^{3}H-fucose-labeled molecules in the periphery as opposed to the more internal parts of the nucleus where much of the peripheral H/E interface label did not really derive from molecules at the H/E interface but somewhere close by while that observed more internally did derive from molecules at the internal H/E interface.

The nucleolus showed some labeling but not as nearly as much as with labeled galactose.

In the rest of the cell, the radioautographic reaction after ^{3}H-fucose was similar to that seen after ^{3}H-galactose, although less intense. Once again labeling of the rER was observed, and it may be noted that substantial addition of fucose to glycoproteins in the rER has also been reported in some other cell types (Pelletier et al., 1973, Magnar et al, 1992). As mentioned with ^{3}H-galactose, this leaves open the possibility that incorporation of ^{3}H-fucose into trans-membrane proteins of the nuclear envelope could account in part for reaction over the nuclear periphery.

3) N-Acetylglucosamine

Cells incubated ^{3}H-glcNAc exhibited considerably less overall radioautographic reaction than after ^{3}H-galactose or even ^{3}H-fucose, but their nuclei accounted for the highest percentage (almost 50%) of the total cell label. These grains were located over the periphery as well as interior regions including the nucelolus. A paricularly high proportion of grains was found over the heterochromatin/euchromatin

interface, perhaps representing incorporation of ^{3}H-glcNAc label into the novel O-linked glcNac-containing transcription factors described by Tijan and Jackson (1988). At the nuclear periphery, part of the labeling might be accounted for by the presence of newly synthesized O-linked glcNAc-containing nuclear pore complex glycoproteins (Hart et al., 1989).

In the cytoplasm, the Golgi apparatus and the plasma membrane were relatively unlabeled. Some reaction appeared over rER as expected, since this sugar is traditionally believed to be incorporated in this compartment. Other cytoplasmic reaction was found over mitochondria and the remainder of the cytoplasm. As mentioned in Chapter 1, there has been other radioautographic and biochemical evidence suggesting incorporation of sugars into mitochondrial glycoproteins. In addition, substantial labeling of mitochondria was observed in hepatocytes exposed to ^{3}H-glcNAc (Figs. 30-31 in Chapter 2). The reaction over the remainder of the cytoplasm could represent cytosolic O-linked glcNAc-containing glycoproteins (Hart et al., 1989).

4) Mannose

Cells incubated with ^{3}H-man exhibited less overall radioautographic reaction than with ^{3}H-fucose, but substantially more than with ^{3}H-glcNAc. With this precursor, much of this nuclear reaction was peripheral, perhaps representing incorporation of mannose resiaues into transmembrane glycoproteins in the nuclear envelope, since

this compartment is an extension of the rER. Silver grains were still observed over the euchromatin and the nucleolus but in greatly decreased numbers. The same phenomenum of a huge difference in grain assignment to the H/E interface relative to the type of grain development was noted with ^{3}H-mannose as with ^{3}H-fucose. Again, the loss of assigned label was higher at the peripherally-located H/E interface than internally which may reflect a heterogeny in the labeled molecules between internal regions of the nucleus and the periphery.

In the cytoplasm, a fairly heavy reaction was observed, as expected, over the rER and the Golgi apparatus.

5) N-Acetylmannosamine

Cells incubated with ^{3}H-manNAc exhibited the least amount of overall cellular reaction among all the precursors used, and considerably longer exposure times were required to obtain substantial reactions. This low level of incorporation was previously observed in intact tissues (Bennett et al., 1981). Interestingly the nuclear/cytoplasmic ratio of labeling was almost as high as that observed for ^{3}H-fucose, with the nucleus accounting for almost 17% of the total cell label. The nuclear reaction with ^{3}H-manNAc was generally more internally located within the nucleus than with ^{3}H-fuc or ^{3}H-man. Within the nucleoplasm, there was substantial labeling of the nucleolus and of the heterochromatin/euchromatin interface which again might be related to some regulatory function. The H/E interface labeling (10.3%) has to be considered somewhat

guardedly, however, because it was only determined from radioautographs with chemical development of silver grains. It may be recalled that a dramatic drop was seen in the percentage of label at the H/E interface with labeled mannose and fucose using the physical fine grain method vs the chemical method.

In the cytoplasm a high proportion of the observed grains lay over the Golgi apparatus (a traditional site of incorporation of this precursor), while many other grains appeared over the plasma membrane and lysosomes.

6) Sulphate

Our EM radioautographs indicated that much of the label appeared to be associated with nuclei. The distribution of nuclear label as assessed qualitatively was similar to that observed for the tritiated sugars. However, it is noted that the assignment distance for grains produced in detecting radiolabeled sulphate is much greater than for tritium so any conclusions reached are much more tentative.

B) Dividing Cells

In dividing cells, it has become well established that the compartments of the nucleus undergo several changes. In early prophase, the euchromatin decreases in quantity while the heterochromatin condenses and takes the form of well defined chromosomes. In early metaphase, the nuclear envelope fragments into small vesicles which disperse throughout the cytoplasm where they have the appearance of endoplasmic

reticulum. Similarly the nuclear pore components and nuclear lamina become dispersed in the cytoplasm. The nucleolus disassembles, but remnants of the granular region persists into the metaphase stage. In anaphase the chromosomes (which have replicated) separate and move to opposite poles of the cell. In telophase, the nuclear envelope initially reforms around individual chromosomes. The chromosomes become partially decondensed, resulting in the reappearance of euchromatin. RNA synthesis resumes and the nucleolus reforms.

In the present study, the chromosomes were labeled at all stages of the mitotis. This was especially true in cells incubated with ^{3}H-glcNAc and ^{3}H-gal, while in cells incubated with ^{3}H-man only a very light reaction was observed over the chromosomes. This correlates with the situation in interphase cells in which cells incubated with ^{3}H-glcNAc and ^{3}H-gal had high percentages of nucleoplasmic labeling while cells incuabated with ^{3}H-man exhibited the lightest nucleoplasmic reaction. Labeling of chromosomes had also been observed in mitotic duodenal crypt cells <u>in vivo</u> after exposure to ^{3}H-galactose (Figs. 24-26).

In the case of myeloma cells incubated with ^{3}H-glcNAc, much of the chromosomal labeling is possibly due to the presence of the O-linked glcNac-containing transcription factors (Jackson and Tijan, 1988) and other O-linked glcNac-containing proteins reported in chromosomes (Kelly and Hart, 1989).

In the mitotic cell there is a physiological separation of the nuclear envelope from the heterochromatin of the chromosomes. In the cells of the present study, the forming chromosomes tended to pull away from the nuclear envelope during late prophase. This provided an opportunity to visualize the radioautographic reaction over the heterochromatin and the nuclear envelope as entities separated by more than one assignment distance, something that had not been possible to do in interphase cells due to their close proximity. In cells incubated with ^{3}H-man, the forming chromosomes were only very lightly labeled in late prophase, while the nuclear envelope remained substantially labeled (Fig. 78). This suggests that the peripheral reaction observed over nuclei in interphase cells incubated with ^{3}H-man (Fig. 77) may have been mainly due to label within the nuclear membrane. In cells incubated with ^{3}H-glcNAc (Fig. 71), on the other hand, substantial reaction was observed over both the forming chromosomes and the nuclear envelope in late prophase, suggesting that both compartments had probably contributed to the peripheral nuclear reaction observed in interphase cells (Fig. 70).

C) Apoptotic cells

In cells undergoing apototic cell death, both the nucleus and cytoplasm undergo a series of distinctive changes leading to the fragmentation of the cell and eventual phagocytosis by other cells (Wyllie et al., 1980). A number of cells observed

in our cultures exhibited changes which indicated that they were undergoing this type of cell death. The interest in these cells, in terms of the present thesis, was due to the fact that certain compartments of the nucleus (which are normally always closely adjacent to one another) became segregated and/or enlarged such that silver grains could be assigned exclusively to these compartments. Although the cells are in a pathological state, information obtained from them may give some insight into the localization of label in healthy cells.

In early stages of apoptosis, the heterochromatin became marginated into large compact masses adjacent to the nuclear envelope, and in these regions the envelope was often extended into protuberances. In this situation it was often possible to assign silver grains exclusively to the heterochromatin compartment. This compartment was thus seen to be moderately but distinctly labeled in myeloma cells incubated with ^{3}H-fucose (Figs. 84-86), and strongly labeled in myeloma cells incubated with ^{3}H-galactose (Fig. 87). The presence of many silver grains assigned exclusively to heterochromatin in apoptotic cells provides evidence that this compartment definitely contains labeled molecules. Thus in interpreting the reaction over the periphery of normal cells, it must be conceded that the peripheral heterochromatin is a viable candidate as a possible source of the labeled molecules.

In the middle stages of apoptosis, the nuclear envelope often became dilated in certain regions and (in cells

incubated with ^{3}H-fucose) exhibited reaction over its outer nuclear membrane and over the expanded nuclear space (Fig. 85). This reaction was very similar in appearance to that over dilated profiles of rER in the cytoplasm. In this situation it was possible to assign some silver grains exclusively to the perinuclear space. In the same fashion, grains over the outer nuclear membrane could be considered as due to label in the membrane or adjacent lumen or cytosol, but not to peripheral chromatin. At later stages of apoptosis, the nuclear heterochromatin fragmented into several round masses, and these were only partially enveloped by expanded profiles of nuclear envelope (Fig. 86). Again both the profiles of nuclear envelope and nearby profiles of rER exhibited substantial reaction over their lumen and limiting membrane. This situation is similar to that seen in certain frog dorsal root ganglion Schwann cells that appeared to be undergoing apoptosis (Fig. 3). After incubation with ^{3}H-fucose, these cells similarly exhibited substantial reaction over dilated regions of the nuclear envelope (including the perinuclear space) and also over dilated profiles of rER. As mentioned above, fucose is not commonly incorporated into glycoproteins in the rER or nuclear envelope, but substantial addition of fucose to glycoproteins in the rER has been reported in some cell types (Pelletier et al., 1973, Magnar et al, 1992). Using the same reasoning as applied to heterochromatin above, the exclusive assignment of silver grains to the perinuclear space

(and perhaps the outer nuclear membrane) in these pathological cells provides evidence that these compartments are viable candidates as possible sources of the labeled molecules when interpreting radioautographic reaction over the nuclear periphery in normal cells.

II) Short Incubations

When myeloma cells were exposed to ^{3}H-glcNAc, ^{3}H-galactose or ^{3}H-mannose for much shorter time periods, e.g. 1 hr, the cells still showed significant radioautographic reaction over their nuclei, suggesting that labeled glycoprotein molecules reach the nucleus rapidly after being glycosylated. The overall intensity of radioautographic reaction was less than that observed in 18 hr incubations suggesting that some of the glycoproteins do not have extremely short turnover times and thus accumulated as labeled molecules in the nucleus with time. Still, in the case of cells exposed to ^{3}H-glcNAc and ^{3}H-galactose, significant nuclear labeling was observed after a 10 min incubation, and in the case of ^{3}H-glcNAc, even after a two min incubation. These results are not entirely surprising in that significant nuclear reactions had been observed in cells of intact tissues after short exposure times, e.g. bile ductule cells and gall bladder epithelial cells after 5 min exposure to ^{3}H-gal (Figs. 27,28), duodenal columnar cells, hepatocytes, bile ductule cells, and pancreatic duct and Islet cells, after 5 min exposure to ^{3}H-glucosamine (Figs. 29-33), and colonic columnar cells, hepatocytes and kidney tubules

cells after ^{3}H-manNAc (Figs. 39,41, and 42). In the literature, some nuclear pore glycoproteins containing O-linked glcNAc residues also have been reported to be glycosylated within 5 minutes (Davis and Blobel, 1987).

C. Biosynthesis of Nucleoplasmic Glycoproteins

If it is postulated that the nucleoplasmic label in our studies does represent glycoproteins (this will be further discussed in Chapter 4), then the above experiments provide some evidence concerning the biosynthesis of these molecules. It has been established that synthesis of the polypeptide chains of cytosolic and nucleoplasmic proteins may only occur on ribosomes in the cytosol. The fact that nuclei in the present study are labeled as little as 2 min after exposure to labeled sugars (e.g. ^{3}H-glcNAc), indicates either that the glycoprotein molecules must migrate to the nucleoplasm very rapidly after having incorporated these sugars in the cytoplasm or that the labeled sugars are in fact added in the nucleoplasm itself. There are some reports in the literature of nuclear glycosyltransferase activities (Martin and Louisot, 1975; Bertillier and Got, 1980; Fayet et al, 1988). These activities have not been rigorously localized, however, and to date there is no conclusive evidence for nucleoplasmic glycosyltransferases.

In the cytoplasm, all of the traditionally described glycosyltransferase enzymes are located on the luminal surface of rER and Golgi apparatus membranes. Newly synthesized

glycoproteins leaving the Golgi apparatus usually take at least 5-10 min to reach nearby destination such as the lateral plasma membranes (Bennett and Leblond, 1976). The very rapid appearance of labeled glycoproteins in the nucleoplasm after addition of labeled sugars is one factor that suggests that these sugars are not being added by the above traditional enzymes. A second factor, as mentioned in the general introduction of chapter 1, is that there are difficult conceptual problems in visualizing a process whereby a glycoprotein glycosylated in this site would be able to cross the limiting membrane to gain access to the cytosol and nucleoplasm.

If the cytoplasmic glycosylation of proteins destined for the nucleus does not occur on the luminal side of the rER or Golgi apparatus, then it must occur in the cytosol. The idea of glycosyltransferases existing in the cytosol which can add sugars to proteins has recently been gaining acceptance. This concept in itself is not entirely new, since at least one glycosyltransferase enzyme, i.e. glycogen synthase has been known for years to exist in the cytosol. However, it has only recently been established that glycogen itself is in fact a glycoprotein, and other enzymes involved in its initial synthesis are also located in the cytosol (discussed in Chapter 1).

The work of Davis and Blobel (1986,1987) provided some of the first evidence that 0-linked glcNAc-containing nuclear

pore glycoproteins might be glycosylated in the cytosol. Their p62 glycoprotein was found to attain most of its glcNAc residues within 5 minutes of the initial synthesis of its polypeptide chain. This led the authors to conclude that the glcNAc residues were added on to the protein in the cytosol, perhaps even co-translationally. These particular glycoproteins were shown to take a long time to migrate to the nuclear pore complexes ($t_{1/2}$ of ~6 hr), confirming that the initial glycosylation had occurred in the cytosol. A further addition of a small amount of glcNAc residues occurred following this migration of the glycoprotein to the pore complexes. It could thus be implied that addition of these latest glcNAc residues would have to be mediated by glycosyltransferases on both the cytosolic and nucleoplasmic sides of the nuclear pore complex. In our radioautographic studies, the peripheral labeling of nuclei in myeloma cells after 2 min exposure to ^{3}H-glcNAc could perhaps reflect this latter addition of ^{3}H-glcNAc residues to p62 or similar nuclear pore glycoproteins.

In the past two years, more definitive evidence has been obtained for the presence of cytosolic glycosyltransferase molecules mediating the O-linked addition of glcNAc residues to proteins. A rat liver cytosolic uridine diphospho-N-acetylglucosamine:peptidebeta-N-acetylglucosminyltransferase has been described by Haltiwanger et al. (1992), and translated recombinant p62 nuclear pore protein has been

efficiently glycosylated by reticulocyte lysate cell-free translation systems (Starr and Hanover, 1990).

Apart from the glycogen story, there has not been a great deal of evidence for cytosolic glycosyltransferases adding sugars other than O-linked glcNAc. Nonetheless, Srisomsap et al. (1988) have recently provided evidence of a glucose-1-phosphotransferase with its active site on the cytosolic side of rat liver microsomes that catalyzes the addition of UDP-glucose onto an O-linked mannose-containing acceptor. Gonzales-Yanes et al. (1992) have reported the presence of a cytosolic fucosyltransferase which adds fucose to a cytosolic fucoprotein, possibly via an O-linkage. Lastly, Shaper et al. (1988) have provided evidence that murine ß-1,4-galactosyl-transferase mRNA transcripts are produced in two lengths. The sequences of these transcripts suggested to the authors that there may be two functionally different forms of ß-galactosyl-transferase, one with its active site on the lumenal side of Golgi saccules and the other with its active site on a cytosolic side. Such a cytosolic site has not as yet been directly demonstrated however.

The tunicamycin experiments in the present study provide some information regarding the nature of nucleoplasmic proteins in myeloma and YAC cells exposed to ^{3}H-galactose. Light microscope radioautographs showed that while the cytoplasmic labeling was decreased with tunicamycin treatment (especially in YAC cells), the nuclear reaction remained

unaffected. Since tunicamycin is known to inhibit the synthesis of N-linked side chains of glycoproteins (Elbein, 1987), the present results suggest that the label observed in the nuclei of these cells has not been incorporated into traditional N-linked side chains. It is possible that the label is in O-linked side chains or in some novel type of glycoprotein whose synthesis is not inhibited by tunicamycin treatment.

D. Turnover of Nucleoplasmic Glycoproteins

In 18 hr incubation studies of the present work, it is assumed that free ^{3}H-sugars remained abundant in the medium throughout the incubation. Under these conditions, no information could be obtained about turnover times of nucleoplasmic glycoproteins since synthesis of new labeled proteins could be expected to continue until the end of the incubation.

On the other hand, when myeloma cells were pulsed with ^{3}H-fucose for 4 hours, and chased for 18 hrs in unlabeled medium containing cold fucose, one could expect that synthesis of new labeled proteins would effectively cease soon after the end of the 4 hour pulse. While the intensity of cytoplasmic reaction was found to substantially decrease at the end of the 18 hr chase (as compared to that at the beginning of the chase), the intensity of reaction over the nucleus remained almost at the level at the beginning of the chase. These results suggest that at least some of the nucleoplasmic

labeled with ^{3}H-fucose proteins have a relatively long turnover rate. Since both peripheral and internal nucleoplasmic regions remained labeled, it would appear that proteins in both sites shared this feature.

In pulse-chase studies with ^{3}H-galactose, the nuclei exhibited somewhat less reaction at the end of the chase than at the beginning. Nonetheless, the results also imply a fairly long turnover time for at least some nucleoplasmic proteins labeled with ^{3}H-galactose.

E. Summary of Chapter 3

To summarize the findings of this chapter, we have provided evidence of prominent nucleoplasmic labeling in three lines of cultured cells after administration of tritiated sugars or radiolabeled sulphate. Chromosomes of mitotic cells also were substantially labeled except in the case of ^{3}H-mannose. Quantitative analysis of the distribution of label within the nucleoplasm of myeloma cells indicated that the euchromatin was routinely the most heavily labeled compartment. The heterochromatin, nucleoli, and nuclear envelope also exhibited varying degrees of labeling. In cells undergoing apoptotic cell death, some normally attenuated nuclear compartments (e.g. heterochromatin) expanded, and it could be seen that many grains were exclusively localized to them. In cells incubated for short time intervals, substantial labeling was observed over nuclei as little as 2 minutes after exposure after ^{3}H-glcNAc or 10 min after exposure to ^{3}H-

galactose indicates either that the glycoprotein molecules must migrate to the nucleoplasm very rapidly after having incorporated these sugars in the cytoplasm or that the labeled sugars are in fact added in the nucleoplasm itself. Pulse-chase studies carried out on myeloma cells with either ^{3}H-galactose or ^{3}H-fucose indicated that at least some nucleoplasmic glycoproteins turnover slowly. Tunicamycin experiments provided evidence that the nuclear label observed in YAC lymphoma and myeloma cells exposed to ^{3}H-galactose had not been incorporated into N-linked glycoprotein side chains.

One question posed at the beginning of this study was whether not these cells would exhibit substantial nuclear labeling when exposed to ^{3}H-sugars, such that they could be used for further biochemical studies. This was indeed found to be the case, and in Chapter 4, the results of studies on isolated nuclear fractions from cells exposed to tritiated sugars were carried out to provide confirmatory information concerning the source of the nuclear label as well as the nature of the labeled molecules.

Table 2 - Percentage (%) Distribution of Silver Grains over Cultured Myeloma Cell Compartments after 18 hr. Exposure to ^{3}H-Sugars

Isotope	Devel	Exposure	Cyto	Nucleus	Nuclear Subcompartments	No. of Grains Counted
^{3}H-Gal	Chem	32 d	77.2	22.3	NE= 2.1 H= 4.7 E=11.6 n= 4.0 *H/E= 9.1	3958
^{3}H-Gal	Phys	60 d	80.5	19.5	NE= 1.3 H= 5.1 E= 9.1 n= 4.0 *H/E= 6.1	4016
^{3}H-Fuc	Chem	32 d	79.8	17.7	NE= 2.4 H= 3.9 E= 8.9 n= 2.4 *H/E= 9.3	2012
^{3}H-Fuc	Phys	11 mo	86.1	14.1	NE= 2.0 H= 4.7 E= 7.1 n= >1.0 *H/E= 1.7	1611
^{3}H-GlcNAc	Chem	32 d	51.1	48.9	NE= 4.2 H=12.1 E= 21.7 n= 11.0 *H/E= 24.3	695
^{3}H-Man	Chem	32 d	84.6	15.4	NE= 3.2 H= 4.9 E= 6.0 n= 1.4 *H/E= 10.5	1560
^{3}H-Man	Phys	11 mo	85.9	13.9	NE= 3.1 H= 3.6 E= 6.4 n= >1.0 *H/E= 2.5	1054
^{3}H-ManNAc	Chem	11 mo	83.7	16.3	NE= 1.9 H= 4.7 E= 5.7 n= 4.0 *H/E= 10.3	570

NE= Nuclear Envelope
H= Heterochromatin
E= Euchromatin
n= Nucleolus
*H/E= Hetero/Euchromatin Interface

Table 3 - Percentage (%) Distribution of Silver Grains over Cultured YAC Lymphoma Cell Compartments after 18 hr. Exposure to ^{3}H-Sugars

Isotope	Devel	Exposure	Cyto	Nucleus	Nuclear Subcompartments	No. of Grains Counted
^{3}H-Gal	Phys	89 d	71.7	28.3	NE= 2.4 H= 8.3 E= 11.3 n= 6.2 *H/E= 9.5	3301
^{3}H-Fuc	Chem	89 d	83.6	16.6	NE= 3.4 H= 5.7 E= 5.8 n= 1.7 *H/E= 11.2	1601

Table 4 - Percentage (%) Distribution of Silver Grains over Cultured Friend Erytholeukemia Cell Compartments after 18 hr Exposure to ^{3}H-Sugars

Isotope	Devel	Exposure	Cyto	Nucleus	Nuclear Subcompartments	No. of Grains Counted
^{3}H-Gal	Phys	36 d	78.3	21.3	NE= 2.1 H= 5.9 E= 10.2 n= 3.1 *H/E= 8.9	1481
^{3}H-Fuc	Chem	36 d	80.3	19.7	NE= 3.0 H= 6.3 E= 6.2 n= 4.2 *H/E= 9.0	1220

NE= Nuclear Envelope
H= Heterochromatin
E= Euchromatin
n= Nucleolus
*H/E= Hetero/Euchromatin Interface

Fig. 47 EM radiautograph of a myeloma cell in interphase, after incubation with ^{3}H-galactose for 18 hr.

A heavy reaction occurs over the nucleus (N), with grains over both peripheral and internal regions. The nucleolus (n) is heavily labeled. In the cytoplasm, reaction is especially prominent over the Golgi apparatus (G) and the plasma membrane (pm). Some grains appear over rough endoplasmic reticulum (rER).

Exposure: 1 mo (Chem. dev.) x 12,000

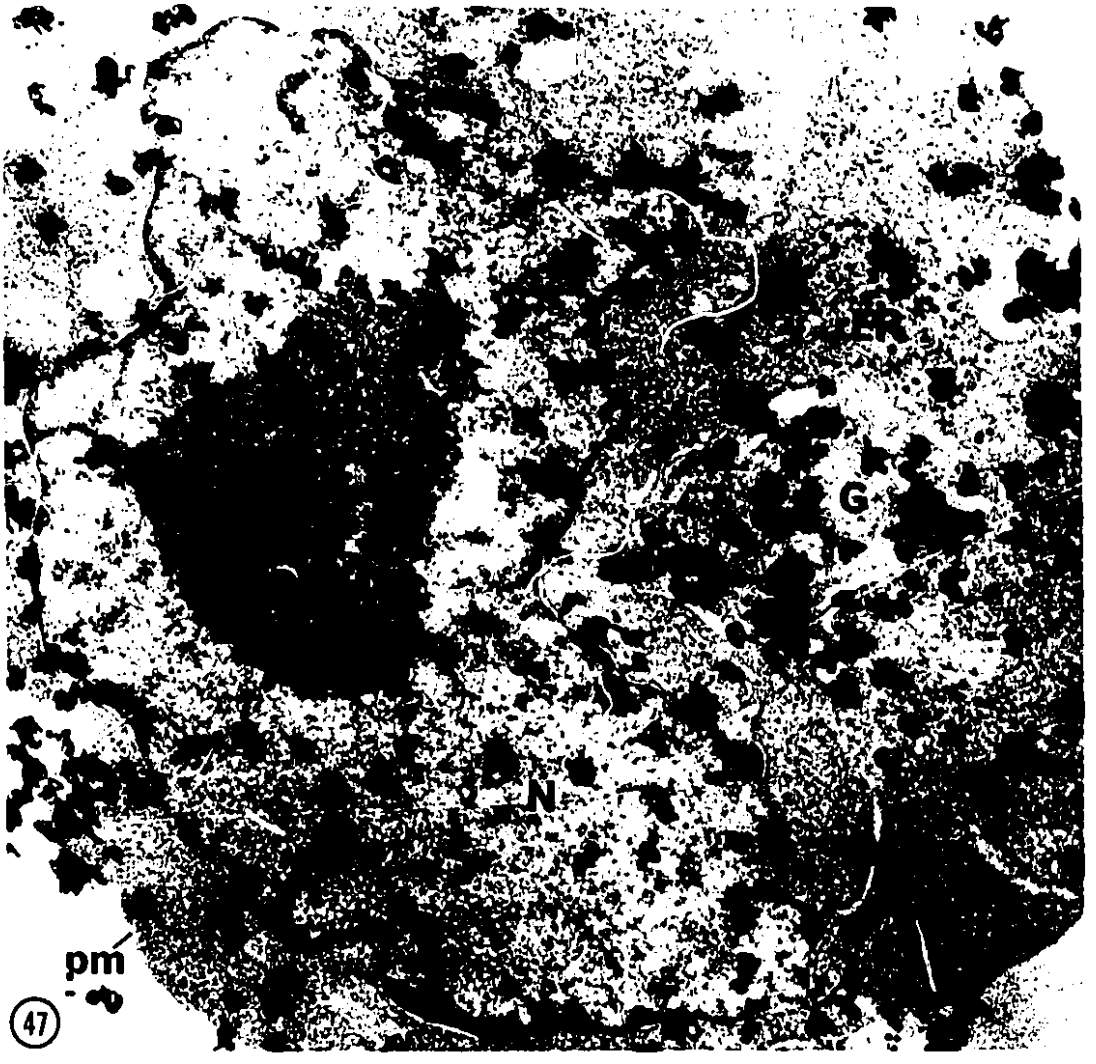

G
N
pm
47

Fig. 48 EM radioautograph of a myeloma cell in interphase, after incubation with ^{3}H-galactose for 18 hr.

The nucleus (N) exhibits a prominent reaction in which grains are especially localized over the nucleolus (n) and its associated heterochromatin. Many grains are localized over the nuclear periphery, and some are situated over the euchromatin (E). In the cytoplasm, intense reaction is associated with the Golgi apparatus (G),lysosomes (L), and the plasma membrane (pm). Some grains are over rough endoplasmic reticulum (rER).

Exposure: 11 mo (Phys. dev.) x 12,000

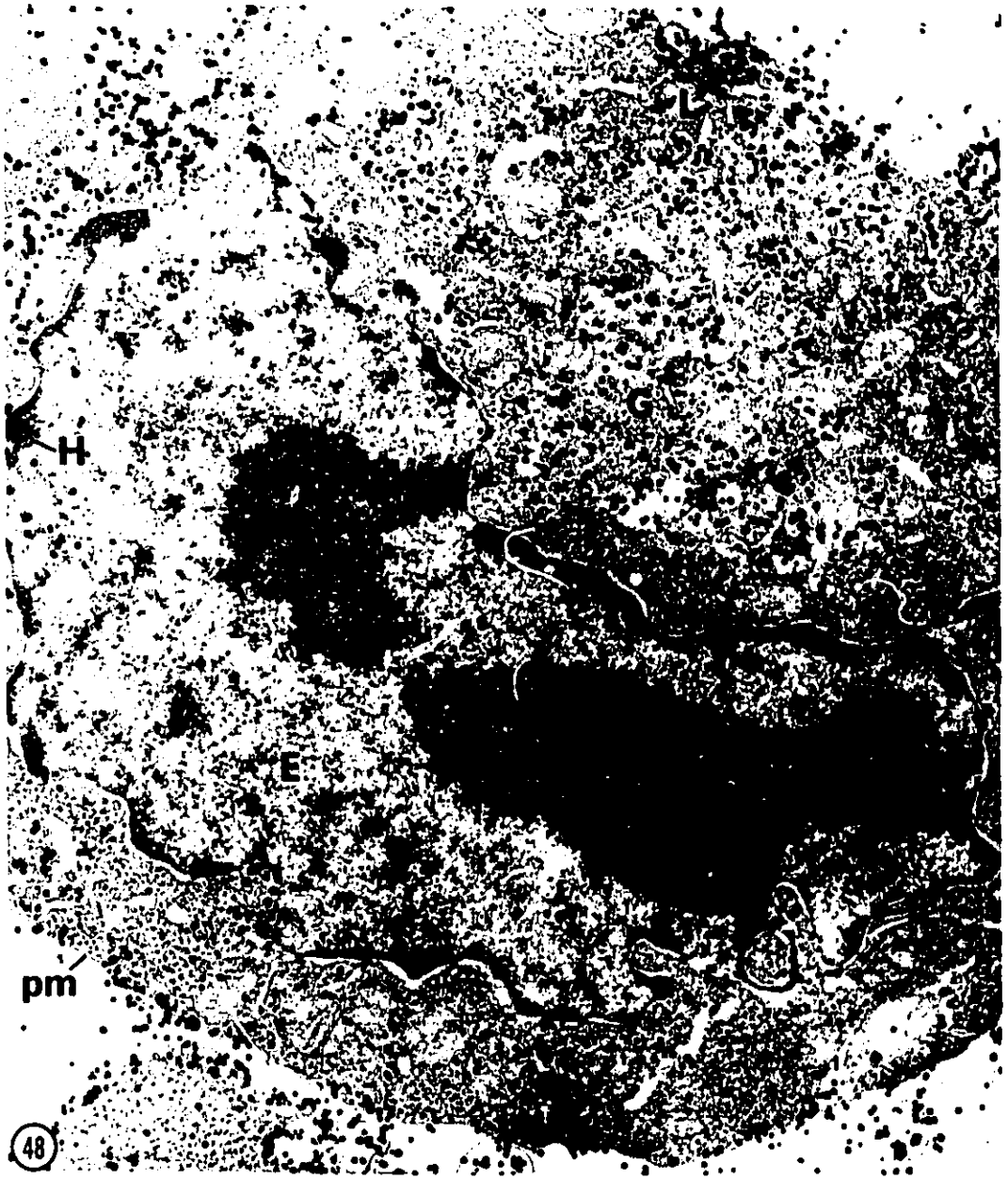

H
E
pm
48

Fig. 49 EM radioautograph of a myeloma cell in metaphase, after incubation with ^{3}H-galactose for 18 hr.

Many grains are localized over the condensed chromosomes (cc), and some are at the periphery of a nucleolar remnant (n). The cytoplasm exhibits a diffuse reaction, with many grains over the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000


pm
n
49

Fig. 50 EM radioautograph of a myeloma cell in anaphase, after incubation with ^{3}H-galactose for 18 hr.

Isolated chromosomes (cc) are still present, but they have become associated with patches of nuclear envelope (NE) in some regions. In other regions the chromosomes are still devoid of envelope (arrowhead). In the cytoplasm, heavy reaction is seen over over lysosomes (L) and the plasma membrane (pm). Lighter reaction covers the remainder of the cytoplasm. There is a heavy reaction over the condensed chromosomes. Some grains are associated with the nuclear envelope.

Exposure: 11 mo (Phys. dev.) x 12,000


pm
NE
L
50

Fig. 51 EM radioautograph of a myeloma cell in telophase, incubated with ^{3}H-galactose for 18 hr.

In this cell, fairly heavy reaction occurs over lysosomes (L), the and the plasma membrane (pm), and lighter reaction is observed over the remainder of the cytoplasm. Over the nucleus, a substantial number of silver grains are associated with the heterochromatin (H). Other grains are found over the nuclear envelope (NE), euchromatin (E) and a forming nucleolus (n).

Exposure: 11 mo (Phys. dev.) x 12,000


pm
E
NE
51

Fig. 52 EM radioautograph of an interphase YAC cell after incubation with ^{3}H-galactose for 18 hr.

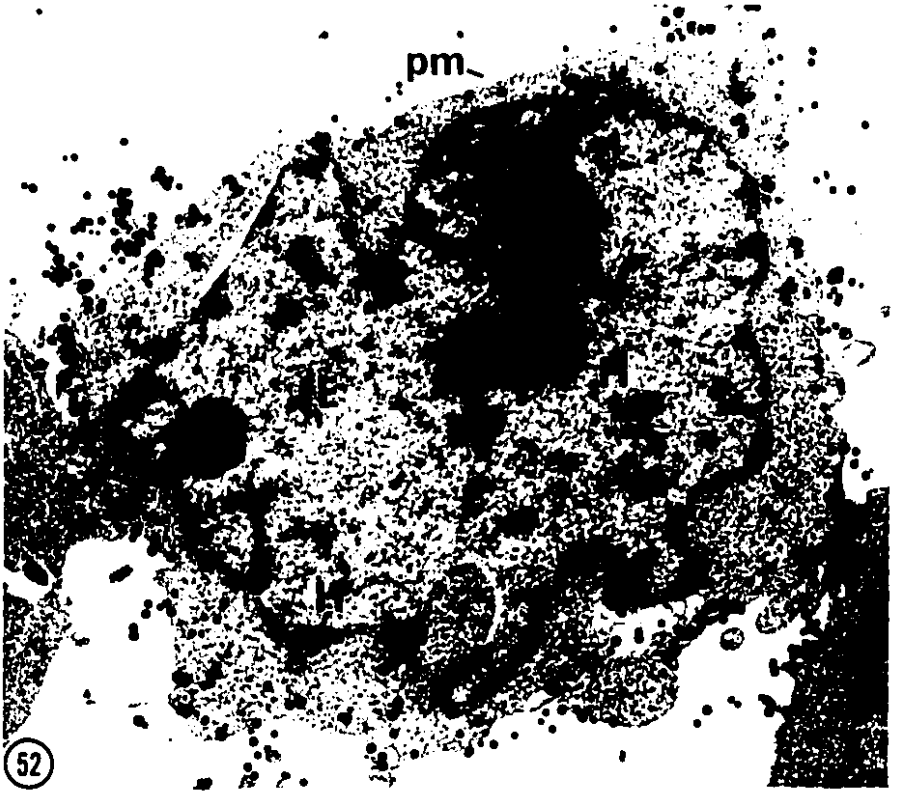
The nucleus exhibits a reaction of moderate intensity in which many grains are associated with nucleoli (n) and their adjacent heterochromatin (H). The rest of the grains are localized near or over the nuclear envelope (NE) and the euchromatin (E). In the cytoplasm, a heavy reaction occurs the plasma membrane (pm), with lighter reaction over the remainder of the cytoplasm.

Exposure: 3 mo (Phys. dev.) x 12,000

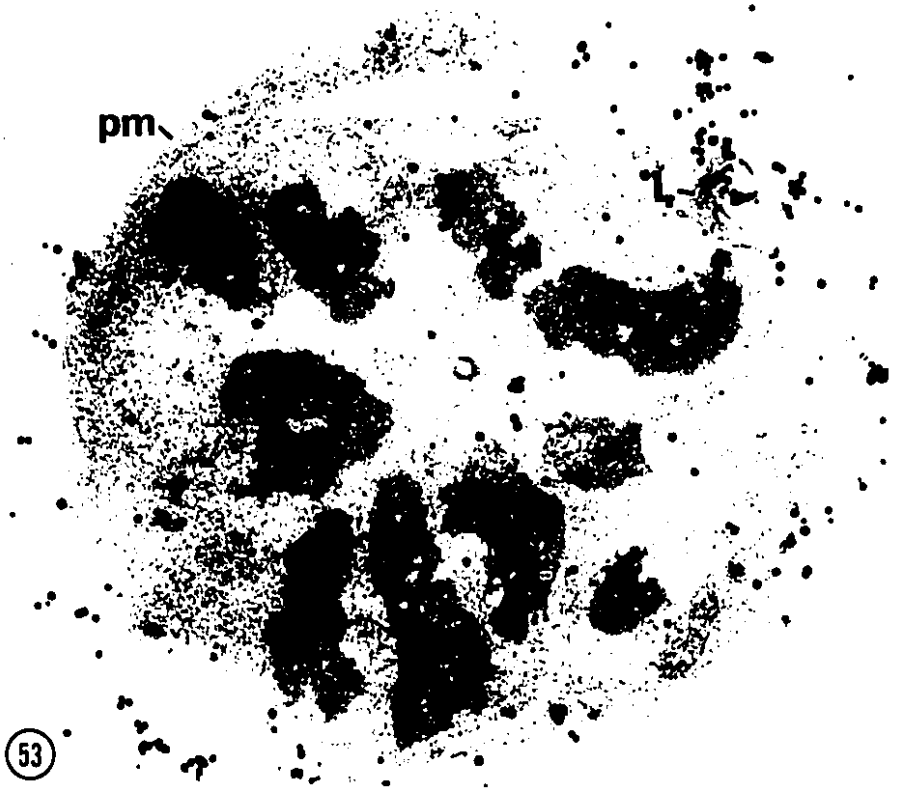
Fig. 53 EM radioautograph of a YAC cell in metaphase, after incubation with ^{3}H-galactose for 18 hr.

In this cell, a moderately light reaction occurs over the cytoplasm, in which most of the silver grains are found over lysosomes (L) and the plasma membrane (pm). The condensed chromosomes (cc), on the other hand, exhibit a striking reaction in which silver grains are both centrally and peripherally located.

Exposure: 3 mo (Phys. dev.) x 12,000



52



53

Fig. 54 EM radioautograph of a Friend cell in interphase, after incubation with ^{3}H-galactose for 18 hr.

The nucleus (N) exhibits a moderate reaction, with many grains localized over the periphery and others associated with nucleoli (n). The cytoplasmic reaction is much stronger than that over the nucleus, with intense reaction over the Golgi region (G). Other grains are situated over the endoplasmic reticulum (rER) and the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000

54

Fig. 55 EM radioautograph of a Friend cell in late prophase, after incubation with ^{3}H-galactose for 18 hr.

The condensed chromosomes (cc), which in this cell type have a filamentous appearance, have pulled away from the nuclear envelope (NE). In some locations, the nuclear envelope has disappeared (arrowhead). The nucleus exhibits substantial reaction, in which the grains tend to be localized over the chromosomes. Some grains may be associated with nucleolar remnants (n). In the cytoplasm, grains are diffusely scattered, with many over the plasma membrane (pm).

Exposure: 5 mo (Chem. dev.) x 12,000


pm
NE
55

Fig. 56 LM radioautograph of myeloma cells after incubation with ^{3}H-galactose for 1 hr.

Many cells exhibit very strong reaction over their Golgi region (G), and the cell surface is labeled (arrowhead). Many grains are also observed over the nuclei, where they tend to be associated with nucleoli (arrow).

Exposure: 15 wk x 1,000

Fig. 57 LM radioautograph of myeloma cells after incubation with ^{3}H-galactose for 10 min.

The overall reaction is similar to that observed at 1 hr, with Golgi reactions (G) and cell surface labeling (arrowhead). Discrete labeling of the nuclei can be observed, with many of the grains again associated with the nucleoli (arrow).

Exposure: 15 wk x 1,000

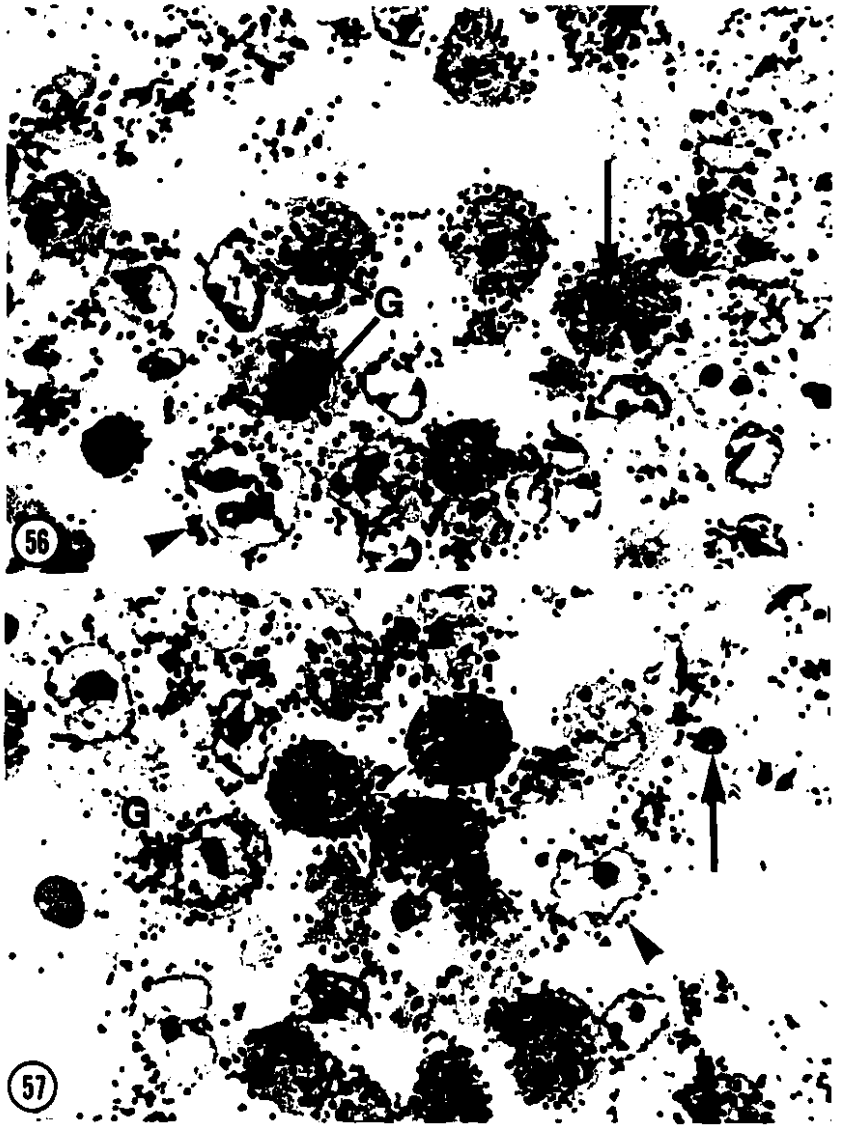

G
56
G
57

Fig. 58 LM radioautograph of myeloma cells after incubation with ^{3}H-galactose for 4 hr.

The cells exhibit a fairly heavy reaction, with grains over their cell surface (arrowhead), cytoplasm and nucleus. Within the latter compartment, many grains are over nucleoli (arrow).

Exposure: 2 mo x 1,000

Fig. 59 LM radioautograph of myeloma cells after incubation with ^{3}H-galactose for 4 hr, followed by 18 hr in unlabeled medium.

There is some overall decrease in labeling of both the cytoplasm and the nuclei. Many grains remain associated with the cell surface (arrowhead).

Exposure: 2 mo x 1,000

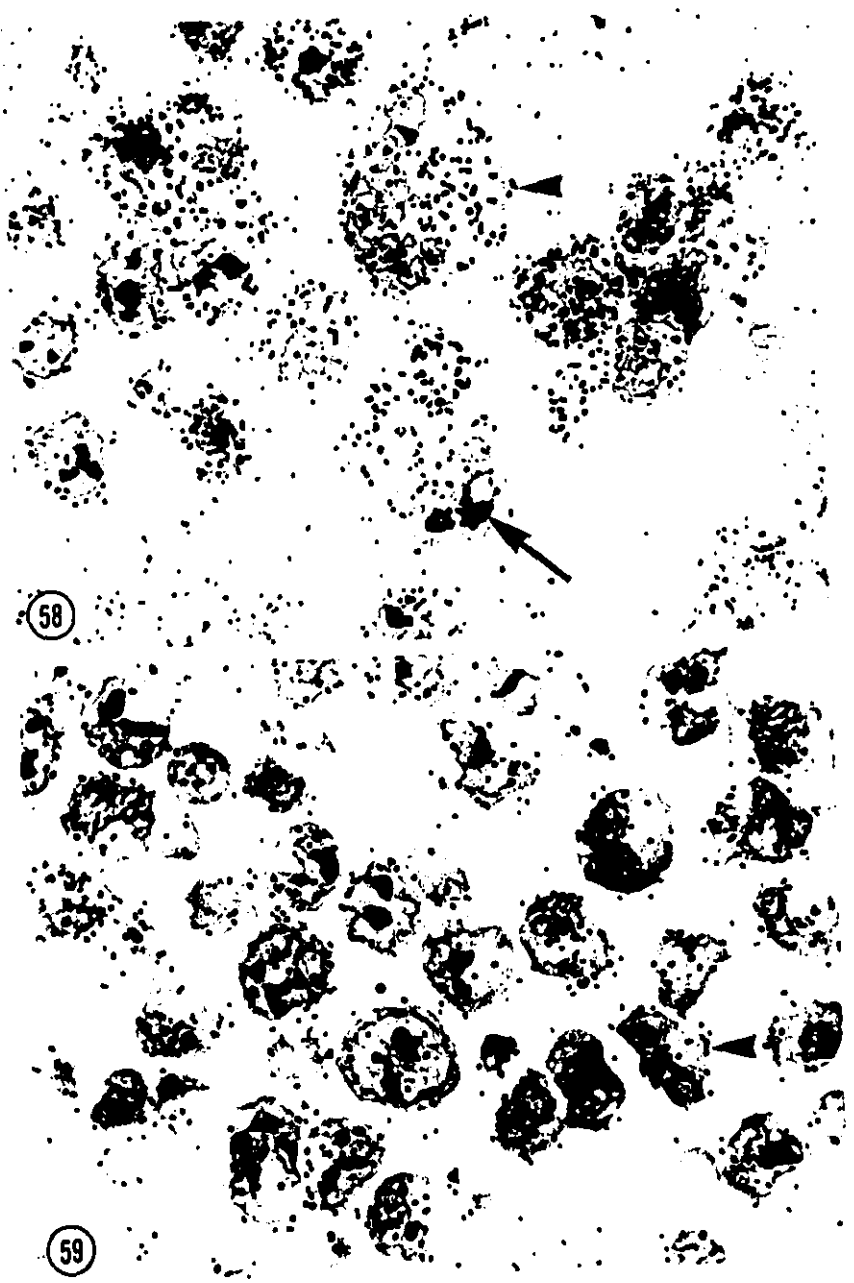

58
59

Fig. 60 LM radioautograph of YAC cells after incubation with ^{3}H-galactose for 4 hr.

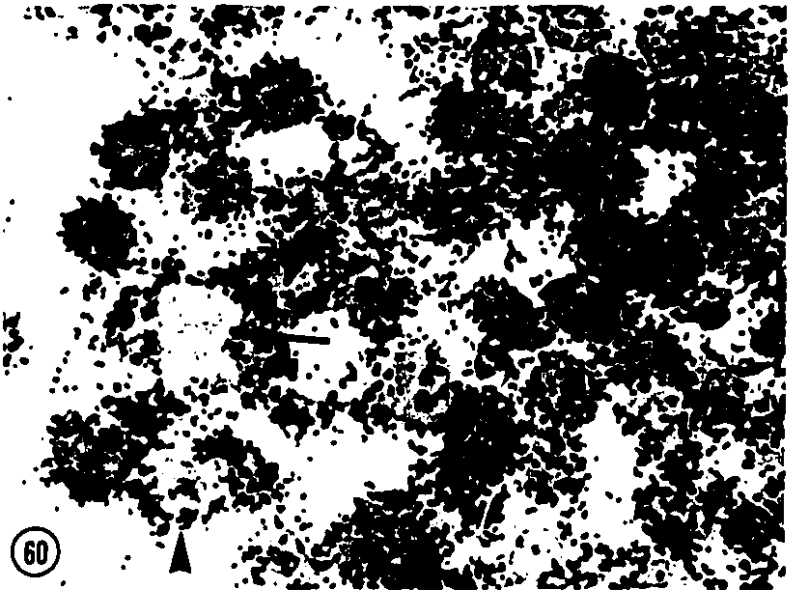
These cells exhibit a very heavy cytoplasmic reaction, with many grains over the cell surface (arrowhead). A substantial number of grains is observed over nuclei (arrow), but the reaction tends to be overshadowed by the heavier cytoplasmic reaction.

Exposure: 1 mo x 1,000

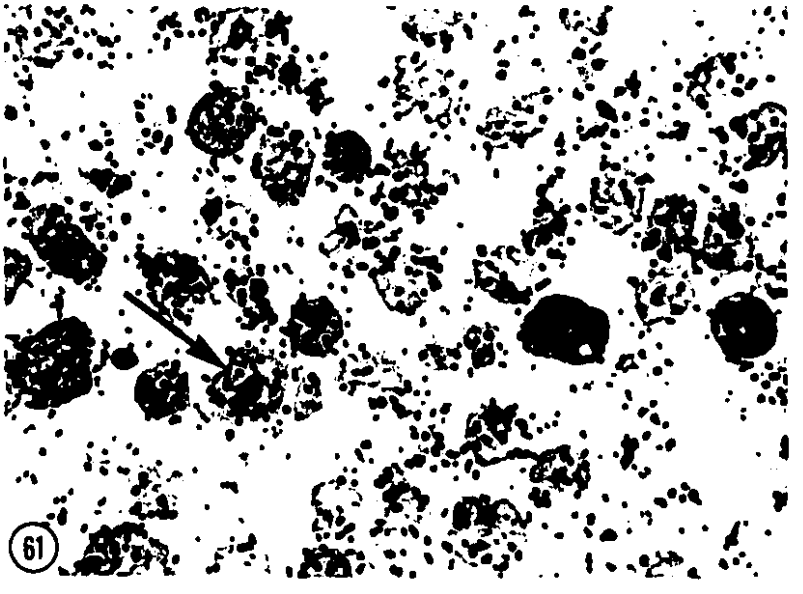
Fig. 61 LM radioautograph of YAC cells after incubation with ^{3}H-galactose for 4 hr in the presence of tunicamycin.

In these cells, the very heavy cytoplasmic and cell surface reaction observed in Fig. 60 is greatly decreased. On the other hand, many silver grains continue to overlie the nuclei (arrow), and these are frequently associated with nucleoli.

Exposure: 1 mo x 1,000



60



61

Fig. 62 EM radioautograph of a myeloma cell in interphase, after incubation with ^{3}H-fucose for 18 hr.

In this cell, moderate reaction occurs over both the cytoplasm and nucleus (N). In the cytoplasm, many of the silver grains are associated with the Golgi apparatus (G), lysosomes (L), and the plasma membrane (pm). Over the nucleus, several grains are associated with the nuclear periphery but others are found over the more internal euchromatin and nucleoli (n).

Exposure: 1 mo (Chem. dev.) x 12,000

62

Fig. 63 EM radioautograph of a dividing myeloma cell in metaphase, after incubation with ^{3}H-fucose for 18 hr.

In this cell, the nuclear envelope has disappeared. A light reaction is observed over the condensed chromosomes (cc), and occasional grains are associated with remnants of the nucleolus (n). In the cytoplasm, heavy reaction is observed over lysosomes (L) and the plasma membrane (pm). Other silver grains are scattered over the remainder of the cytoplasm.

Exposure: 11 mo (Phys. dev.) x 12,000

(63)

Fig. 64 EM radioautograph of interphase YAC cells after incubation with ^{3}H-fucose for 18 hr.

The cells exhibit a light nuclear reaction, with some grains found over nucleoli (n) and others near the nuclear envelope (NE). In the cytoplasm, there is labeling over the Golgi apparatus (G), lysosomes (L), and the plasma membrane (pm).

Exposure: 3 mo (Chem. dev.) x 12,000

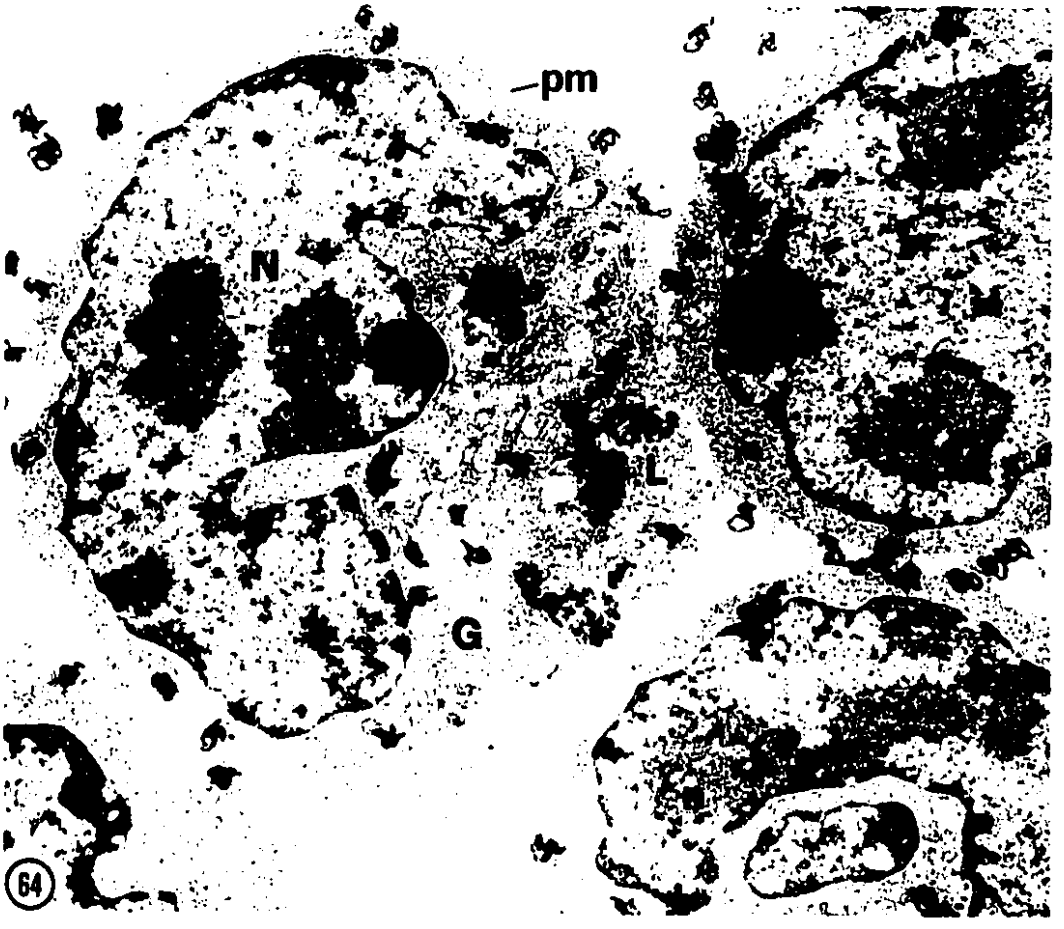
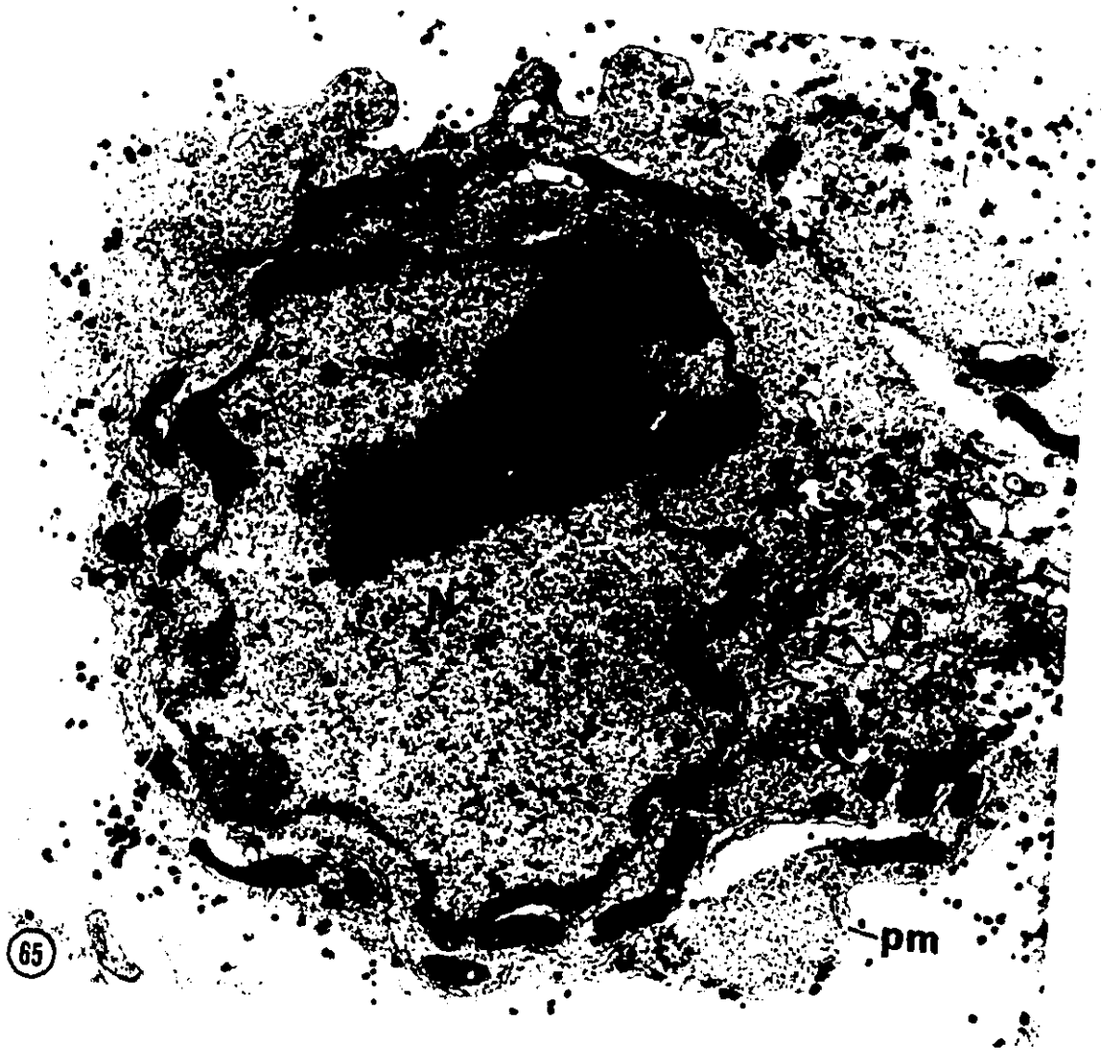

pm
N
L
G
64

Fig. 65 Electron microscope radioautograph of an Friend cell in interphase, after incubation with ^{3}H-fucose for 18 hr.

There is a light nuclear reaction with grains over the periphery of the nucleolus (n) and its associated heterochromatin, the euchromatin (E), and the nuclear periphery. In the cytoplasm, the majority of silver grains are seen over the Golgi apparatus (G) and the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000



(65)

Fig. 66 Electron microscope radioautograph of a Friend cell in prophase after incubation with ^{3}H-fucose for 18 hr.

In this cell, the nuclear envelope is still present (NE), but the chromosomes have condensed (cc) and pulled away from the envelope. These exhibit light labeling, and other nuclear grains are associated with the nucleolus (n). In the cytoplasm, a very heavy reaction occurs over the well developed Golgi apparatus (G), as well as over lysosomes (L) and the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000

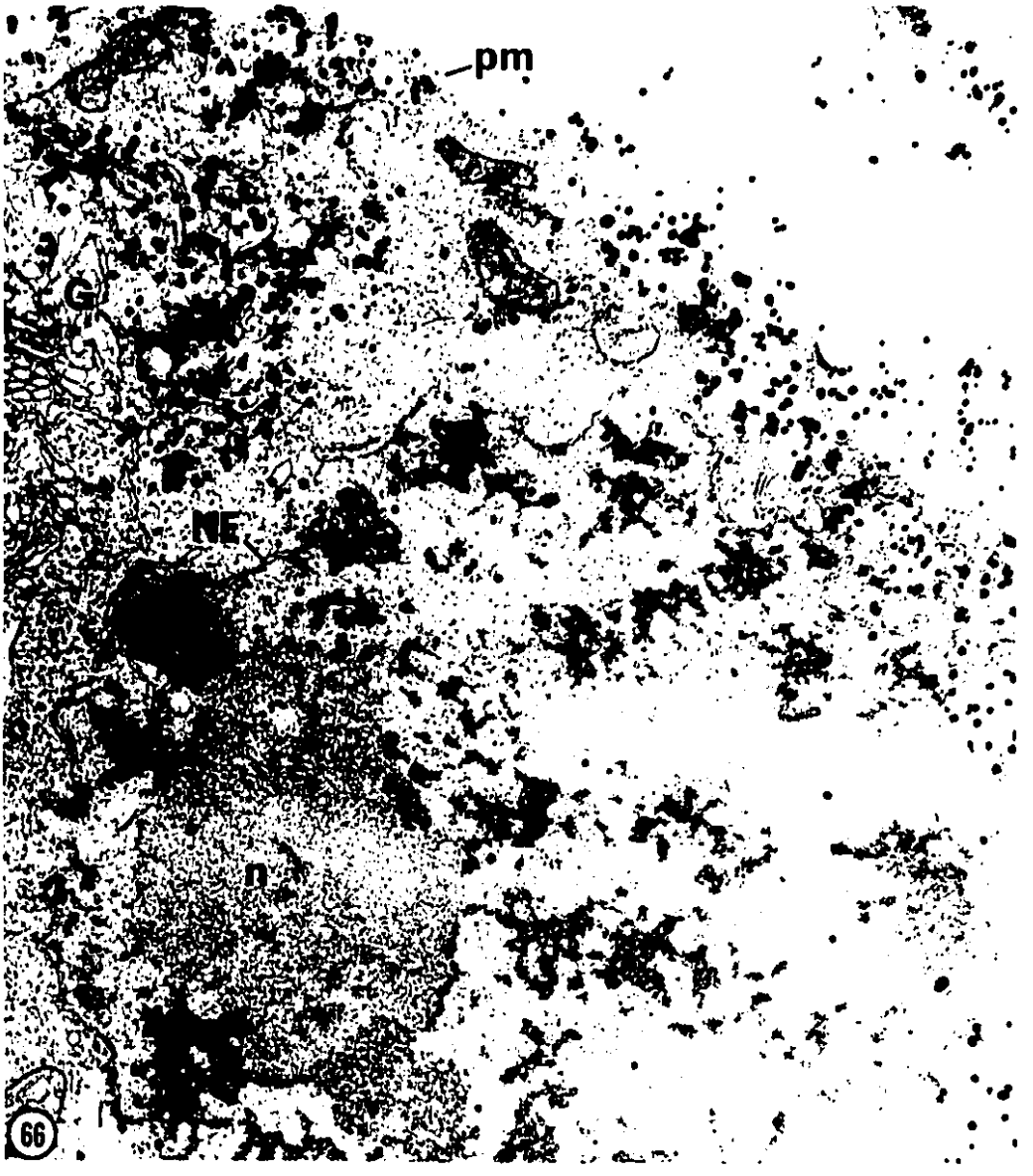

pm
G
NE
n
66

Fig. 67 LM radioautograph of myeloma cells after incubation with ^{3}H-fucose for 4 hr.

These cells exhibit fairly strong cytoplasmic and cell surface reaction, but in addition, discrete labeling of the nuclei is observed, with many grains associated with nucleoli (arrows).

Exposure: 4 mo x 1,000

Fig. 68 LM radioautograph of myeloma cells after incubation with ^{3}H-fucose for 4 hr, followed by incubation in unlabeled medium for 6 hr.

The cells exhibit a reaction very similar to that observed at 4 hr in Fig. 67. Thus, there is heavy cytoplasmic reaction, accompanied by nuclear labeling, in which grains are frequently associated with nucleoli (arrows).

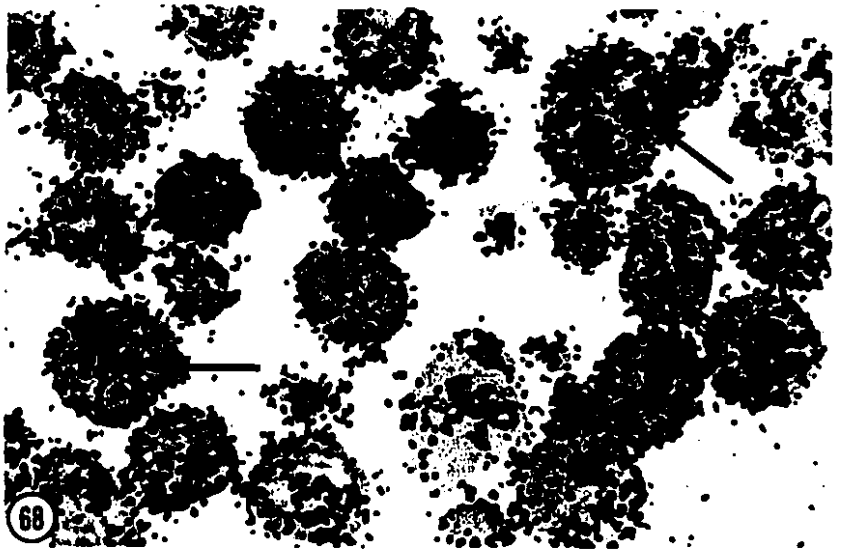
Exposure: 4 mo x 1,000

Fig. 69 LM radioautograph of myeloma cells after incubation with ^{3}H-fucose for 4 hr, followed by incubation in unlabeled medium for 18 hr.

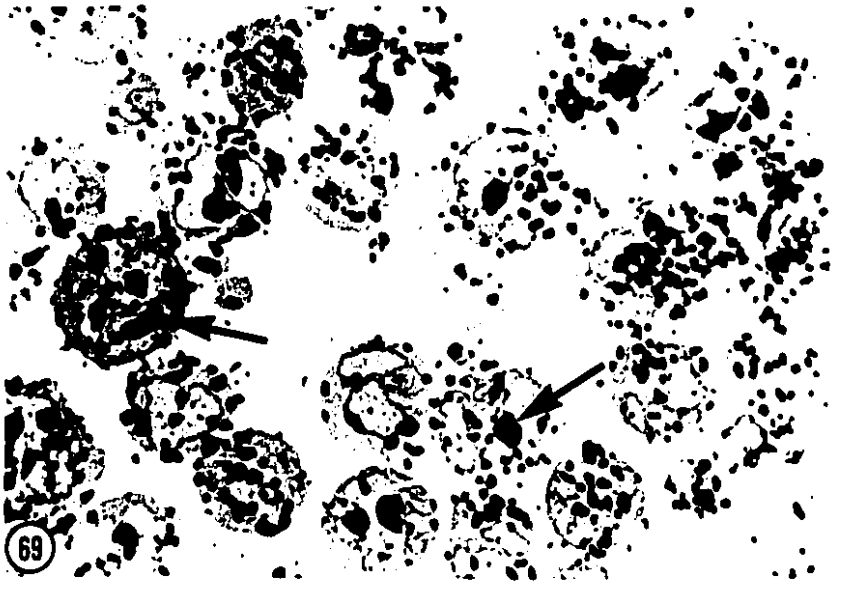
In these cells, the heavy cytoplasmic and cell surface reaction observed in Fig. 68 has dramatically decreased, but the nuclear labeling remains substantial. Again, many of these nuclear grains are associated with nucleoli (arrows).

Exposure: 4 mo x 1,000

67



68



69

Fig. 70 EM radioautograph of a myeloma cell in interphase, after incubation with ^{3}H-glcNAc for 18 hr.

A heavy reaction occurs over both the cytoplasm and nucleus. Over the nucleus, some grains are over the nuclear periphery, but many more are over the internal euchromatin (E) and the nucleoli (n). The diffuse cytoplasmic labeleing includes grains over endoplasmic reticulum (rER), the Golgi apparatus (G), and to a lesser extent, the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 12,000

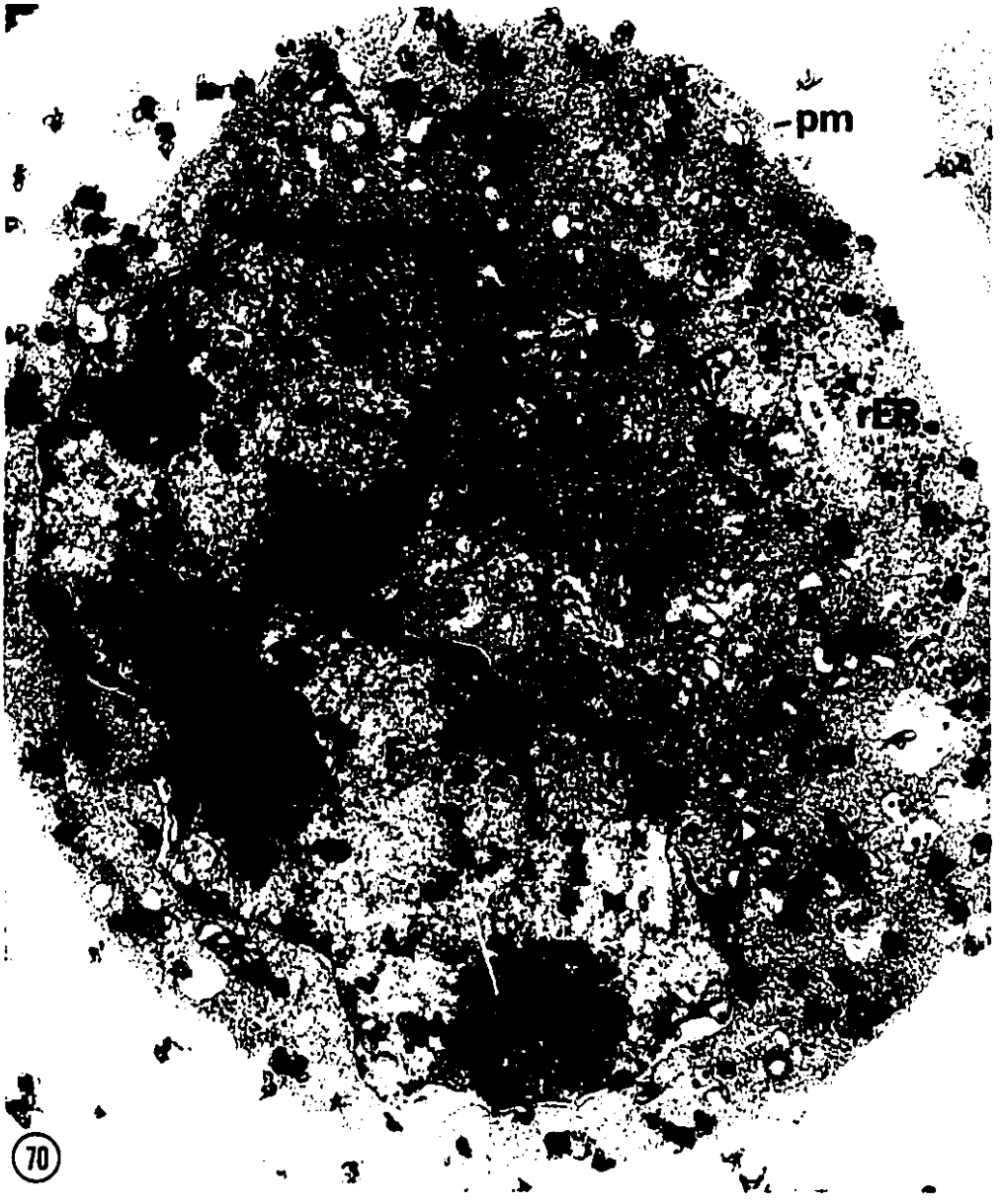
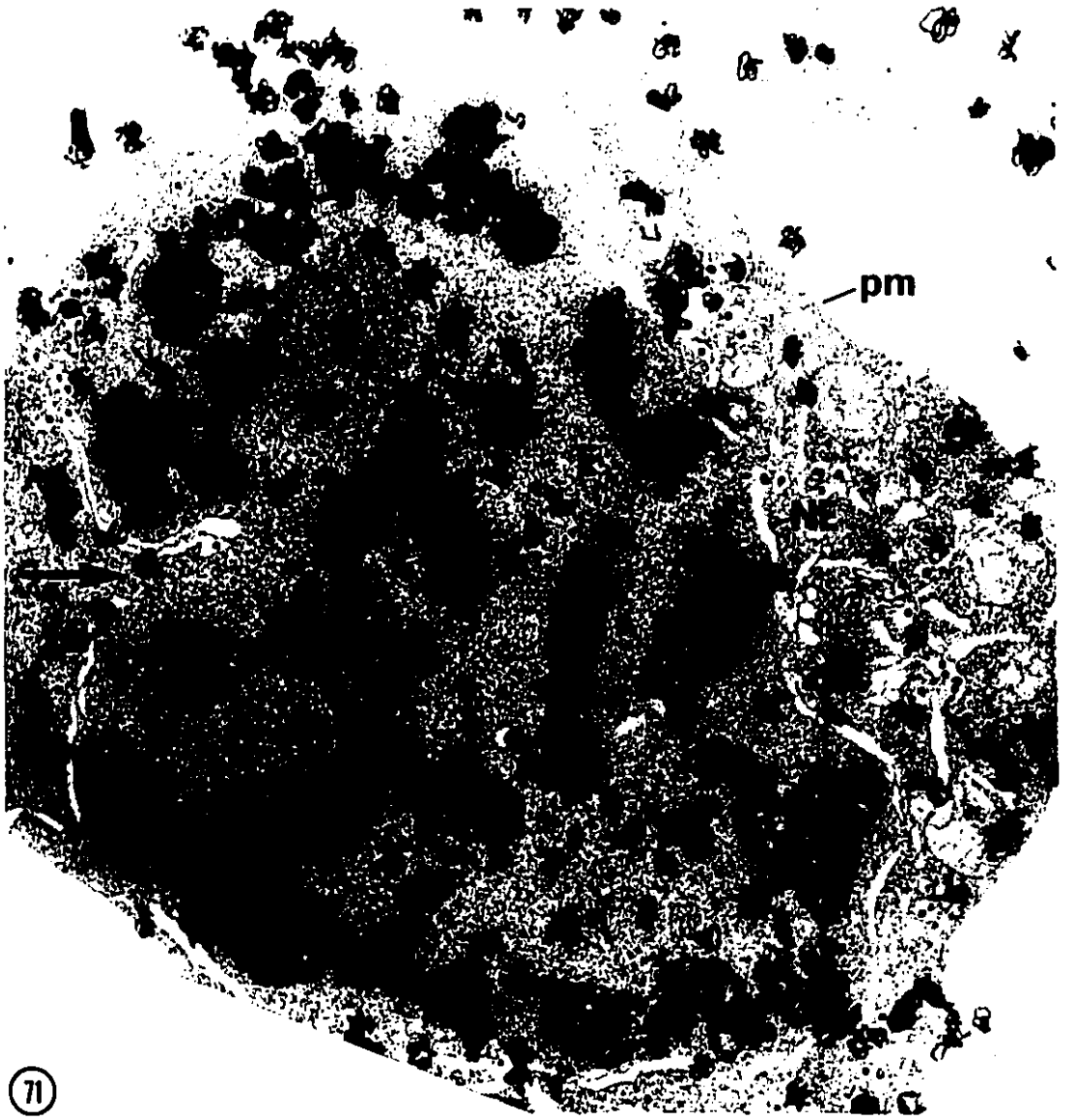

pm
rER
70

Fig. 71 EM radioautograph of a myeloma cell in late prophase, after incubation with ^{3}H-glcNAc for 18 hr.

In this cell, the nuclear envelope (NE) is beginning to breakdown and in some regions has disappeared (arrow). The condensing chromosomes (cc) have pulled away from the nuclear envelope and exhibit heavy reaction. Some grains remain associated with the euchromatin (E), others with a nucleolar remnant (n), still others with the nuclear envelope. The cytoplasm exhibits a diffuse reaction, with some grains over the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 12,000

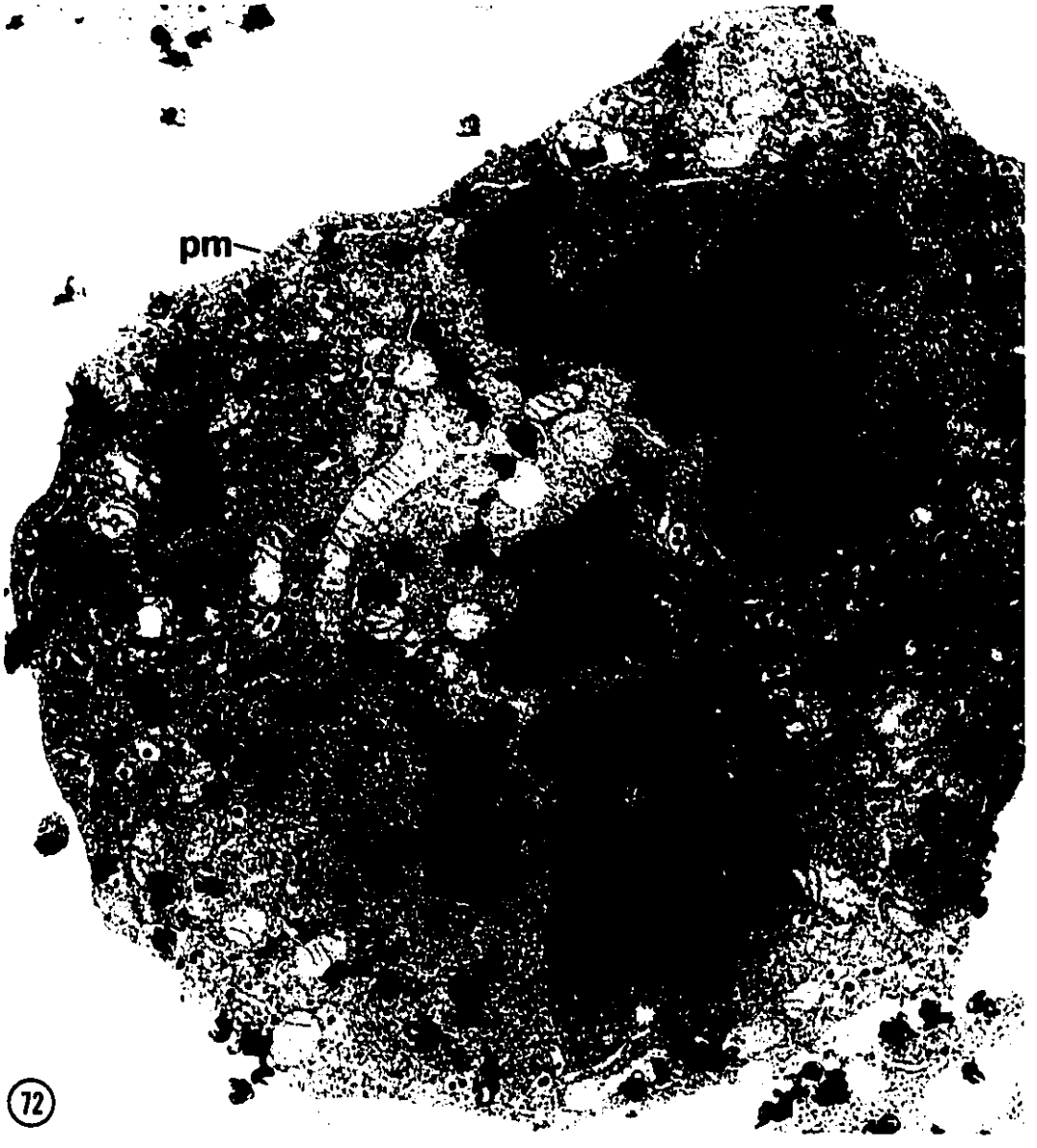


71

Fig. 72 EM radioautograph of a myeloma cell in anaphase, after incubation with ^{3}H-N-acetylglucosamine for 18 hr.

In this cell, a heavy reaction continues to cover the condensed chromosomes (cc), in which the grains are both centrally and peripherally located. The cytoplasm is diffusely labelled, and a light reaction occurs over the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 12,000

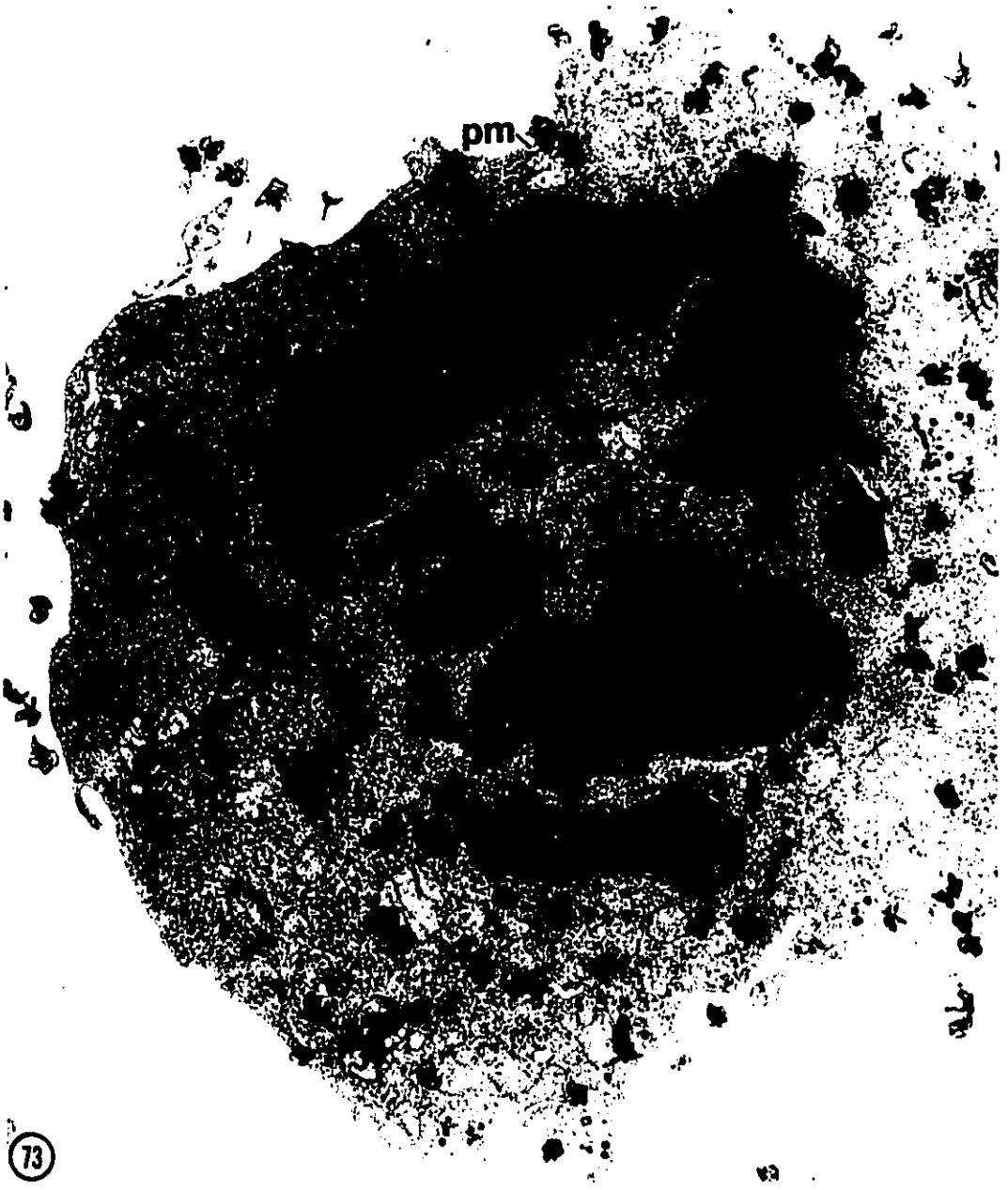


72

Fig. 73 EM radioautograph of a dividing myeloma cell in early telophase, after incubation with ^{3}H-glcNAc for 18 hr.

In this cell, the nuclear envelope (NE) has reformed in many areas, and some regions of euchromatin (E) have differentiated from the heterochromatin (H). A heavy reaction occurs over the nucleus, in which more grains appear to be associated with the heterochromatin and euchromatin than with the nuclear envelope. The cytoplasm contues to exhibit a diffuse reaction, with some grains over the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 12,000



73

Fig. 74 LM radioautograph of myeloma cells after 1 hr incubation with ^{3}H-glcNAc.

A discrete reaction is observed over the nuclei of these cells, with some of the grains associated with nucleoli (arrows). Only a light reaction is seen over the cytoplasm.

Exposure: 15 wk x 1,000

Fig. 75 LM radioautograph of myeloma cells after 10 min incubation with ^{3}H-N-acetylglucosamine.

Although the overall reaction is lighter than that observed in Fig. 74, the cells exhibit nuclear reactions, and many grains are assocaited with nucleoli (arrows).

Exposure: 15 wk x 1,000

Fig. 76 LM radioautograph of myeloma cells after 2 min incubation with ^{3}H-N-acetylglucosamine.

Even at this early time interval, definite nuclear reactions are observed, with some grains over nucleoli (arrows). The overall reaction is not less than observed in Fig. 75 because a higher dose ^{3}H-N-acetylglucosamine was administered.

Exposure: 4 mo x 1,000

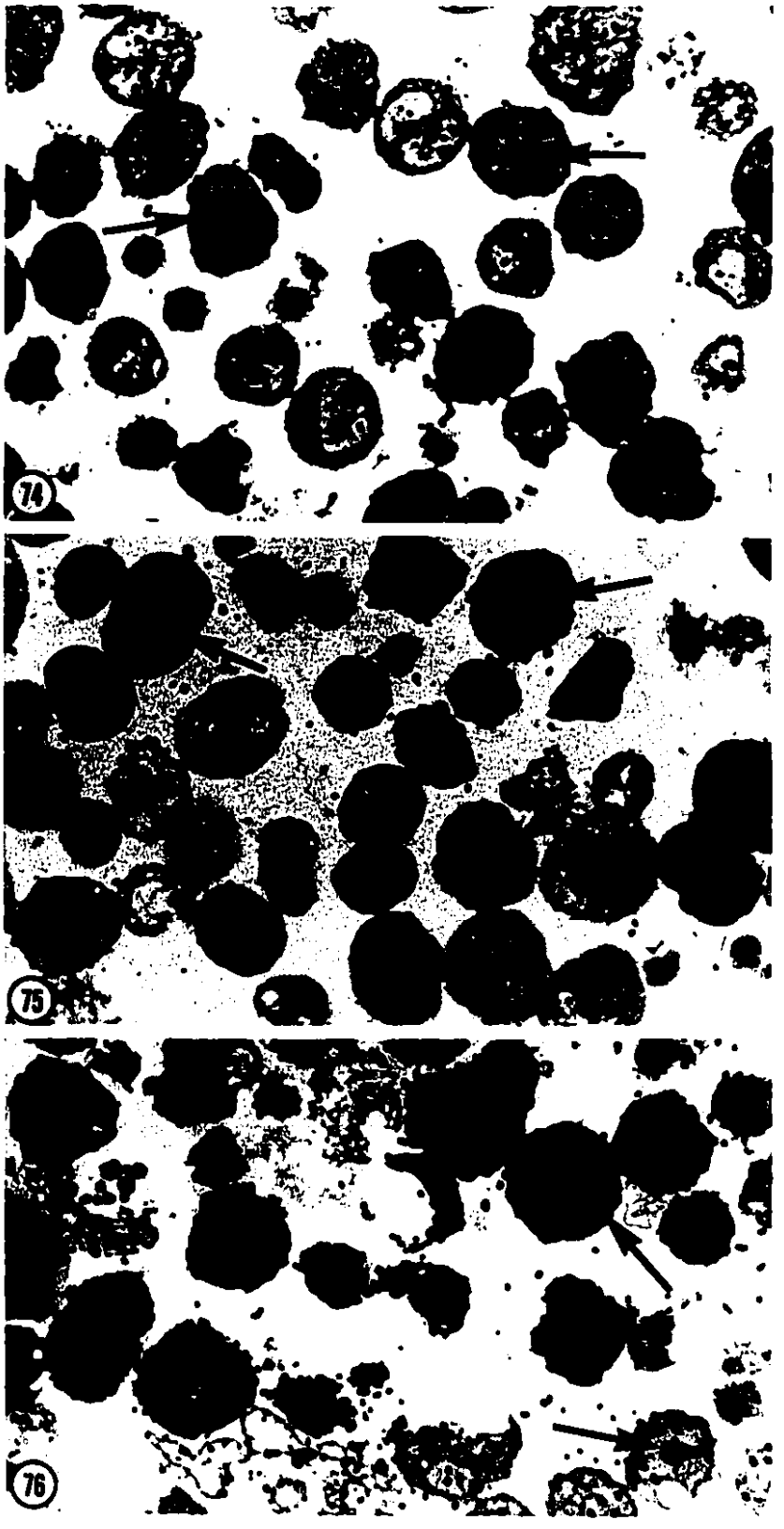

74
75
76

Fig. 77 EM radioautograph of a myeloma cell in interphase, after incubation with ^{3}H-mannose for 18 hr.

The nucleus (N) exhibits only a light reaction, with some grains located peripherally and others internally, sometimes in association with nucleoli (N). In the cytoplasm, there is a fairly heavy reaction over the Golgi apparatus (G), and lighter reaction over rough endoplasmic reticulum (rER). Only occasional grains are observed over the plasma membrane.

Exposure: 11 mo (Phys. dev.) x 12,000

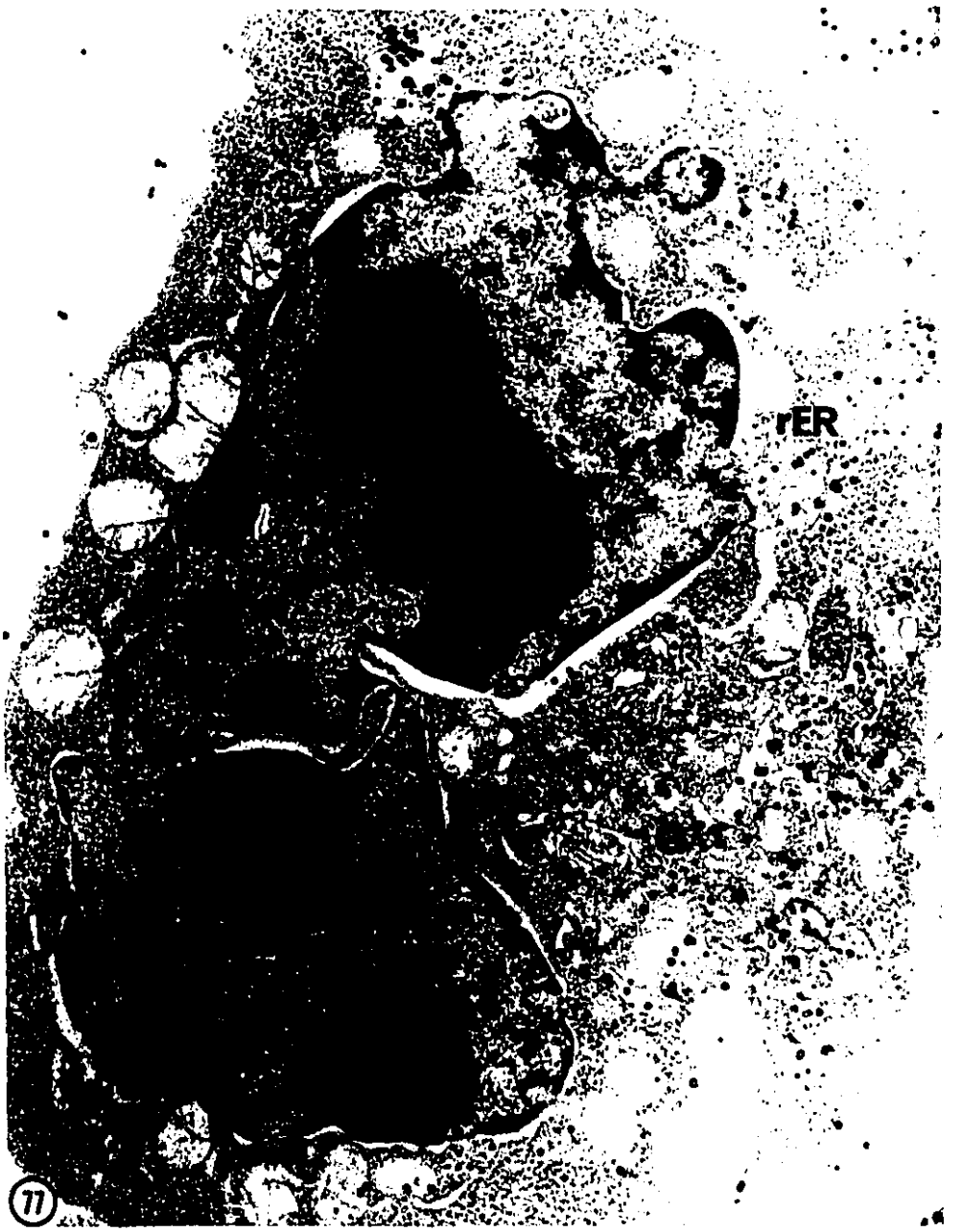

rER
11

Fig. 78 EM radioautograph of a myeloma cell in prophase, after incubation with ^{3}H-mannose for 18 hr.

In this cell, the condensing chromosomes (cc) have pulled away from the nuclear envelope (NE), which has disappeared in some locations (arrowhead). Only a very light reaction is observed in which grains occur over the condensed chromosomes or over nucleolar remnants (n). Somewhat greater numbers of grains are associated with the nuclear envelope. In the cytoplasm, one observes a heavier reaction in which many silver grains are over cisternae of endoplasmic reticulum (ER), lysosomes (L). Some grains occur over the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000

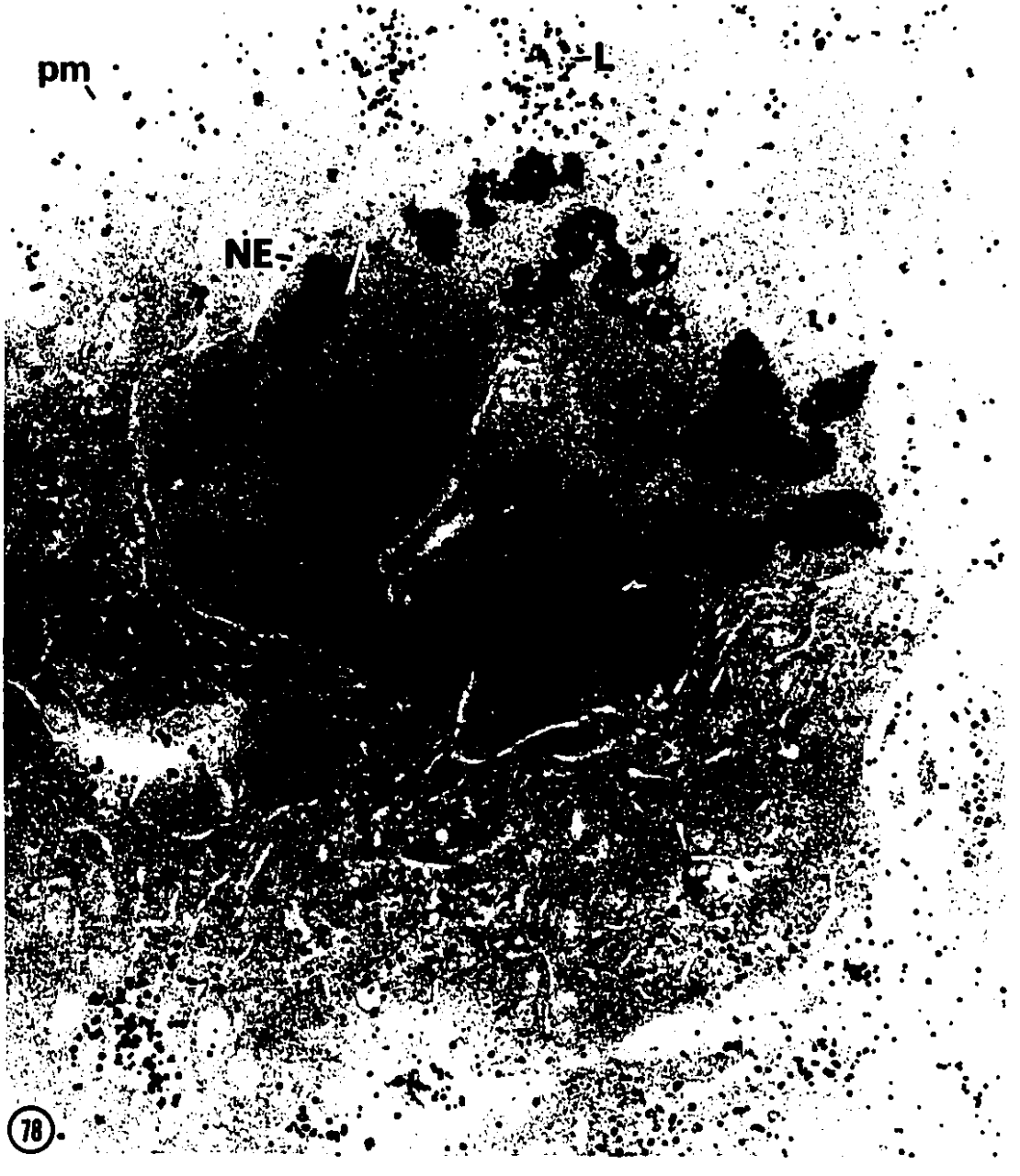

pm
L
NE
78

Fig. 79 LM radioautograph of myeloma cells after incubation with ^{3}H-mannose for 1 hr.

The cells exhibit a moderate reaction over both their cytoplasm and nucleus. In the nucleus, many of these grains are over the nuclear periphery (arrow).

Exposure: 15 wk x 1,000

Fig. 80 LM radioautograph of myeloma cells after incubation with ^{3}H-mannose for 10 min.

At this time interval, no significant reaction is observed over the cytoplasm or nucleus of the cells.

Exposure: 15 wk x 1,000

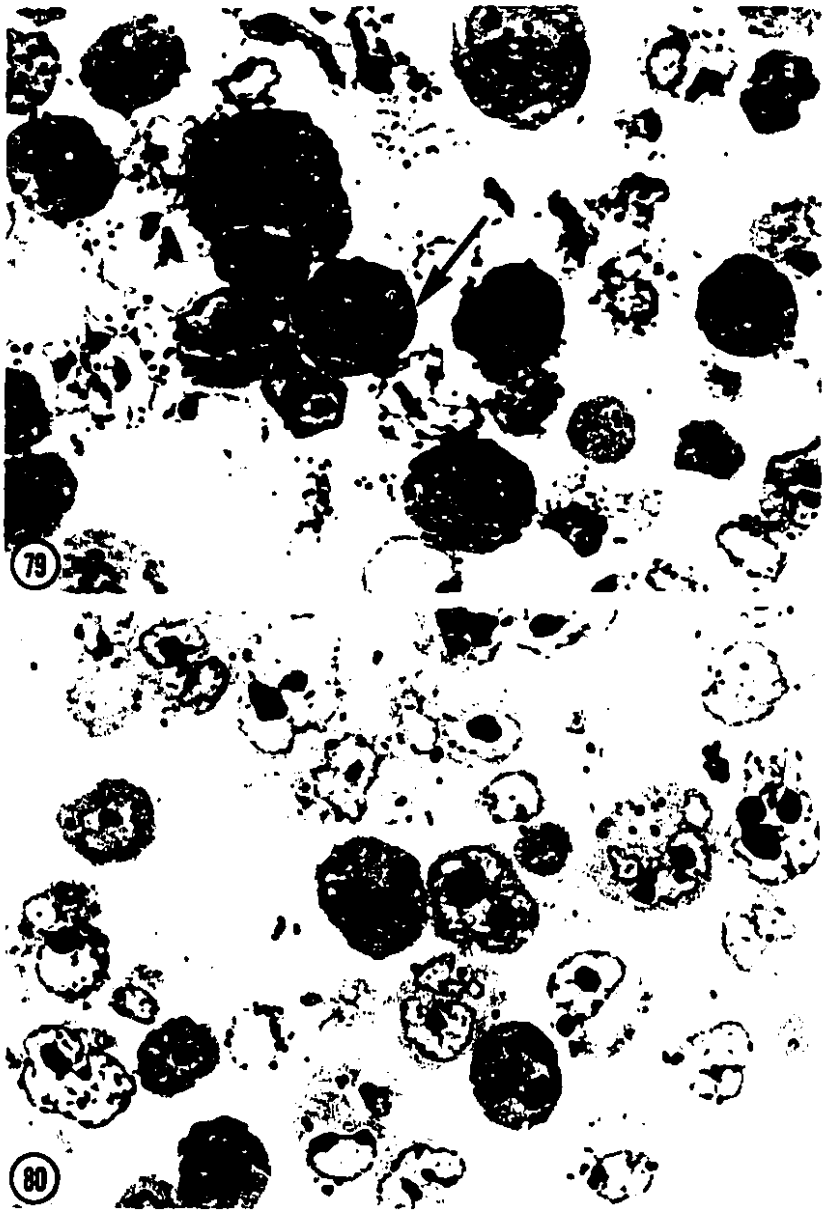
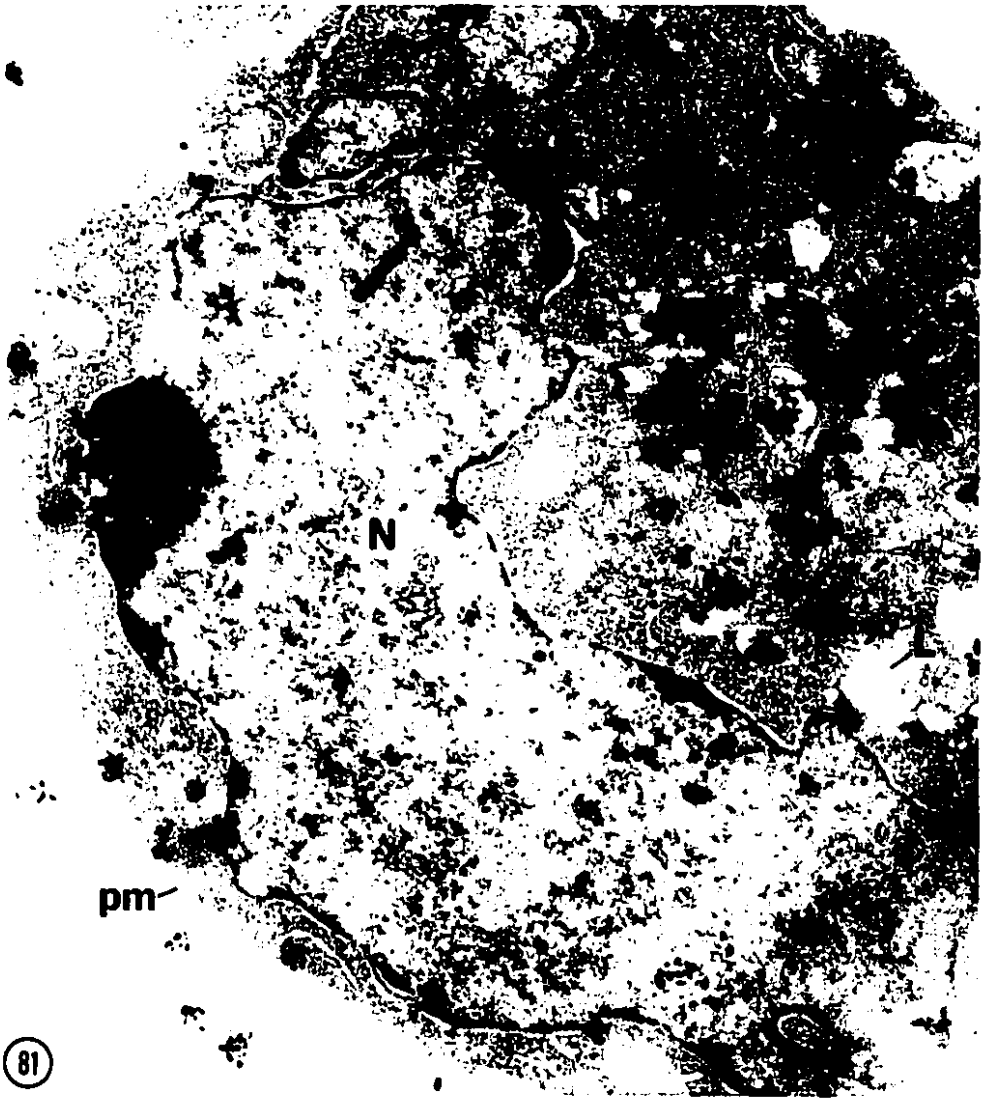

79
80

Fig. 81 EM radioautograph of a myeloma cell in interphase after incubation with ^{3}H-manNAc for 18 hr.

Only a moderate reaction occurs over the nucleus of this cell, even after this long exposure time. A few grains are localized over the nuclear envelope (NE) and its associated heterochromatin (H). Others are over euchromatin (E), and associated with nucleoli (n). In the cytoplasm, the Golgi apparatus (G) exhibits a substantial reaction, while a few grains occur over the remainder of the cytoplasm and the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 9,000

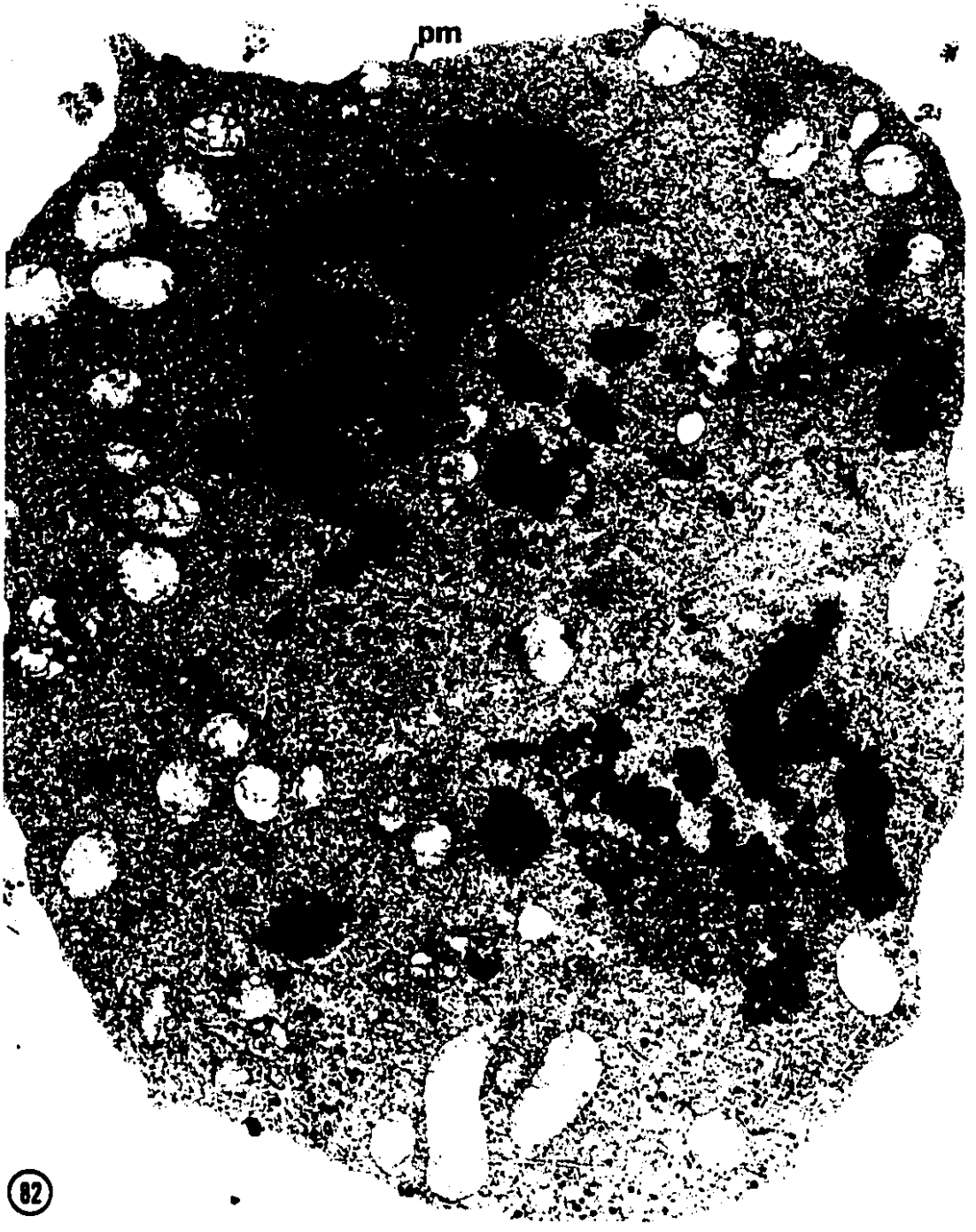


81

Fig. 82 EM radioautograph of an myeloma cell in metaphase, after incubation with ^{3}H-manNAc for 18 hr.

This cell exhibits a very light overall reaction. Some grains are observed over condensed chromosomes (cc), while the nucleolar remnants are largely unlabeled (n). In the cytoplasm, the heaviest reaction is over lysosomes (L). Occasional grains occur over the plasma membrane (pm).

Exposure: 11 mo (Chem. dev.) x 9,000



82

Fig. 83 EM radioautograph of a dividing myeloma cell in late telophase, after incubation with ^{3}H-manNAc for 18 hr.

In this cell, the nuclear envelope (NE) has reformed, and the heterochromatin of the condensed chromosomes is closely applied to it. The nucleus exhibits a light reaction with most of the grains over the peripheral condensed chromatin. The nucleoli (n) are unlabeled. Very little reaction is observed over the cytoplasm. In contrast to the situation in interphase cells (Fig. 81), the Golgi apparatus (G) has incorporated no label. (E: euchromatin)

Exposure: 11 mo (Chem. dev.) x 9,000


E
83

Fig 83a. Electron microscope radioautograph of a myeloma cell in interphase after exposure to ^{35}S-sulphate for 18 hrs.

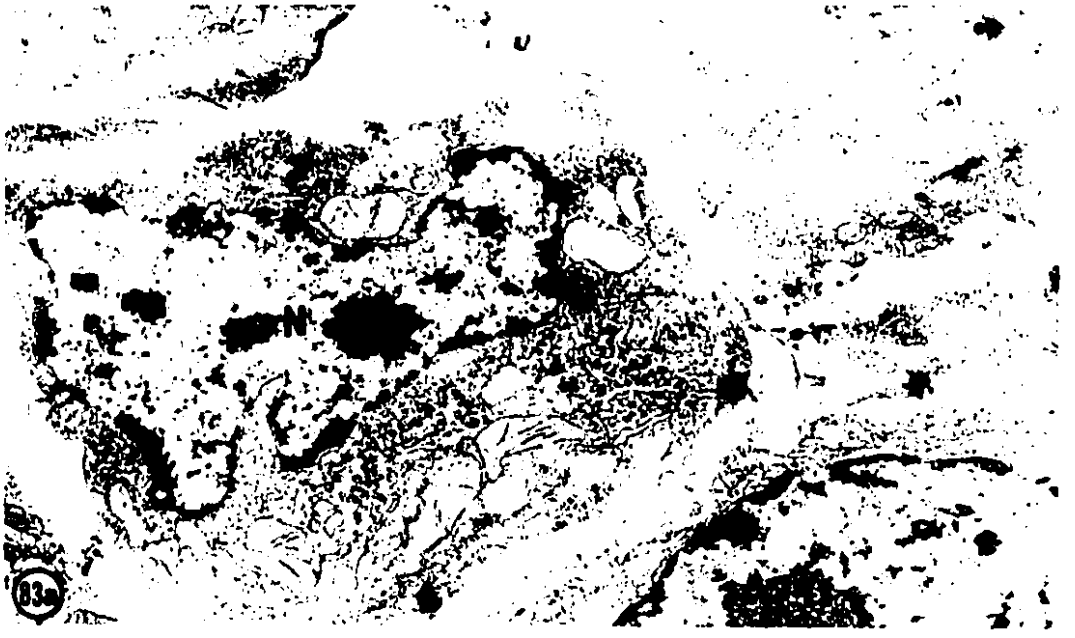
A very light overall reaction is observed in these cells at the EM level but many of the grains appear to be associated with the nuclei (N). A few grains are seen at the periphery of the nucleus close to the nuclear envelope while others are seen more internally. Away from the nucleus, grains are mostly observed at the cell surface with only the occasional grain located in the cytoplasm.

Exposure: 3 mo (Chem. dev.) x 9,000

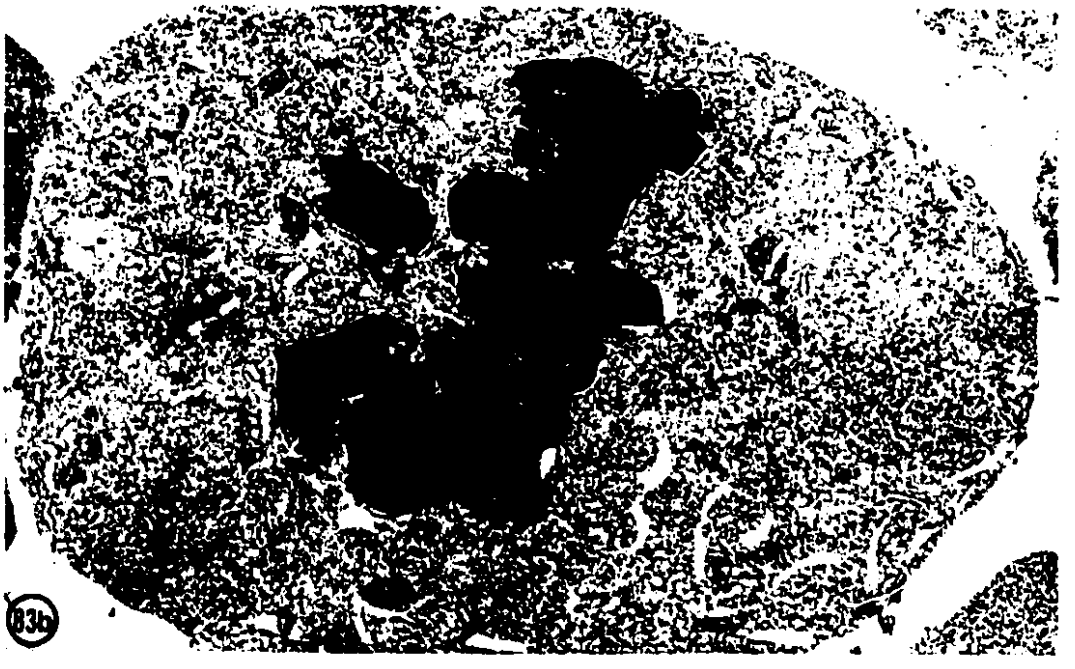
b. Electron microscope radioautograph of a myeloma cell in late prophase which was exposed to ^{35}S-sulphate for 18 hr.

Grains appear to be localized to the periphery of the condensed chromosomes (cc).

Exposure: 3 mo (Chem. dev.) x 9,000



83a



83b

Fig. 84. Electron microscope radioautograph of a myeloma cell in the early stages of apoptosis, after incubation with ^{3}H-fucose for 18 hr.

The heterochromatin (H) has become stongly marginated against certain regions of the nuclear envelope (NE) which tend to be evaginated. Most of the euchromatin stains lightly (E), but some other parts have a granular appearance (Gr). Large masses of nucleolar material are seen (n). A light reaction occurs over all of these nuclear components, and in the case of the heterochromatin, some grains are sufficiently removed from the nuclear envelope and euchromatin to be assigned exclusively to the heterochromatin compartment. The cytoplasm exhibits a fairly heavy reaction in which many of the grains are over lysosomes (L), profiles of rough endoplasmic reticulum (rER), and the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000

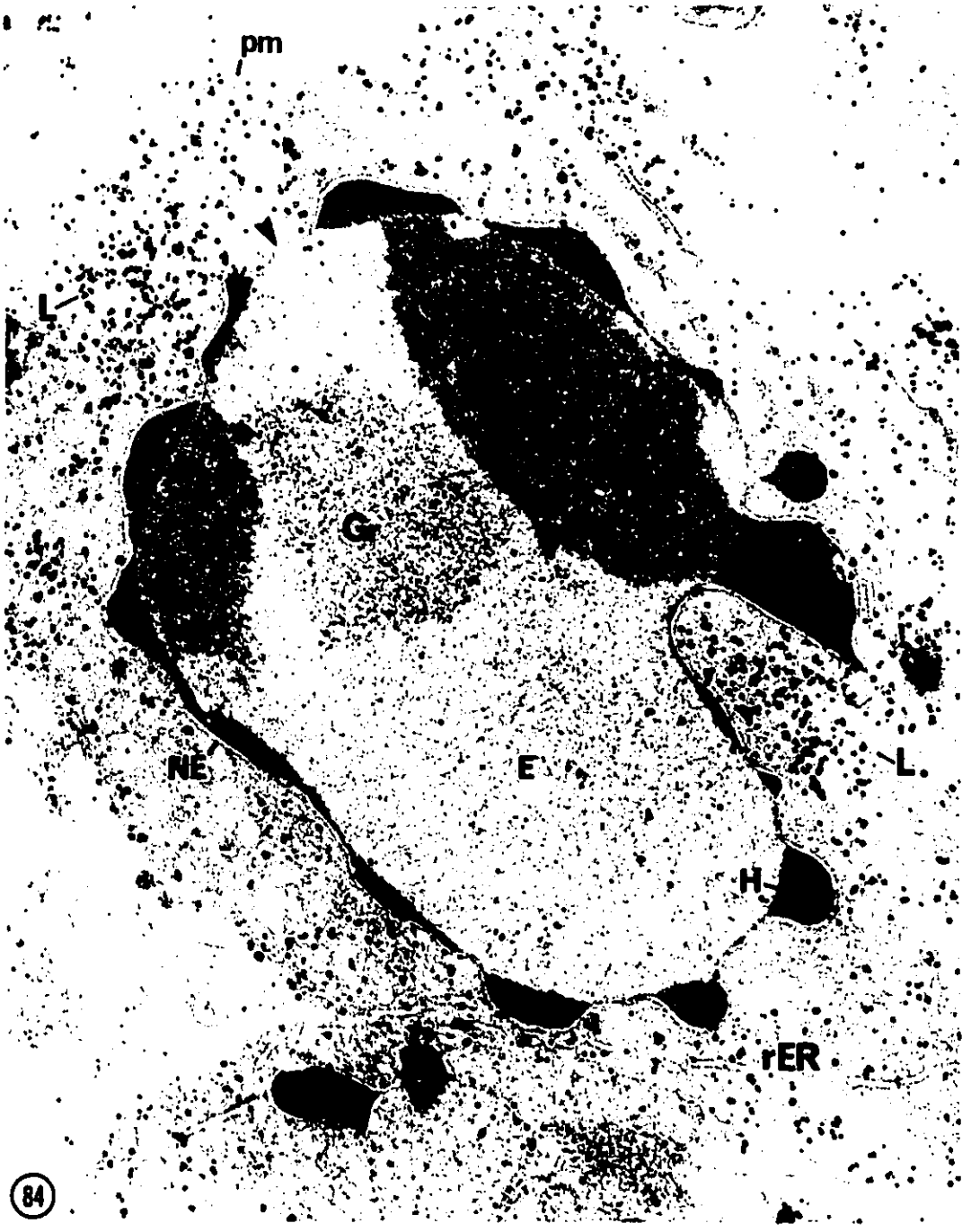
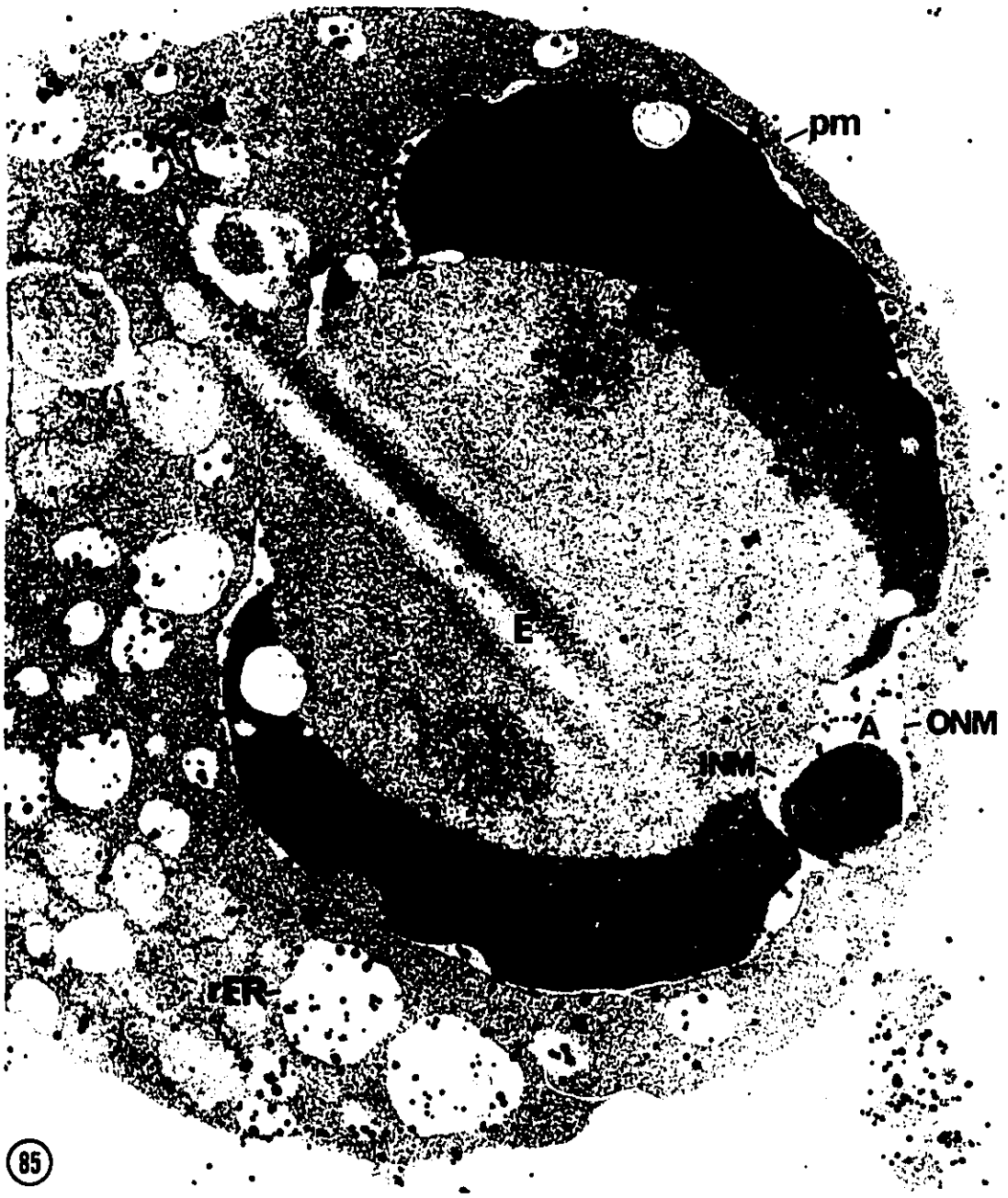

pm
L
G
NE
E
L
H
rER
84

Fig. 85 EM radioautograph of a myeloma cell in a later stage of apoptosis, after incubation with ^{3}H-fucose for 18 hr.

The heterochromatin (H) has condensed into large masses against the nuclear envelope (NE). Most of the euchromatin stains lightly (E), with granular patches (Gr). Some nucleolar material remains (n). In some locations, as seen at (A), the perinuclear space between the outer nuclear membrane (ONM) and the inner nuclear membrane has become greatly enlarged, in a manner similar to that seen in Schwann cells undergoing apoptosis in Fig. 3. A moderate reaction covers all of the nuclear compartments. At the enlargements of the perinuclear space, reaction appears over the space, as well as over the inner and outer nuclear membranes. In the cytoplasm are heavily-labeled ribosome-studded profiles of rough endoplasmic reticulum (rER); these resemble the nuclear envelope enlargement seen at A and may be in continuity with such enlargements. They often contain small spherical particles, a feature shared by the nuclear envelope (arrow), as well as the endoplasmic reticulum of normal myeloma cells (Figs. 77-78).

Exposure: 11 mo (Phys. dev.) x 12,000



(85)

Fig. 86 EM radioautograph of a myeloma cell in a late stage of apoptosis, after incubation with ^{3}H-fucose for 18 hr.

The heterochromatin has fragmented into several spherical masses. Most of these remain associated, over part of their surface, with a fragment of expanded nuclear envelope (NE). These latter are identical in appearance to expanded profiles of endoplasmic reticulum (rER) seen throughout the cytoplasm. A small amount of euchromatin with granular material remains (Gr). The heterochromatin masses are lightly labeled, and a few grains are over the granular region. In the cytpolasm, the heaviest reaction overlies the profiles of nuclear envelope, rough endoplasmic reticulum and lysosomes (L). Some grains are at the plasma membrane (pm).

Exposure: 11 mo (Phys. dev.) x 12,000

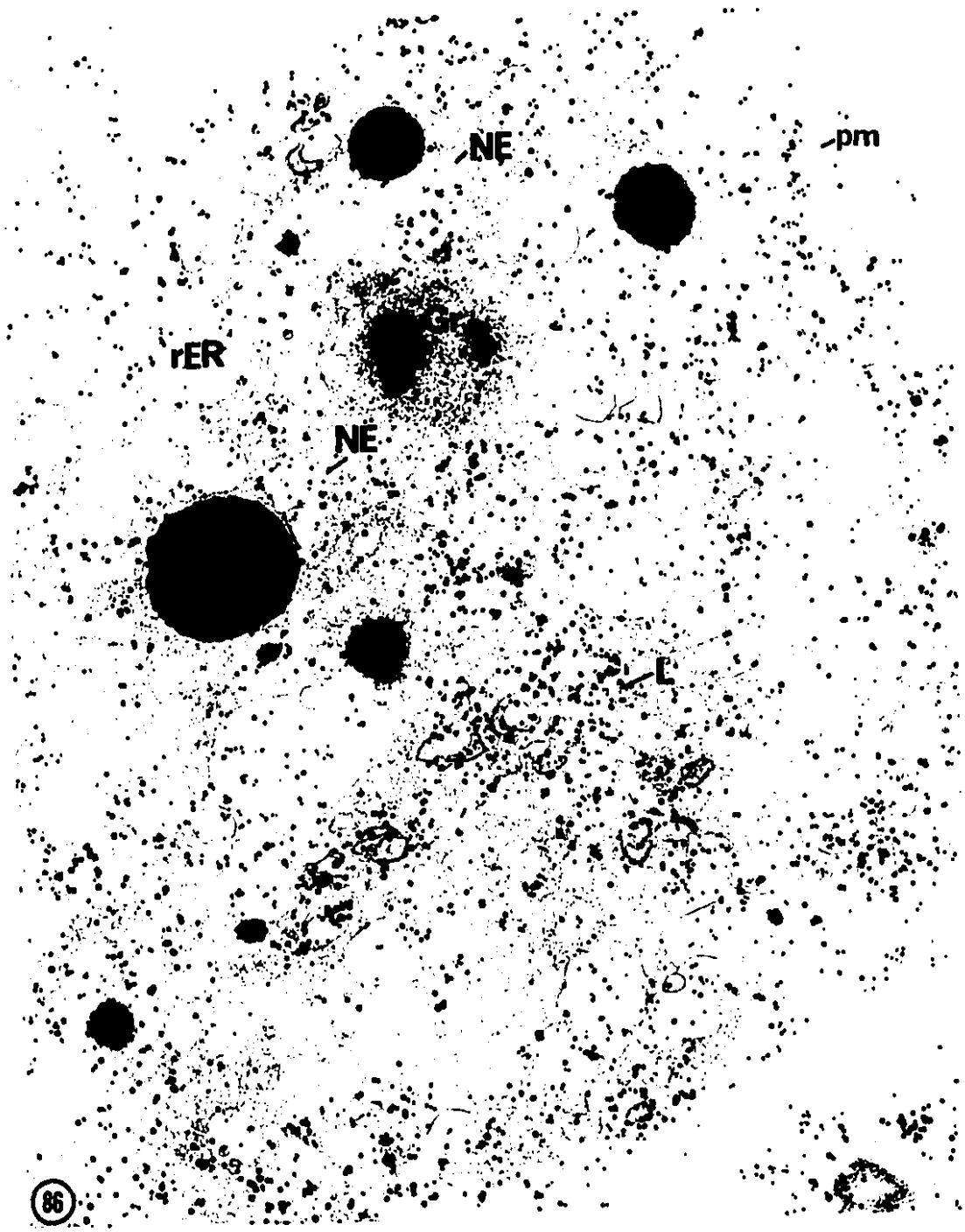

NE
pm
rER
NE
L
86

Fig. 87. EM radioautograph of a myeloma cell in a middle stage of apoptosis, after incubation with ^{3}H-galactose for 18 hr.

The heterochromatin (H) has become stongly marginated against very evaginated regions of the nuclear envelope (NE). In other regions the nuclear envelope has disappeared (arrowhead). More centrally in the nucleus there is lightly staining euchromatin (E) with granular regions (Gr). Some nucleolar material (n) is also present. The heterochromatin exhibits a fairly strong reaction, and many of the grains over this compartment are some distance removed from the nuclear envelope, allowing them to be assigned exclusively to heterochromatin. A substantial number of grains are found over nucleolar material (n), but fewer grains occur over the euchromatin. In the cytoplasm, very heavy reaction covers lysosomes (L) and the plasma membrane (pm), while lighter reaction is seen over the endoplasmic reticulum (rER).

Exposure: 11 mo (Phys. dev.) x 12,000


E
H
rER
NE
E
pm
87

Fig. 88. EM radioautograph of a Friend erythroleukemia cell in a middle stage of apoptosis, after incubation with ^{3}H-galactose for 18 hr.

In this cell, the heterochromatin (H) has strongly marginated to the nuclear periphery. The nuclear envelope appears to have largely disappeared, the cell gives the appearance of extruding its nucleus. A light reaction is observed over the heterochromatin and the euchromatin (E). The nucleolus (n) is unlabeled except for grains at its periphery. The cytoplasm exhibits a fairly heavy reaction, in which many of the grains overlie lysosomes (L) and profiles of rough endoplasmic reticulum (rER).

Exposure: 11 mo (Phys. dev.) x 9,000


E
88

Chapter 4 - Biochemical characterization of nucleoplasmic glycoproteins in cultured myeloma cells

I. Introduction

As described in the introduction to Chapter 3, to determine more conclusively the extent to which the radioautographic reaction over the nucleoplasm of cells exposed to ^{3}H-sugars was indeed due to labeled molecules residing in the nucleoplasm, we needed to fractionate similarly labeled cells and examine the radioautographic reaction over the isolated nuclei as compared to that observed over the nuclei of undisrupted cells. Our findings from the previous chapter that cultured cells exhibited substantial nuclear reactions after the cells were exposed to ^{3}H-sugars has allowed us to use these cells as a model to formally address this issue. This chapter describes experiments in which cultured myeloma cells were exposed to ^{3}H-sugars, fractionated, and then the nuclear labeling of the isolated nuclei was compared to that of the nuclei of undisrupted cells.

Additionally, based on the preliminary indications from the above experiments that at least some of the radioautographic reaction over the cultured cell nuclei was retained in isolated nuclei, we also decided to carry out further radioautographic experiments to try to obtain more information about those labeled intranuclear molecules. Experiments in which stripped nuclei fractions prepared from myeloma cells exposed to ^{3}H-galactose were subfractionated by low and high salt-extraction and then examined radioautographically, are also described in this chapter.

The second basic question left unanswered by our radioautographic studies relates to the specific nature of the labeled nucleoplasmic molecules. An acknowledged limitation of the radioautographic method is that it does not provide a great deal of information in this regard. Certain tentative conclusions could nevertheless be made from past experience and thus it was suggested in Chapter 2 that the labeled molecules were glycoproteins.

It is known that small molecules (e.g. amino acids, sugars, nucleotide sugars) are lost in routine radioautographic processing. Therefore it was highly likely that the label seen in radioautographs resided in macromolecules. It is also possible to selectively remove certain types of molecules through enzymatic treatment of sections prior to radioautographic processing. Thus, when sections of a variety of cell types (which had been exposed to ^{3}H-mannose) were treated with salivary amylase, DNase or RNase, and processed for radioautography, the nucleoplasmic labeling in these cells was unaffected, suggesting that the retained label had not been incorporated into glycogen or into deoxyribose or ribose residues of DNA or RNA (Bennett, unpublished results).

The known specificity of the administered ^{3}H-sugars also gives strong clues as to the fate of the label. A variety of biochemical studies have been carried out which have characterized the labeled molecules after cells were exposed to radiolabeled sugars or sulphate. Many of these studies are

discussed in Appendix 1. To summarize these specificities, ^{3}H-fucose has been shown to be a highly specific precursor for fucose residues of glycoconjugates (glycoproteins and glycolipids), with little catabolism or conversion to other labeled sugars. ^{3}H-galactose and ^{3}H-glcNAc are considered to be fairly good overall precursors for galactose and glcNAc residues, respectively, and in many cases are highly specific for those residues in glycoconjugates, including cultured myeloma cells (Melchers, 1971). ^{3}H-mannose has been shown to be incorporated into mainly mannose residues of glycoconjugates in a number of cell types (although there is sometimes significant conversion to other labeled sugars for this precursor, making RAG results more difficult to interpret than with the other labeled sugars). ^{3}H-manNAc has been shown to be variable in its specificity for sialic acid (especially in cultured cells), but is highly specific for sialic acid residues in numerous cell types. Lastly, ^{35}S-sulphate has been shown to be a very good to excellent precursor for sulphated glycosaminoglycans of proteoglycans.

The fact that in our radioautographic studies the pattern of observed label distribution in myeloma cells was different after administration of each ^{3}H-sugar suggested that there had been no extensive interconversion of the administered sugars. Along the same lines, when a prominent Golgi reaction was observed in a myeloma cell exposed to ^{3}H-fucose, this suggested from past evidence that the label was being

incorporated into glycoproteins in the Golgi apparatus. When the same cell exhibited substantial nucleoplasmic labeling, it could be proposed that, unless the cell metabolized ^{3}H-fucose differently in the nucleus, the label represented glycoproteins at this site as well.

Taken together, all of the factors described above provide clues as to the nature of the labeled molecules in our radioautographs. The data is nonetheless only suggestive, and constitutes no formal proof of the glycoprotein nature of the molecules. To provide this proof it was realized that concurrent biochemical studies were necessary. These studies are described in the present chapter. Included are experiments analysing the labeled molecules of nuclear fractions from ^{3}H-sugar-labeled myeloma cells as well as lectin binding studies on proteins from similar fractions.

II. Materials and Methods

A. Isolation of Myeloma Cell Nuclei and Nuclear Subfractions

Myeloma cells were incubated for 18 hrs with 5 μCi/ml of either ^{3}H-galactose, ^{3}H-fucose or ^{3}H-mannose, and then washed 3 times with ice-cold PBS. The nuclei were isolated from cells by the procedure of Mellon and Bhorjee (1982) with some modifications. The washed cells were incubated on ice for 15 min in hypotonic TECK buffer (10 mM Tris-HCl, 1 mM EDTA, 3 mM $CaCl_2$, 10 mM KCl pH 7.8 at 4°C) with the following protease inhibitors: 0.5 mM phenylmethylsulfanylfluoride (PMSF) (added immediately prior to use from a 0.5 M stock in ethanol), 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml antipain, and 100 units/ml aprotinin (all from Sigma). The swollen cells were then lysed by 10-15 up and down strokes with a tight-fitting (A-type) Dounce homogenizer in 20 volumes of the same buffer. When 95% or greater cell lysis was achieved, as observed by staining of samples with toluidine blue, the cell homogenate was centrifuged at 600 x g for 10 min to produce a pellet of intact nuclei and a supernatant which was discarded. The intact nuclei were washed by resuspension in the same TECK buffer, followed by 2 more up and down strokes with the homogenizer, and centrifugation as above. This washing procedure was repeated twice more, and the pellet was resuspended in TECK buffer. In some cases intact nuclei were then spun through a cushion of 2.0 M sucrose in the same buffer. To remove the nuclear envelope as well as any

remaining cytoplasmic tags, the intact nuclei fraction was treated with detergent by adding Triton X-100 to the TECK buffer to a final concentration of 0.5%. This was followed by a further centrifugation at 600 x g for 10 min to produce a pellet of nuclear envelope-depleted "stripped nuclei". The stripped nuclei fraction was then washed twice in TECK buffer lacking detergent.

In cells exposed to ^{3}H-galactose, further subfractionation of nuclei was carried out using the procedure of Berezney and Coffey (1977) with some modifications. Fractions of stripped nuclei were incubated for 1 hr at 4°C with 250 μg/ml DNAse (from bovine pancreas-grade II, Boehringer Mannheim) in TMP buffer (10 mM Tris, 5 mM $MgCl_2$, 1 mM PMSF, pH 7.4) containing 0.25 M sucrose and then centrifuged at 800 x g for 10 min. In some cases 250 μg/ml RNAse was included with the DNase. The supernatants (DNase or DNase-RNase extracts) were saved for later analyis while the pellets were washed with and finally resuspended in TMP buffer. The nuclei in suspension were then extracted for 20 min with a low salt buffer (0.15 M NaCl in TMP, added drop by drop with vortexing, from a 0.30 M NaCl stock). The suspensions were centrifuged at 800 x g for 10 min to produce a supernatant (low salt extract) which was saved for further analysis and a pellet of low salt-extracted nuclei. Pellets of low salt-extracted nuclei were resuspended in TMP and extracted for 20 min with a high salt buffer (2.0 M NaCl in TMP, added drop by drop with vortexing to the

nuclear suspensions from a 4.0 M NaCl stock), and then centrifuged as above. The supernatants (high salt extract) were again saved for further analysis. This procedure was repeated to produce final pellets of "residual nuclei" representing the nuclear matrix.

B. Assessment of Possible Cytoplasmic Contamination of Nuclear Fractions

The purity of the nuclear fractions obtained from the above methods, in terms of the relative presence or not of cytoplasmic contaminants was assessed by the following two approaches:

I) Morphological Analysis

Each of the fractions (intact nuclei, stripped nuclei, low salt extracted nuclei, and residual nuclei) was examined by electron microscopy to determine the extent to which cytoplasmic tags or nuclear envelope components were present.

II) Mixing Experiments

To test the possibility that some of the nucleoplasmic labeling observed in the stripped nuclei fraction was derived from cytoplasmic molecules which relocated to the nucleoplasm during the initial homogenization step, Mixing Experiment 1 was carried out (Fig. 93): a cytoplasmic fraction from cells incubated with ^{3}H-galactose (C^{hot}) was mixed with an intact nuclei fraction from unincubated cells (IN^{cold}). From this suspension, an intact nuclear fraction was then re-isolated (IN^{mixed}), and treated with detergent to yield a stripped nuclei fraction (SN^{mixed}). Aliquots of these stripped nuclei fractions

were examined radioautographically at the LM level, and compared with the stripped nuclei fraction (SN^{hot}) of the cells actually incubated with ^3H-galactose.

To test the possibility that some of the nucleoplasmic labeling observed in the stripped nuclei was derived from molecules in the cytoplasmic tags or nuclear envelope which relocated to the nucleoplasm during the detergent treatment step of the isolation procedure, Mixing Experiment 2 was carried out (Fig. 94). Briefly, a nuclear envelope detergent extract from cells incubated with ^3H-galactose (DE^{hot}) was mixed with a stripped nuclei fraction from unincubated cells (SN^{cold}). From this suspension, a stripped nuclei fraction was then re-isolated (SN^{mixed}), and compared radioautographically with the stripped nuclei fraction of cells actually incubated with ^3H-galactose (SN^{hot}).

C. Radioautography on Labeled Whole Cells and Nuclear Fractions

Aliquots of the whole cell and nuclear fractions (intact nuclei, stripped nuclei, low salt-extracted nuclei, residual nuclei) from the above experiments were fixed, pelleted, and prepared for LM and/or EM radioautography as described in the Materials and Methods of Chapter 3.

D. Determination of the Percentage of Total Cellular Macromolecular Label in Stripped Nuclei Fractions

Aliquots of whole cell homogenates and stripped nuclei fractions from cells labeled with ^3H-gal, ^3H-fuc or ^3H-man were treated with 10% TCA at 4°C for 30 min, and then centifuged at

10,000 x g for 10 min to separate macromolecules (TCA precipitable) from TCA soluble molecules. The pellets of TCA precipitable material were then solubilized by the addition of 0.5 ml of 0.5 M NaOH and overnight incubation at 70°C. The solubilized pellets were then counted by liquid scintillation, using Optifluor scintillation cocktail (Packard Co.) as the scintillant in a Packard Tri-carb 460 scintillation counter. From a knowledge of the volume of each aliquot relative to the total volume of the fraction, the percentage of the total cellular macromolecular label found in the nuclear fractions could be calculated.

To determine biochemically if any of the label in the nuclear fractions resided in RNA, aliquots of the high salt extract fraction or residual nuclear fraction derived from ^{3}H-gal-labeled cells were first incubated with 250 μg/ml RNase A (Boeringer Mannheim) for 3 hr at 4°C before TCA precipitation. The RNase was heated at 80°C as before for 10 min immediately prior to use to inactivate any contaminating proteases.

E. Determination of Macromolecular Label /μg Protein in Nuclear Fractions

The macromolecular counts per μg protein in the nuclear fractions of myeloma cells after incubation with different ^{3}H-sugars were determined to measure the relative degree of incorporation of these sugars into nucleoplasmic macromolecules, thus providing complementary evidence to that provided by radioautography.

The total amount of protein in each of the nuclear

fractions was determined by the method of Bradford et al. (1976) or Lowry et al. (1951) using BSA as a standard. The macromolecular label was determined as above in aliquots of these fractions containing a known amount of protein (10 μg).

F. Determination of Molecular Weight of Labeled Proteins by SDS-PAGE

To separate labeled proteins according to their molecular weight, nuclear fractions derived from cells radiolabeled with ^{3}H-sugars were subjected to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) using the procedure of Laemmli (1971). Briefly, samples of the labeled nuclear fractions were sonicated for 90 seconds with a Bronwill Biosonik IV probe sonicator (VWR Scientific) as described by Glass et al. (1981) in sample preparation buffer containing 2% SDS, 0.0125 M Tris-HCl (pH 6.8), 22.2% glycerol, 0.001% bromophenol blue and 5% 2-mercaptoethanol, and boiled for 5 minutes. The samples were run on either 8% slab gels or 5-15% gradient polyacrylamide gels, for 12 hrs at a constant 100 volts or for 5 hrs at 30 mA constant current. The gels were stained with Coomassie blue dye to visualize the protein bands.

Some gels of stripped nuclei fractions from cells labeled with ^{3}H-sugars were then processed for fluorography. Briefly, gels were impregnated with EN^{3}HANCE (Dupont Chemicals), dried on 3 mm Whatman filter paper, placed in a film cassette (Picker Instruments) against Hyperfilm (Amersham), and exposed from 1-5 months at -70°C.

Other gels were cut into 2 mm slices which were then placed in scintillation tubes. These slices were solubilized with a small amount of 30% H_2O_2, incubated overnight at 70°C, and then counted using liquid scintillation as described above.

The radiactivity in gels of nuclear fractions derived from further subfractionating stripped nuclei of ^3H-gal-labeled myeloma cells was assessed by liquid scintillation counting only.

G. Identification of Sugar Residues in Glycoproteins in SDS Gels by Lectin Blotting

Proteins of nuclear fractions were separated by SDS-PAGE as described above and transferred to nitrocelluose membranes using the protocol of Towbin (1983) with slight modifications. Briefly, the gels were placed in a protein transfer buffer which contained 25 mM Tris and 0.192 M glycine (without methanol)(pH 8.3), and were gently agitated for one hr with two changes of buffer. The proteins in the gel were then transferred onto nitrocellulose membranes using a GENIE electrophoretic blotter (Idea Scientific Company). The time of transfer used was 2½-3 hrs at 400 mA, with an average voltage of 14-15 V.

The nitrocellulose "blots" were then stained with Ponceau S dye (Sigma Chemical Co.) to visually assess the quality of the protein transfer. The blots were subsequently rinsed briefly in 50 mM Tris-buffered saline (TBS) pH 7.4 to remove the dye and then probed with lectins using either of two

protocols:

Some blots were incubated in a blocking step for 1 hr in 50 mM TBS (pH 7.4) containing either 1% deglycosylated BSA (Glass et al., 1981 from BSA Fraction V, Sigma) or 2% polyvinylpyrrolidone (Bartles et al., 1985) in TTBS (TBS with 0.1% Tween 20 detergent, Sigma). This was followed by a 30 min incubation with 5 μg/ml of biotinylated (RCA I) (Sigma) in TTBS. After three washes (10 min each) in the same buffer, the blots were then incubated for 30 min in a 1 μg/ml solution of Extravidin-peroxidase (Sigma) in TTBS. Following 3 more washes (for 10 min each) in TTBS and one final wash in TBS without Tween, the blots were incubated for 20 min in the dark with 100 mM Tris pH 7.4 containing 0.06% 4-chloronapthol (Sigma) and 0.02% H_2O_2, washed in distilled water, and dried between two sheets of Whatman filter paper.

For some blots of nuclear matrix proteins, lectin-digoxigenin-antidigoxigenin-alkaline phosphatase (AP) conjugates (Boehringer Mannhein) were used. Blots were blocked for 1 hr with 0.5% of the supplied blocking agent in TBS (pH 7.4) containing 0.1% Tween 20 (TTBS). This was followed by 2 washes for 10 min each in the same buffer and then by incubations for 1 hr with different lectin-digoxigenin complexes. To identify terminal galactose residues, 5 μg/ml RCA I or 10 μg/ml PNA lectins were used. For terminal sialic acid residues 10 μg/ml MAA lectin was used, while for fucose residues 10 μg/ml AAA lectin was used. All lectin incubations

were done in TTBS, and followed by 3x10 min washes with the same buffer. The blots were then incubated with antidigoxigenin-AP (750 U/ml) for one hr in TTBS, washed 3 times in the same buffer, and finally colorimetrically developed using 0.01% nitroblue tetrazolium (NBT) and 0.005% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 50 mM glycinate at pH 9.5.

To confirm the specificity of the lectin binding, companion control blots were included with all experiments. 0.2 M B-D-galactose was added to RCA I and PNA, 200 μM fetuin was added to MAA and 0.2 M fucose was added to AAA lectin. Lactosylated-bovine serum albumin (Lac-BSA) was included as a positive control on some blots incubated with RCA I.

To probe for the presence of N-glycosidic linkages of sugar to protein in the glycoproteins of the nuclear matrix fraction, samples from that fraction were treated with N-glycanase F before being electrophoretically separated on gels and later probed with RCA I-digoxigen conjugates as described above. Briefly, 25 μg samples of nuclear matrix fractions were incubated in 50 mM Tris-HCl buffer pH 7.4 containing 0.1 units of N-glycanase F (Sigma) at 37°C for 2 hrs. 1 mM PMSF was included as a protease inhibitor. Companion samples were incubated under the same conditions without the enzyme. As a positive control, 1 μg samples of fetuin (a glycoprotein known to have N-linked sugars) were incubated under the same conditions as above and included on the blots with the samples of nuclear matrix proteins later probed with RCA I.

III. Results

A. Radioautographic Studies on Isolated Nuclear Fractions

I) Isolated Intact and Stripped (envelope-depleted) Nuclei

A) ^{3}H-Galactose Experiments

Isolated intact nuclei derived from myeloma cells which had been exposed to ^{3}H-galactose (Fig. 90) exhibited an radioautographic reaction of nearly the same magnitude as that seen over the nuclei of the whole cells (Fig. 89). The silver grains were distributed over the nuclear envelope, heterochromatin, euchromatin, and the nucleolus in a manner similar to that observed in the whole cells.

Similarly, isolated stripped nuclei (Figs. 91+92), whose nuclear membranes had been removed by detergent, exhibited a radioautographic reaction which was nearly as intense as that seen over the isolated intact nuclei. The distribution of label was also similar to that of the isolated intact nuclei.

In some fields, recognizeable isolated chromosomes were observed amongst the isolated nuclei, and these were also labeled, with the radioautographic grains localized mostly to their edges (Fig. 92).

In the first set of mixing experiments (to assess the extent of contribution of relocated cytoplasmic label to the reaction observed over isolated stripped nuclei, Fig. 93), the radioautographic reaction observed over the nuclei of the stripped "mixed" nuclei fraction (SN^{mixed})(Fig. 95) was insignificant compared with that observed over the stripped

nuclei of cells actually incubated with ^{3}H-galactose (SN^{hot})(Fig. 96).

In the second set of mixing experiments (to assess the extent of contribution of relocated label of the detergent-solubilized nuclear envelope to the RAG reaction over the stripped nuclei, Fig. 94), the reaction over nuclei of the "mixed" stripped nuclei fraction (SN^{mixed})(Fig. 97) was again insignificant compared with that over the stripped nuclei fraction of cells actually incubated with ^{3}H-galactose (SN^{hot})(Fig. 98). In both mixing experiments, the macromolecular label contained in the SN^{mixed} and SN^{hot} fractions was also measured by scintillation counting of TCA precipitable label. (These results are presented in the Biochemical Results section below).

B)^{3}H-Fucose Experiments

Radioautographic studies on isolated stripped nuclei fractions from myeloma cells incubated with ^{3}H-fucose were carried out only at the light microscope level, since the amount of incorporation of this sugar was considerably less than that seen with ^{3}H-galactose. Nonetheless it could be readily be observed that the stripped nuclei (Fig. 100) exhibited a reaction almost equal in intensity to that seen over the nucleus of the whole cells (Fig. 99). As in the whole cells, the silver grains were observed over the periphery of the nucleus as well as over internal regions including the nucleoli.

C) ^{3}H-Mannose Experiments

As in the case of the ^{3}H-fucose experiments, radioautographic studies on isolated stripped nuclei fractions of myeloma cells incubated with ^{3}H-mannose were carried out only at the light microscope level due to the relatively low level of incorporation of this sugar. The stripped nuclei (Fig. 102) exhibited a somewhat lighter reaction than that observed with ^{3}H-fucose (Fig. 100), but the reaction was equal in intensity to that seen over the nucleus of the whole cells incubated with ^{3}H-mannose (Fig. 101). As in the whole cells, the silver grains were largely localized to the nuclear periphery although some were observed over nucleoli (Fig. 102).

II) Radioautographic Studies on Salt-extracted Nuclei

A) Low Salt-extracted Nuclei

When stripped nuclei fractions were treated with DNase and extracted with 0.15 M NaCl, the nuclei became more condensed and showed some signs of fragmentation. These nuclei appeared to contain mainly heterochromatin and nucleoli, with little visable euchromatin. However, the nuclei continued to be heavily labeled, with grains over both the peripheral and central regions of the nucleus including nucleoli (Fig. 105).

B) High Salt-extracted Residual Nuclei (Nuclear Matrix)

When the above fraction was further extracted with 2.0 M NaCl, even more fragmentation of nuclei was observed. The undisrupted nuclei appeared to have lost a substantial portion of their internal content although residual nucleoli could

still be observed. Nonetheless, these structures were often highly labeled with radioautographic grains over both peripheral and internal areas. Internally, the grains appeared to be over both the filamentous elements of the internal matrix and the residual nucleoli (Fig. 106). Grains were also observed over profiles of circular laminae which were seemingly devoid of content (Fig. 107).

B. Biochemical Studies on Nuclear Fractions

I) Macromolecular Label in Nuclear Fractions of Myeloma Cells exposed to ^{3}H-Sugars

A) Stripped Nuclei

The results of experiments to determine the percentage of the total cellular macromolecular label present in stripped nuclei of myeloma cells labeled with ^{3}H-galactose, ^{3}H-fucose, or ^{3}H-mannose are shown in Table 5. In myeloma cells incubated with ^{3}H-galactose, an average of 10.9% of the total cellular TCA precipitable counts were present in stripped nuclei fractions. No significant difference in this value was found when nuclei were spun through a 2.0 M cushion before removal of their nuclear membranes.

The stripped nuclei fraction isolated from cells incubated with ^{3}H-fucose was found to contain an average of 5.9% of the total celluar TCA-precipitable counts. The same fraction derived from myeloma cells incubated with ^{3}H-mannose was found to contain only 2.9% of the total cellular TCA-precipitable counts.

Determinations of the macromolecular counts per μg protein

in the stripped nuclei fractions of myeloma cells exposed to different ^{3}H-sugars are also shown in Table 5. It was found that stripped nuclei from cells incubated with ^{3}H-galactose contained an average of 7,200 dpm/10 μg protein, based on three experiments. Nuclei of cells exposed to ^{3}H-fucose had an average of 2,600 dpm/10 μg protein based on two experiments, while those from cells exposed to ^{3}H-mannose had only 1,100 dpm/10 μg protein, also based on two experiments.

Macromolecular counts per μg protein were also determined in the different nuclear fractions of the mixing experiments (in companion experiments to the RAG studies described earlier in the RAG results section above). In both types of mixing experiments (see Figs. 93 and 94), TCA precipitation of protein samples from the various fractions showed that the SN^{mixed} (whose label would have been derived from non-nucleoplasmic sources of radioactivity) never contained more than 4% of the radioactivity of the SN^{hot} based on the results of three experiments.

B) Salt-extracted Nuclei

Low salt extracts derived from treating the stripped nuclei of ^{3}H-gal-labeled myeloma cells with 0.15 M NaCl were found to contain an average of 3,100 dpm/10 μg protein (Table 5). The high salt extracts (after treatment of the nuclei with 2.0 M NaCl) contained 5,700 dpm/10 μg protein. The residual nuclei fraction was found to contain the highest average amount of radioactivity of all the nuclear fractions at 12,500

dpm/10 μg protein.

Extracts derived from the treatment of stripped nuclei of ^{3}H-galactose-labeled myeloma cells with DNase and/or RNase before salt extraction were found to contain less than 5% of the total TCA precipitable counts of the stripped nuclei.

Samples of the high salt extract incubated for three hours at 4°C with 250 μg/ml RNase A, and then subjected to TCA precipitation, had an average of 88% of the precipitable counts of untreated high salt extracts, with the other 12% of the counts of the RNase-treated samples shifting to the TCA soluble fraction. When samples of the residual nuclei fraction were incubated under the same conditions, the TCA-precipitable counts in the RNase-treated samples had an average of 83% of the counts of untreated samples, with the other 17% of the counts of the treated samples shifting to the TCA soluble fraction.

III) Determination of Molecular Weight of Labeled Proteins by SDS-PAGE

A) ^{3}H-Galactose Experiments

In fluorographs of gels containing proteins from the stripped nuclei fraction of myeloma cells incubated with ^{3}H-galactose, a number of radioactive bands were observed at one month exposure (Fig. 103-panel 3). A band in the high molecular weight region corresponded to a protein with a molecular weight of ~75 kd, while other prominent bands were observed in the very low molecular protein range close to the migrating front of the gel. A third faint radioactive band in

the range of 180 kd was usually, but not always observed.

Liquid scintillation counting of the 2 mm gel slices from lanes of nuclear fractions of ^{3}H-gal-labeled cells also indicated the presence of labeled proteins in the stripped nuclei fraction (Fig. 104a). As expected, a peak of radioactivity was observed at 75 kd, corresponding to the radioactive band of the same approximate molecular weight on fluorographs. Small peaks at 180 kd and a number of other molecular weights between 30-100 kd were also observed. The latter small peaks were not detected on the fluorographs at the 1 month exposure time. A fairly large peak was observed in the very low molecular weight region as well, seeming to correspond to the labeled bands in the fluorograph of very low molecular weight.

In low salt nuclear extracts, liquid scintillation counting of radioactivity in gel slices revealed a peak at ~75 kd, but it was much smaller that of the stripped nuclei fraction (Fig. 108b). This fraction contained few other easily identifiable peaks of radioactivity with the exception of a distinct peak at very low molecular weight in the same position as the low molecular weight peak in stripped nuclei fractions (Fig. 104a).

The high salt nuclear extract showed no distinct peak of radioactivity in gel slices in the vicinity of 75 kd but there was a moderate peak of radioactivity observed at ~95 kd (Fig. 104c). A very large peak was again observed at very low

molecular weight while a small amount of radioactivity was observed to be present in very small peaks at a variety of molecular weights between 30-100 kd.

In the residual nuclei fraction (Fig. 108d), on the other hand, there was a very prominent radioactive peak at the ~75 kd molecular weight which contained approximately twice the label observed in the stripped nuclei fraction. Other noticeable peaks of radioactivity were routinely observed between 30-100 kd, most noteably at 28-30 kd, 48-50 kd and 62-66 kd. The largest peak of the lane by far was again at very low molecular weight.

B) ^{3}H-Fucose Experiments

In fluorographs of gels containing proteins of stripped nuclei fractions from ^{3}H-fucose-labeled myeloma cells, only one radioactive band was observed at an exposure of 3 months which corresponded to a protein of ~75 kd molecular weight, as was observed with ^{3}H-galactose-labeled fractions.

Liquid scintillation counting of the 2 mm gel slices of the stripped nuclei fraction (Fig. 104b) showed a small but distinct peak in the molecular weight region near 75 kd. Other peaks were detected which did not appear on fluorographs including a fairly large peak in the very low molecular weight region, and a few scattered small peaks, most noteably at ~97 kd, and ~180 kd.

C) ^{3}H-Mannose Experiments

Fluorographs of gels exposed for as long as 5 months

exhibited only one radioactive band in lanes of stripped nuclei fraction proteins from cells labeled with ^{3}H-mannose (Fig. 103-panel 1). Once again, this band corresponded to a protein of ~75 kd molecular weight, as had been observed with both ^{3}H-galactose and ^{3}H-fucose.

Liquid scintillation counting of the 2 mm gel slices of the stripped nuclei fraction (Fig. 104c) showed a small peak in the molecular weight region near 75 kd. A somewhat larger peak in the very low molecular weight region was detected which surprisingly did not appear as a band on fluorographs. A number of very small peaks just above the level of background radioactivity were also seen but these were also not detected on fluorographs.

IV) Lectin Blotting Experiments

When nitrocellulose blots of separated stripped nuclei proteins were probed with biotinylated RCA I conjugates (RCA-biotin + extravidin-peroxidase), a number of bands were observed including a single band at ~75 kd, a pair of bands migrating at 67-68 kd and a lighter single band ~53 kd (Lane 3, Fig. 109). Lactosyl-BSA neoglycoprotein, which was run as a positive control for RCA I, showed a intense signal at ~80 kd, confirming the specificity of the probe (Lane 2). In negative control preparations (Lanes 4+5), it was observed that addition of 0.2 M galactose to the RCA I before incubation completely abolished the reaction.

Nitrocellulose blots of separated nuclear matrix (residual

nuclei) fraction proteins also showed a number of lightly reactive bands when probed with biotinylated RCA I. These bands were at molecular weights of approximately 48 kd, 60 kd, 67 kd, 75 kd, 92 kd and 120 kd (Lane 3 of Fig 110). The specificity of the probe was again confirmed by a strong labeling of the lactosylated-BSA protein of ~80 kd (Lane 2). As above, the addition of 0.2 M galactose to the RCA I probe prior to incubation almost completely abolished the reaction (Lanes 4+5).

Proteins from these nuclear matrix fractions probed with RCA I-digoxigenin conjugates (i.e lectin-digoxigenin + anti-digoxigenin-AP) showed labeled bands more or less similar to the above (in Fig 110) but the reaction was, on the whole, stronger than that above (lane 6 of Fig. 111a). A strongly reactive band was observed at a molecular weight of ~68 kd while there appeared to be a pair of bands between 72-76 kd, a single band at ~80 kd, and some diffuse reactivity between 94-98 kd, and just over 100 kd. At lower molecular weight, a band was also observed at 48 kd. Prior treatment of the samples with N-glycanase enzyme (to remove N-linked oligosaccharide side chains from proteins) (lane 5) resulted in the disappearance of the bands between 72-76 kd, the band at 80 kd and the reactivity at 94-98 kd and over 100 kd. The band at 68 kd, on the other hand, was completely unaltered in its intensity, as was the light band at 48 kd.

In positive controls for the N-glycanase F reaction,

untreated asialofetuin exhibited a strong but somewhat diffuse RCA I-reactive band at ~60 kd (lane 1). After N-glycanase F treatment the RCA I binding of this protein was drastically reduced (Lane 2), which was the expected finding if the enzyme was indeed active and the N-linked oligosaccharides of the protein were cleaved. As a negative control for all the RCA I labeling, the addition of 0.2 M galactose to the RCA I probe prior to incubation completely abolished the reaction (Lanes 3+4, 7+8).

When samples from the residual nuclei fractions on nitrocellulose blots were probed with digoxigenin-labeled MAA lectin (specific for sialic acid), a number of reactive bands were observed. These had molecular weights similar to those observed with RCA I, including a cluster of distinct bands between 72-76 kd, as well as a light band at ~48 (lane 1 of Fig. 111b). On the other hand, the prominent RCA I-reactive band at 68 kd (lane 5 of Fig. 111a) did not appear to be labeled with MAA. In negative controls, when 200 μM fetuin was added to the MAA prior to incubation, the reaction was abolished, indicating the specificity of the lectin for sialic acid (lane 2 of Fig. 111b).

The reaction observed on blots of residual nuclei preparations probed with the fucose-specific AAA-digoxigenin was quite strong, where there appeared to be a number of reactive bands (Fig. 112a). These were detectable at ~63-65 kd, 75 kd, 80 kd, 92 kd, 95 kd, 105 kd, and 120 kd. In this

preparation, as in some others lectin blots carried out, there was considerable streaking of separated proteins where reactivity could be observed between bands. However, control blots where 0.2 M fucose was included in the incubation with AAA were completely devoid of reaction indicating that reaction was specific.

The reaction observed after identical blots of residual nuclei preparations were probed with PNA was much less intense as that seen with RCA I or AAA lectins. Single reactive bands were observeable at ~48 kd and ~125 kd, as was a doublet at 62-64 kd. Little if any PNA binding was observed in the region between 70-90 kd where the other lectins showed the greatest amount of binding. Controls blots where 0.2 M D-galactose was included in the incubation were again almost totally devoid of reactivity.

IV. Discussion

A. Localisation of Labeled Molecules Responsible for Nucleoplasmic Radioautographic Reactions

I) Observations with ^{3}H-galactose

In the present study, EM radioautographic results showed that the RAG reaction appearing over nuclei of undisrupted myeloma cells labeled with ^{3}H-galactose was also observed over nuclei isolated from these cells. The reaction in this latter case was largely unaltered both in its intensity and pattern of grain distribution from that seen in the undisrupted cell (i.e. both internal and peripheral labeling of the nucleus was observed).

The internal labeling was predicted since previous analysis of the distribution of grains in RAG reactions over nuclei of Schwann cells had strongly suggested that at least some of the reaction over internal regions must be of nucleoplasmic origin (Bennett et al., 1986).

In this earlier work, it had been harder to determine which cellular compartments might contain the sources of label causing reaction over the periphery of the nuclei. Such compartments could include the peripheral nucleoplasm (such as the nuclear pore complexes), the membranes of the nuclear envelope, and the adjacent cytoplasm. The results of the present study suggest that the latter compartment can be eliminated as a significant source of this labeling since peripheral reaction observed over the isolated intact nuclei (in the absence of substantial cytoplasmic tags) was almost as

intense as that seen in the nuclei of undisrupted cells.

When the isolated intact nuclei had the membranous components of their nuclear envelope removed by detergent treatment, the RAG reaction over the resulting stripped nuclei was similar in intensity and grain distribution to that seen over intact nuclei and the nuclei of undisrupted whole cells. Companion biochemical studies revealed considerable amounts of labeled macromolecules (~11% of the total cellular TCA-precipitable label) in the fraction of stripped nuclei isolated from cells incubated with ^{3}H-gal, and smaller amounts in the case of cells incubated with ^{3}H-fuc (5.9%) and ^{3}H-mannose (2.9%). Since EM examination shows that the membranes of the nuclear envelope are almost completely removed from these nuclei, this suggests that most of the peripheral RAG reaction is due to labeled molecules in the peripheral nucleoplasm. Occasional grains were seen over flocculent material outside of the nuclei (possibly remnants of the nuclear envelope) but this material was not highly labeled.

These above conclusions assume that no significant contamination of fractions occurs during the isolation procedures. It could be argued that some of the nucleoplasmic labeling observed in the intact nuclei or even stripped nuclei fractions of the above experiments was derived from cytoplasmic molecules which relocated to the nuclei during the initial homogenization step. For example, Staufenbiel and Deppert (1983) reported that nuclear fractions from cultured

cells were frequently contaminated with cytoskeletal elements resulting from the initial steps of their nuclear isolation procedure. While there are few reports of glycosylated cytosketeletal elements, there is thus the possibility that labeled molecules may be coming from other cytoplasmic sources in the same way. In our study, this possibility was examined in Mixing Experiment 1 (see Fig. 93) in which a cytoplasmic fraction (C^{hot}) from cells incubated with ^{3}H-galactose was mixed with an intact nuclear fraction from unincubated cells. When nuclei from this mixture were re-isolated and stripped of their nuclear envelope, the RAG reaction over the stripped nuclear fraction (SN^{mixed}) was insignificant compared with that observed over the stripped nuclei of the cells actually incubated with ^{3}H-galactose (SN^{hot}). In companion biochemical studies, samples of the above "mixed" and "hot" stripped nuclei were compared in terms of TCA-precipitable label, and it was found that the radioactivity in the "mixed" sample was never greater than 4% of that of the "hot". These results strongly suggest that labeled cytoplasmic molecules made no significant contribution to the RAG reaction observed over the nucleoplasm of intact cells incubated with ^{3}H-galactose.

It was also possible that some of the label over the nucleoplasm of stripped nuclei might have been derived from the membranes of the nuclear envelope. One would not expect this to be a major source since in the RAG studies of Chapter 3, less than 7% of the nuclear label was assigned to the

nuclear envelope. Nonetheless, some labeled nuclear membrane molecules could have translocated to the nucleoplasm after nuclear membranes were solubilized in the detergent step. Smith et al. (1985), for example, provided evidence that Con A-binding glycoproteins of nuclear membranes of rat liver cells were present in stripped nuclei fractions and retained in the nuclei through subsequent extraction steps such that they were still found in nuclear matrix fractions. The possibility of similar contamination of our stripped nuclei fractions from nuclear membranes was examined in Mixing Experiment 2 (Fig. 94), in which a detergent extract of the nuclear membranes of ^{3}H-gal-labeled cells (DE^{hot}) was mixed with a preparation of nuclei already stripped of their nuclear membranes from unlabeled cells (SN^{cold}). When stripped nuclei from this mixture were re-isolated, the RAG reaction over these nuclei (SN^{mixed}) was again insignificant compared with that observed over the stripped nuclei of the cells incubated with ^{3}H-galactose (SN^{hot}). In concurrent biochemical studies, samples of the above "mixed" and "hot" stripped nuclei were compared in terms of TCA-precipitable label, and it was found that the radioactivity in the "mixed" sample was only a tiny fraction of the "hot". The above results suggest that labeled molecules in the nuclear membranes made no significant contribution to the radioautographic reaction observed over the nucleoplasm of whole cells incubated with ^{3}H-galactose.

As described in the results, labeled isolated chromosomes

were observed amongst the stripped nuclei. These observations further suggest that the RAG reaction seen over chromosomes of undisrupted mitotic cells was indeed due to labeled molecules associated with the chromosomes and not from moieties of the adjacent cytoplasm, since the cytoplasm is lacking in the stripped nuclei preparations.

EM radioautography carried out on aliquots of nuclei after low salt and high salt extractions indicated that the nuclear labeling initially observed over the isolated stripped nuclei of cells exposed to ^{3}H-galactose appeared to be largely retained in the residual salt-resistant nuclear structures representing the nuclear matrix. In agreement with these results, later TCA precipitation studies indicated an enrichment of labeled macromolecules in nuclear matrix fractions relative to the stripped nuclei fractions (Table 5). Some of the structures in the nuclear matrix fraction resembled the nuclear matrices described by Berezney (1991), which contained an internal scaffold-like component surrounded by nuclear laminae and pore complexes. Other structures resembled the "nuclear ghosts" described by Kaufmann et al. (1981) in some of their nuclear matrix preparations, in which the nuclear lamina was present but the internal component was lacking.

Some controversy has existed in the past as to what formally constitutes the nuclear matrix. While a great variety of functional properties have been attributed to this

compartment operationally from work done on biochemical "nuclear matrix" fractions (summarized in Berezney, 1991), there has been considerable debate as to whether or not the nuclear matrix comprises a bona fide scaffold-like nuclear substructure which is integral to determining the three-dimensional organization of the nucleus (see Nigg et al., 1988; Berezney, 1991). Recent evidence indicates that such an internal nuclear component almost certainly does exist in some form and that there are a unique set of proteins ascribable to it which are distinct from the lamin proteins of the nuclear matrix periphery. These proteins have been collectively termed the "matrins" (Nakayasu and Berezney, 1991).

The confusion regarding the presence or absence of an internal nuclear matrix component would appear to be at least partly due to the fact that the integrity of this portion of the matrix appears to be highly sensitive to the use of RNase and sulfhydryl reducing agents which have been used in some isolation procedures (Kaufmann et al., 1981). When these agents are used, the internal matrices are totally disrupted and the extracted residual nuclei take on the appearance of roughly circular profiles of almost entirely empty laminae and pore complexes referred to the "nuclear ghosts". In our isolation procedure, while we did not include RNase or reducing agents, structures resembling the "nuclear ghosts" were observed. Disruption of the internal matrices in these instances might have been due to mechanical stress resulting

from the repeated pelleting and resuspending of the extracted nuclei. Devoid of much of their content, including almost all DNA, it is likely these structures are very sensitive to mechanical stress.

Both "nuclear ghosts" and internal nuclear matrices were labeled in our study, suggesting that some of the labeled molecules are constituents of an internal RNA-protein nuclear matrix while others are associated with the peripheral lamina and nuclear pore complexes.

II) Observations with ^{3}H-fucose and ^{3}H-mannose

Radioautographic studies of isolated nuclear fractions of myeloma cells exposed to ^{3}H-fucose or ^{3}H-mannose were carried out only at the light microscope level since the observed labeling was considerably less than that seen after exposure to ^{3}H-galactose. Nonetheless, it could be clearly seen that stripped nuclei isolated from myeloma cells which had been incubated with ^{3}H-fucose (Fig. 100) or ^{3}H-mannose (Fig.102) exhibited an reaction nearly equal in intensity to that seen over the nucleus of the undisrupted cells (Figs. 99 and 101 respectively). As in the case of ^{3}H-galactose, these results suggest that the nuclear reaction observed over whole cells exposed to the above sugars is due to labeled molecules residing in the nucleoplasm itself. This was true even in cells exposed to ^{3}H-mannose where most of the reaction over the isolated stripped nuclei was peripheral.

It may be noted that, in myeloma cells exposed to any of

the ^{3}H-sugars, the percent of total cellular macromolecular label in the nucleoplasmic compartment determined by the TCA precipitation experiments was appreciably lower than was indicated by the radioautographic studies of Chapter 3 (based on the notion that all the silver grains observed in the cytoplasm and nuclei of radioautographs represented labeled macromolecules). For ^{3}H-galactose, the amount of nucleoplasmic label present according to biochemical studies was 11%, versus 18% determined by quantitative EM radioautography. In cells exposed to ^{3}H-fucose, the respective figures were 5.9% vs 11.9%, and for ^{3}H-mannose 3.0% vs 11%. The reasons for the differences are not obvious but may relate to the fact that when nuclei are isolated in a low viscosity aqueous medium (as in most nuclear isolation procedures including this one), there may be considerable loss of diffusible proteins from the nucleoplasm to the cytoplasm. Paine et al. (1983), for example, have reported that when nuclei were micro-dissected from frozen frog oocytes and placed in such an aqueous medium, up to 95% of the nucleoplasmic proteins diffused into the medium within 4 minutes. DNA polymerase α, for example, is routinely recovered mostly from cytosolic fractions after cell disruption, even though immunohistochemical studies carried out on frozen sections indicate that it is localized to the nucleoplasm in intact cells (Bensch et al., 1982). Paine et al. (1983) conclude that "any proteins which remain in the nucleus following aqueous isolation are likely to be part of

the nuclear matrix or other structural elements, or tightly associated with chromosomes". In our studies, it is possible that while both the biochemical and RAG techniques detected the labeled molecules of "the nuclear matrix or other structural elements", only the RAG technique additionally detected the diffusible labeled nuclear proteins in the undisrupted cells which were selectively retained in the nuclei because of the fixation procedure with glutaraldehyde.

Another possible factor contributing to differences in nucleoplasmic labeling between the biochemical and radioautographic methods is that during the nuclear isolation procedure there is always lysis of some nuclei. Some of the resulting nuclear fragments containing label may not pellet with undisrupted nuclei and might end up in the cytoplasmic fraction.

B. Biochemical Characterization of the Labeled Molecules

I) Macromolecular Nature of the Molecules

As earlier described, TCA precipitation experiments carried out as a first step in the characterization of labeled nucleoplasmic molecules revealed considerable amounts of labeled macromolecules in stripped nuclei isolated from cells exposed to ^{3}H-gal, and smaller amounts in the case of cells exposed ^{3}H-fuc and ^{3}H-man.

It should be noted that in interpreting our radioautographic and biochemical data, the possibility must be considered that administered free ^{3}H-sugars could have bound to endogenous nuclear lectins in cells, accounting for some of

the nucleoplasmic label. Such endogenous lectins were discussed in Chapter 1, and include such molecules as the galactose-specific nuclear lectin CBP 35 (Jia and Wang, 1983; Moutsatsos et al., 1986,1987; Liang and Wang, 1988). However, there is no evidence in the literature that administered free monosaccharides bind to these endogenous lectins <u>in vivo</u>, especially since their affinity for free monosaccharides is usually much lower than for more complex oligosaccharides (see Wang et al. 1992). Furthermore, even if binding of a free sugar were to occur, there is no evidence that such a sugar would remain bound to the lectin through the steps of histological or biochemical processing used in our studies. One might expect that the conditions resulting from the use of TCA would cause dissociation of a sugar from a lectin, either because of the effects of the precipitation reaction on the conformational structure of the lectin, or the effects of the drop in pH on the ability of the lectin to bind sugar.

Our previous experiments on frog Schwann cells exposed to ^{3}H-fucose (Bennett et al., 1986), provided three types of evidence that the nuclear labeling observed in radioautographs was not due to lectin binding of the free ^{3}H-sugar. The labeling was found to be inhibited by prolonged exposure of the cells to cycloheximide, indicating that it had been metabolically incorporated. Furthermore, when sections of Lowicryl K4M-embedded cells were exposed to free ^{3}H-fucose and processed for radioautography, no significant reaction was

observed. Finally, the distribution of nuclear labeling at later time intervals after administration of the ^{3}H-sugar was different from that seen at earlier intervals. This would be an unexpected finding if the label were simply bound to a nuclear lectin, whose distribution should bear no relationship to the time of precursor administration.

II) Biochemical Characterization of Individual Proteins

Having established that the nuclear label is macromolecular in form, such label could theoretically reside in lipids, DNA, RNA or proteins. Glycolipids are traditionally thought to occur in membranes rather than in the cytosol or nucleoplasm. Phospholipids do exist in the nucleoplasm (Cocco et al., 1980; Maraldi et al., 1992) but there are no reports of their being glycosylated.

It is unlikely that significant label resided in RNA and/or DNA for three reasons: 1) DNase-RNase extracts derived from enzyme treatment of stripped nuclei fractions (from cells exposed to ^{3}H-gal) contained only a very small portion of the total nuclear label (< 5%). Similarly, only a small amount of label of RNase-treated high salt nuclear extracts or nuclear matrix preparations (derived from similarly labeled cells) was shifted from the TCA precipitable to the TCA soluble portions of the samples. The label that was shifted in these latter cases could represent very small labeled peptides (too small to be TCA precipitated) which were associated with RNA and subsequently liberated by the RNase treatment. 2) Extraction

of DNA did not appreciably affect the RAG reaction in low and high salt extracted nuclei. Similarly, LM radioautographs of sections of RNase-treated, stripped nuclei from ^{3}H-gal-labeled cells showed little decrease in reaction over nuclei as compared with that observed over untreated sections (figure not shown). 3) As described in Appendix 1, Melchers (1970) indicated in his study on a different myeloma cell line that very little (< 10%) of the administered ^{3}H-galactose that was incorporated into the cells was converted to other sugars, which would include ribose of DNA and RNA.

The above results thus suggest that the macromolecular label observed in our preparations does not reside in lipids or nucleic acids, but rather exists in sugar residues of glycoprotein molecules.

When stripped nuclei preparations from cells exposed to ^{3}H-gal were treated with SDS and run on polyacrylamide gels, gel slice data revealed several radioactive peaks, indicating the presence of labeled proteins with different molecular weights. Some of these peaks were sufficiently radioactive to show up as bands on fluorographic films (a less sensitive detection technique for radioactive molecules). A detected radioactive band of relatively high molecular weight was consistently observed at ~75 kd. There was also sometimes a band of ~180 kd detected. Two heavy bands were routinely seen at very low molecular weights which could represent degradation products. However, in the work of Kelly and Hart

(1989), several radioactive bands of low molecular weight representing O-linked glcNAc glycoproteins were observed in gel fluorographs, suggesting that the lower bands observed in the present study may well represent bona fide undegraded low molecular weight glycoproteins.

In the case of cells exposed to ^{3}H-fuc and ^{3}H-man, gel slice counts of separated stripped nuclei fractions also revealed the presence of several radioactive peaks at different molecular weights, although these contained much less radioactivity than in the case of ^{3}H-gal. Fluorographs of stripped nuclei proteins from cells exposed to these latter two ^{3}H-sugars showed a common labeled band corresponding to a molecular weight of 75 kd. Labeled bands at very low molecular weight were not observed in fluorogaphs in the case of either ^{3}H-fuc and ^{3}H-man, but moderate peaks of radioactivity appeared in this region in gel slice data. It is possible that the labeled molecules (located near the dye front) migrated off the gel preparations which were processed for fluorography.

In experiments where stripped nuclei of cells exposed to ^{3}H-gal were further subfractionated, gel slice data revealed only a small amount of radioactivity in the 75 kd region in the low and high salt nuclear extracts. The only peak of any real magnitude in gel slices of these two fractions was one at ~95 kd in the high salt extract. In the residual nuclei fraction, on the other hand, a 75 kd peak was quite prominent, containing approximately twice the label observed in the

stripped nuclei fraction. This evidence suggests that the 75 kd ^{3}H-gal-labeled protein observed in the stripped nuclei fractions is a constituent of (or tightly associated with) the nuclear matrix. Other peaks of radioactivity in the residual nuclei fraction suggest that the nuclear matrix also contains several other ^{3}H-gal-labeled proteins.

The results of the lectin blotting experiments served to provide supporting evidence for the presence of different sugar residues on nuclear proteins of myeloma cells, as was first suggested by the earlier RAG studies with ^{3}H-sugars, and further indicated by the preliminary biochemical analysis of the labeled molecules in nuclear fractions in these cells (as just discussed above).

As cells exposed to ^{3}H-galactose showed the greatest radioautographic reaction over nuclei and the highest level of incorporation of label into macromolecules of nuclear fractions, this was the first sugar we decided to assay for in these fractions. RCA I lectin was chosen because of its broad specificity for galactose residues, including the ability to bind to both terminal and penultimate galactose moieties of oligosaccharides (Goldstein et al., 1978).

Our consistent observation of a number of specific RCA I-reactive bands on nitrocellulose transfers containing proteins of stripped nuclei proteins of myeloma cells indicated the presence of galactose residues on several proteins. As might be expected, an RCA I binding protein was detected at ~75 kd,

corresponding in molecular weight to the radiolabeled protein in fluorographs of stripped nuclei proteins of ^{3}H-gal-labeled cells. The other RCA I bands detected at 67-68 kd and 53 kd also appeared to roughly correspond to radiolabeled proteins in similar fractions of ^{3}H-gal-labeled cells, represented in this latter case by peaks of radioactivity in gel slices. These results support the idea that not only was the incorporated ^{3}H-gal label of the biochemical and radioautographic experiments present in glycoproteins, but in galactose residues of glycoproteins.

Numerous RCA I-binding bands indicative of galactosylated proteins were also observed on lectin blots of nuclear matrix fractions. As for the stripped nuclei fractions, several of these RCA I-binding proteins were found at molecular weights where considerable radioactivity was detected in gel slices of nuclear matrix fractions derived from ^{3}H-gal-labeled cells. Of particular note was a RCA I-reactive band at 75 kd which seemed to correspond in molecular weight to the greatest peak of radioactivity in the high molecular weight range. These results would again suggest that the incorporated ^{3}H-gal label in proteins of the nuclear matrix was in galactose residues.

However, it should be pointed out that the amount of incorporated ^{3}H-sugar label detected for any given glycoprotein may not necessarily be comparable to the amount of reaction observed on lectin blots for that protein. A protein may contain a significant amount of sugar residues and be

detected on lectin blots, but if the protein is turning over only very slowly, it might not incorporate sufficient ^{3}H-label to be observed as a prominent peak or band. This might explain why in RCA I lectin blots of nuclear matrix fractions that a protein of 68 kd showed greater lectin binding than the 75 kd protein (Fig 111a, lane 6) but was not as labeled with ^{3}H-gal. The 75 kd glycoprotein might have less total sugar but have a faster turnover rate than the 68 kd moiety, and thus incorporate more ^{3}H-gal label.

As described in the Materials and Methods of this chapter, RCA I-binding proteins on blots of nuclear matrix proteins were revealed by two different detection systems. The earliest experiments were done with biotinylated lectins detected by colorimetrically-developed peroxidase (which was conjugated to biotin-reactive avidin molecules). This system had certain drawbacks in that, in our hands, the reaction on some of the blots started to fade almost immediately following the color development (as was the case in Fig 110). Thus, while the reaction was specific, the intensity of it varied from preparation to preparation. Also, for reasons not completely clear, biotinylated RCA I was the only biotinylated lectin of three we tried which showed a consistently specific reaction on blots containing nuclear proteins. Biotinylated UEA I (for terminal fucose residues) and LCA (for mannose) showed unacceptably high nonspecific binding and thus gave unusable results. Digoxigenin-conjugated lectins, including RCA I, were

used at the very end of these studies. The nonspecific binding was usually low in almost all cases and the bands did not fade which made them easier to see. Despite the differences in the detection systems, however, reactive bands of similar molecular weight were revealed when the same fractions were probed with either RCA I-conjugate, mostly in the range between 60-95 kd (Fig. 110 vs 111a lane 6).

The presence of different glycosidic linkages of sugar to protein among the galactosylated nuclear matrix proteins was indicated when samples of these proteins were treated with N-glycanase F before being probed with RCA I. The lectin binding of the bands between 72-76 kd, as well as most of the reaction at higher molecular weights, was totally abolished. This would indicate that the sugar sidechains of these proteins were N-linked. The finding of N-linked glycoproteins in the nuclear matrix is in agreement with the results of Ferraro et al. (1991) although they did not find any glycoproteins in the molecular weight range of 72-80 kd as we did. The strongly RCA I-reactive band at 68 kd, on the other hand, was completely unaltered in its intensity after similar glycanase treatment, as was the light band at 48 kd. This indicates that these latter glycoproteins are not N-linked. The existence of galactosylated nuclear proteins with sidechains not N-linked to protein might explain the results of our tunicamycin experiments of Chapter 3. It might be recalled the presence of this drug did not abolish nucleoplasmic labeling in YAC

lymphoma and myeloma cells exposed to ^{3}H-galactose which would be only expected if the sugar sidechains were not N-linked. The best characterized nuclear glycoproteins that do not have N-linked sidechains are those containing the O-linked glcNAc residues (see Hart et al., 1989), and it has been shown that these sidechains are good substrates for the addition of galactose in vitro using exogenous galactosyltransferase. It is possible that our glycanase-resistant, RCA I-binding proteins could represent galactosylated forms of this type of glycoprotein in vivo. Thus far, however, with the possible exception of the SV 40 large T antigen (Jarvis and Butel, 1988), there have been no reports in the literature describing O-linked galactosylated glycoproteins either in the nuclear matrix or elsewhere in the nucleus.

The binding pattern observed with the other galactose specific lectin PNA on blots of residual nuclei proteins was different from that found with RCA I. The overall reactivity was much lower with PNA with little detectable reactivity from 70-100 kd where much of the RCA I reactivity was found. This is not totally unexpected in that PNA, unlike RCA I, does not detect galactose residues masked by sialic acid. Our results with sialic acid-specific MAA lectin showed that there were indeed sialylated glycoproteins in this molecular weight range (~72-76 kd). The lack of binding of PNA could also suggest that the RCA I binding proteins contain glcNAc attached to galactose as opposed to galNAc. Another known difference in

sugar specificity between PNA and RCA I is that PNA recognizes galactosylated glycoproteins with galNAc as the penultimate sugar where RCA I preferentially detects proteins with gal attached to glcNAc (Goldstein et al, 1978).

As alluded to above, sialylated glycoproteins in the nuclear matrix fraction were revealed by MAA binding, although there was not as many proteins detected overall as with RCA I. The existence of different glycosidic linkages among the sialic acid-containing glycoproteins in this fraction was also suggested as for those containing galactose. A cluster of MAA-binding glycoproteins proteins at 72-76 kd were similar in molecular weight to RCA I-binding proteins found to be N-glycanase F sensitive. This suggests that there are N-linked glycoproteins in the nuclear matrix containing both galactose and sialic acid. However, the MAA binding (albeit light) observed at 48 kd, appeared to correspond to RCA I-reactivity at a similar molecular weight which was unaffected by N-glycanase F. This would seem to indicate the presence of glycoproteins with galactose and sialic acid that do not have N-linked sidechains. Thus, the presence of sialic did not seem to be dependent on the glycosidic linkage of the sidechains.

The results using the AAA lectin indicated that were several nuclear matrix proteins containing N-linked sidechains with fucose residues. Unlike most fucose-specific lectins, the fucose residues that are recognized by AAA are those attached to core glcNAc of N-linked sidechains of glycoproteins

(Yamashita et al, 1985). As was the case for RCA I, the reactive bands were found to be mostly at relatively high molecular weights between 60-100 kd. The smudgy interband staining observed with this lectin was somewhat disconcerting but the controls in which fucose was added were completely devoid of reaction, and it is notable that several of the AAA-reactive bands were similar in molecular weight to bands in which RCA I binding was abolished by N-glycanase F. These bands were at ~75 kd, 80 kd, and at 92-94 kd. Thus it is likely that the reactivity was specific and the diffuse interband staining was the result of protein streaking in the gels. Such streaking is not uncommon for preparations containing highly insoluble proteins such as those of the nuclear matrix. These results thus provide supporting evidence for the presence of N-linked nuclear matrix glycoproteins in addition to the N-glycanase F experiments.

C. Possible Identities of the Nucleoplasmic Glycoproteins

As discussed above, the cumulative evidence of these studies indicates the presence of a number of different glycoproteins in the nuclei of myeloma cells. In speculating as to whether or not these glycoproteins correspond to nuclear glycoproteins already described in the literature, one can discuss the latter in terms of their sugar residues, their localization in the nucleus, and their known molecular weights.

I) Galactose-containing Glycoproteins

The evidence derived from RAG studies on labeled stripped nuclei of myeloma cells exposed to ^{3}H-gal suggested that much of the label was associated with euchromatin and heterochromatin, probably signifying incorporation into chromosomal and/or nuclear matrix proteins. The only identified chromosomal proteins which have been reported to contain galactose residues are the HMG proteins (Reeves et al., 1981) and these proteins might account for the small amount of radioactivity observed at low molecular weight ~30 kd in gel slices of the stripped nuclear preparations and high salt extracts. It is not surprising that little radioactivity at this molecular weight was observed in the low salt extracts, in that the low salt buffer we used (0.15 M NaCl) would not be expected to extract the HMG proteins. These proteins are extracted with 0.3 M NaCl (Dixon et al., 1985). The radioactivity observed at ~180-200 kd could correspond to labeled DNA polymerase α, as this is close to the molecular weight of the heterodimer form of this protein in some animal species (Hsi et al, 1990). This protein has been reported to be at least partially found in tight association with chromatin in some cells (Eichler et al., 1977; Filpula et al., 1982). Interestingly, one of the subunits of this protein has a reported molecular weight of 76 kd (Filpula et al., 1982) which is close to the largest band of radioactivity of our gel slices and fluorographs.

Our radioautographic findings some ^{3}H-galactose label was

associated with the scaffold-like portion of the nuclear matrix suggests that labeled glycoproteins could belong to the recently reported class of "matrin" molecules described by Nakayasu and Berezney, 1991. It is notable that the amino acid sequence of a cloned matrin contains a predicted asparagine-linked glycosylation site (Hakes and Berezny, 1991). The presence of galactose in nuclear matrix proteins has been described by Coffee and Berezney (1977), and Burrus et al. (1988). However, neither of these studies localized the galactosylated proteins to a particular region of the nuclear matrix, so it is not possible to compare these studies with our radioautographic results. The major isolated RCA I-binding nuclear matrix proteins described by Burrus et al. (1988) had molecular weights of 55 kd and 95 kd. We did observe minor RCA I reactive bands close to these molecular weights (~48 kd and ~95 kd) in our nuclear matrix preparations which may correspond to those proteins. However, the ^{3}H-gal-labeled proteins and RCA I-binding proteins between 68-80 kd of our nuclear matrix fractions do not correspond to any protein they described.

The indication from our RAG studies that the peripheral nuclear matrix contains labeled molecules might indicate the presence of galactosylated lamin or lamin-associated proteins. There was considerable RCA-I binding activity and incorporated ^{3}H-gal label detected in proteins of our nuclear matrix fractions between 60-75 kd which corresponds to the known

molecular weights of lamin molecules. Baglia and Maul (1983) and Ferraro et al. (1989a) provided evidence that lamins are glycosylated though there was no evidence presented in these studies that they contained galactose residues.

Our radioautographic studies also indicated that a portion of the ^{3}H-gal label was associated with nucleoli, including residual nucleoli of nuclear matrix preparations, and thus may represent labeled nucleolar or ribonucleoproteins (RNPs). There have been occasional cytochemical reports of galactose residues associated with proteins of nucleoli (Bramwell et al., 1982, Kan et al., 1987), but no direct biochemical evidence is present in the literature to confirm this. Possible indirect evidence for the presence of galactose residues in RNPs comes from two different studies. Firstly, a very recent cytochemical study showed that a number of different RNPs were localized to the edges of chromosomes during mitosis (Gautier et al., 1992). This location corresponds to a frequently observed site of labeling in our RAGs of dividing cells of all three cultured cell lines exposed to ^{3}H-gal (in Chapter 3). Secondly, recent findings that the galactose-specific lectin CBP35 is associated with RNP complexes and/or sliceosomes (Wang et al., 1992) makes it tempting to speculate that the putative glycoprotein substrate(s) for the lectin is also associated with RNPs although as yet, an <u>in vivo</u> substrate glycoprotein has not been found for this lectin.

II) Fucose-containing Glycoproteins

Our RAG results in nuclei of myeloma cells exposed to ^{3}H-fucose suggested that the nucleoplasmic label was located in the interior regions of the nuclei as well as at the periphery. As with ^{3}H-gal, the internal nuclear labeling suggests that some ^{3}H-fuc was incorporated into chromosomal, internal nuclear matrix and/or nucleolar glycoproteins. Again, the only characterized chromosomal glycoproteins containing fucose thus far described are the HMGs (Reeves et al, 1981). These proteins may account for at least some of the internal nucleoplasmic RAG label as well as the low molecular weight label around 30 kd in our gel slices of ^{3}H-fuc-labeled stripped nuclei proteins.

In terms of fucosylated nuclear matrix proteins (which were indicated by AAA lectin-binding results) the ^{3}H-fucose label could again reside in lamin, matrin and/or nucleolar-associated proteins. Cervoni et al. (1990) have reported fucosylated glycoproteins in nuclear matrix fractions based on the affinity of the proteins for UEA I lectin. These proteins were found to be enriched in nuclear lamina fractions. As described above for ^{3}H-gal, the nuclear lamins have been reported to be glycosylated, and in terms of location may account for at least some of the peripheral nucleoplasmic label observed in RAGs of stripped nuclei from ^{3}H-fuc-labeled myeloma cells. Lamin A has a reported molecular weight of 70-75 kd which corresponds both to the 75 kd observed in our

fluorographs of ^{3}H-fuc-labeled stripped nuclei proteins as well as to an AAA lectin-binding protein in our nuclear matrix fractions. However, pertaining to the lectin binding results, it should be pointed out that the specificities of the fucose-specific UEA I and AAA lectins are not the same and it is difficult to compare results obtained for these two lectins. UEA I has an affinity for peripheral fucose residues of glycoproteins (Goldstein et al, 1978) while as mentioned earlier, the AAA binds to the fucose residues attached to core glcNAc of N-linked glycoproteins (Yamashita et al, 1985).

The presence of AAA-binding proteins in nuclear matrix fractions at molecular weights outside of the range for lamins (ie. 80-120 kd) suggests that fucosylated matrin and/or nucleolar proteins are present in the nuclear matrix. Evidence for fucosylated nucleolar glycoproteins was provided by Kan et al. (1987) who reported binding of UEA I lectin to the edges of the fibrillar cords of nucleoli in cultured liver cells. There was also an unpublished report cited of fucose residues in RNPs (Codogno et., cited by Hubert et al., 1989).

III) Mannose-containing Glycoproteins

Our radioautographical studies showed that the ^{3}H-mannose label was located primarily but not exclusively at the periphery of nuclei. As with ^{3}H-fuc, at least part of the peripheral label could derive from glycosylated lamin molecules of the peripheral nuclear matrix. Our fluorographs of stripped nuclei proteins from cells exposed to ^{3}H-mannose

exhibited one prominent labeled band at 75 kd which is similar in molecular weight to the reported value of lamin A. Ferraro et al. (1989a) have provided evidence that lamins, and especially lamin A, contain mannose residues. Alternatively, some of the peripheral label (at least in whole cells) could be caused by a protein similar to gp190, a mannose-containing glycoprotein associated with the nuclear envelope found to be present in a variety of cells (Gerace et al., 1982; Fisher et al., 1982; Aris and Blobel, 1989).

Some ^{3}H-mannose label in our radioautographs was also observed in association with euchromatin and heterochromatin, suggesting an association of the label with chromosomal and/or internal nuclear matrix proteins. The relatively light reaction observed over chromosomes of dividing cells, on the other hand, indicates that those labeled chromatin molecules retained in the chromosomes may not account for a high proportion of the nuclear reaction observed in interphase cells. The best documented evidence for mannose residues in nonhistone chromosomal proteins is in the case of HMG proteins (Reeves et al., 1981). One of the peaks (albeit very small) in the gel slices of ^{3}H-man-labeled stripped nuclei fractions was found at a molecular weight similar with that of some HMG proteins (~30 kd). Some radioactivity was detected at ~180 kd which might correspond to mannose-containing DNA polymerase α proteins (Hsi et al., 1990). The finding by Welch et al. (1983) that a mannosylated stress protein is at least

partially localized to the nucleoplasm of cells makes it possible that some of the internal ^{3}H-man label might be residing in this type of protein. Heat-shock and stress proteins are ubiquitous in mammalian cells and comprise a family of numerous members. There is evidence that they are produced constitutively at low levels in all mammalian cells. Thus far, however, there is only the one above report that these proteins may be glycosylated.

A number of studies in the literature have described different mannose-containing nuclear matrix proteins (Berezny and Coffey, 1977; D'erme et al., 1987; Carmo-Fonseca, 1988; Ferraro et al., 1989b and Burris et al., 1988). However, no location within the nuclear matrix (internal or peripheral) was indicated for these proteins. It is also noteable that none of glycoproteins mentioned in these studies were close to 75 kd (the molecular weight of the ^{3}H-man-labeled protein detected in our fluorographs and gel slices of labeled stripped nuclei preparations).

IV) GlcNAc-containing glycoproteins

Our radioautographic studies in myeloma cells after exposure to ^{3}H-glcNAc indicated that much of the label was associated with the euchromatin and heterochromatin of the nucleoplasm, again suggesting the presence of glcNAc in chromosomal proteins and/or the internal nuclear matrix. It is probable that a good portion of this label was in O-linked glcNAc-containing glycoproteins such as the RNA polymerase II

transcription factors (Jackson and Tijan, 1988; Lichsteiner and Schibler, 1989; Schroter et al, 1990). Kelly and Hart (1989) provided evidence that ~90% of the glcNAc associated with chromosomal proteins in Drosophila nuclei was present in this form. On the other hand, some nuclear proteins have been reported to contain N-linked sidechains with glcNAc residues, e.g (Reeves et al., 1981; Galland et al, 1989).

There have been a few reports of nuclear matrix glycoproteins containing glcNAc (Schmidt et al., 1984: Carmo-Fonseca 1988). Other work of possible relevance is that of Chaly and coworkers who showed that a monoclonal antibody (PI1) raised against a nuclear matrix fraction in murine lymphocytes labeled structures in both internal parts and peripheral parts of the nuclei of a number of cell types (Chaly et al., 1984,1986). This same antibody was later shown to recognize a 68 kd O-linked glcNAc-bearing nuclear envelope glycoprotein in frog oocytes (Dabauville et al., 1988a). If it is postulated that the antibody is at least partly specific for O-glcNAc residues as has been shown for some other antibodies (Davis et al., 1987; Turner et al, 1990) the internal nucleoplasmic reaction might represent O-glcNAc proteins of the internal nuclear matrix.

Our radioautographic studies also indicated that significant label was associated with the nucleolus (11% of total cellular grains) which could reside in nucleolar and/or ribonucleoproteins. Soulard et al. (1990) have reported the

presence of glcNAc in RNPs.

At least some of the ^{3}H-glcNAc label observed at the periphery of the nuclei in our EM radioautographs is likely present in the O-linked glcNAc-bearing nuclear pore proteins which have been described in numerous studies (Davis and Blobel, 1986; Holt and Hart, 1986; Hogan and Schindler, 1987; Snow et al., 1987).

V) Sialic Acid-containing Glycoproteins

Our radioautographic results suggested that, as was the case for ^{3}H-gal and ^{3}H-glcNAc, most of the incorporated label (i.e. ^{3}H-manNAc) representing putative sialic acid residues was associated with internal elements of the nucleoplasm, including chromatin, nuclear matrix and/or nucleoli. Our lectin blots of residual nuclei proteins showed specific binding of the MAA lectin at several molecular weights, indicating the presence of a number of sialic acid-containing glycoproteins associated with the nuclear matrix, which might include proteins of the residual nucleolus.

In the literature, there have been relatively few reports describing nucleoplasmic glycoproteins with sialic acid residues. This sugar has been reported to be present in chromatin molecules (Spangler et al., 1975; Bologni-Fantin et al.,1989; Vannier-Santos et al., 1991), or other less defined nucleoplasmic molecules (Margolis et al., 1976). Several studies have reported binding of WGA lectin to nucleoplasmic molecules (See Lectin Cytochemistry section of Ch. 1). In some

instances, it is possible that this lectin might be detecting sialic acid residues in addition to glcNAc residues because of its known dual specificity for these sugars.

However, there are no reports as far as we know describing sialylated nuclear matrix glycoproteins. This work thus provides the first evidence for the presence of this sugar in in proteins of this compartment.

VI) Sulphate-containing Glycoproteins

The presence of radioautographic reaction over the nucleoplasm of myeloma cells exposed to ^{35}S-sulfate in our studies, combined with the documented high specificity of ^{35}S-sulphate for sulphate residues of GAG sidechains (See Appendix 1), provides evidence for the presence of sulphated proteoglycans and/or hyaluronic acid molecules in the nucleus.

Nucleoplasmic sulphated proteoglycans have been described in a number of studies either associated with chromatin or simply to the nucleus in general. These include heparin sulphate proteoglycans (Kinoshita et al., 1971; Bhavanandan and Davidson 1975; Margolis et al., 1976; Fedarko et al., 1986 and Ishihara et al., 1987), chondroitin sulphate proteoglycans (Bhavanandan et al., 1975 and Margolis et al., 1976; Furukawa and Terayama, 1977). Sulfated hyaluronic acid molecules have also been descibed in the nucleus (Margolis and et., 1976 and Furukawa and Terayama, 1977), as well as sulfate residues in traditional glycoprotein side chains (Margolis et al., 1976).

D. Functional Significance of Glycosylation of Nucleoplasmic Proteins

The functional importance of nucleoplasmic glycoproteins in cells has been suggested a number of ways in considering the available evidence. First, there has been a direct demonstration of glycosylation of important regulatory chromosomal nuclear proteins such as the HMGs (see Ch. 1, p. 4) and the RNA transcription factors (see p. 5). Secondly, there is evidence that nucleoplasmic glycoproteins are associated with other functionally important compartments such as the nuclear matrix (see p. 14 and the results of this chapter). Thirdly, nuclear glycoproteins or lectins (with putative glycoproteins substrates) have been reported to be differentially expressed in cancerous cells versus their normal differentiated counterparts (see pp. 9-10).

The functional significance of these glycoproteins having been indicated, the immediate question which arises concerns the actual purpose of the glycosyl groups themselves on the nuclear glycoproteins. This is still unclear in many cases but there are indications that glycosylation may serve different purposes on different nuclear proteins. These putative roles and the supporting evidence are presented below.

I) Role of Glycosylation in the Function of Nuclear Regulatory Proteins

There are indications from a number of studies that glycosylation has an active role in the function of particular regulatory nuclear proteins. The best evidence of this comes from studies on the O-linked glcNAc-bearing glycoproteins. Jackson and Tjian (1988) found that binding of WGA lectin to

transcription factor Sp1 caused a 3-4 fold decrease in RNA transcription activation. They further showed that the inhibition of transcriptional activation caused by WGA was not due to an effect of WGA on the ability of Sp1 to bind to DNA, but to a repression of the transcriptional activation function of Sp1. The importance of glycosylation in the transcriptional activation function of Sp1 molecules was also demonstrated by the fact that glycosylated Sp1 derived from HeLa cells is approximately 3-5 fold more efficient at activating transcription than unglycosylated Sp1 synthesized in E. coli cells (Jackson and Tjian, 1988).

A number of recent studies have indicated that O-glcNAc glycosylation of nuclear pore proteins is directly involved in the process of nucleocytoplasmic transport of proteins (Findley et al., 1987; Yoneda et al., 1987; Featherstone et al., 1988; Dabauvalle et al., 1988b; Wolff et al., 1988; Akey and Goldfarb, 1989). WGA was shown to inhibit the uptake of proteins into the nucleus in a number different experimental systems. This appeared to result from the lectin's ability to block the energy-dependent translocation step of nuclear transport and not due to a decrease in available binding sites for the transitory proteins to the nuclear pores and/or the lectin simply blocking the pore itself (Forbes et al., 1988; Newmeyer and Forbes, 1988).

Evidence for a role of other sugars in the function of nuclear proteins comes from the work of Bhattacharya and co-

workers on DNA polymerase α. In their studies, they showed that the addition of lectins Con A or RCA II reduced the activity of DNA polymerase α in their assay system (Bhattacharya et al, 1979; 1981; 1982). This implies that glycosyl groups containing mannose and galactose (or galactosamine) might be directly involved in regulating the function of this protein.

Indirect evidence comes from studies on the cellular lectin CBP 35. CBP 35 has been reported to be associated with hnRNP complexes and snRNP complexes in nuclei (Laing and Wang, 1988). Inclusion of galactose-containing saccharides (or antibodies to CBP 35) in cell free mRNA splicing assays was shown to inhibit formation of spliced RNA products (Wang et al., 1992). Thus, both the lectin and the glycosyl groups of a putative glycoprotein substrate are directly implicated as being important in RNA processing.

It is not clear at the molecular level how protein glycosylation in the above examples might exert its influence on function. The most straight forward idea is that the sugar sidechains form or contribute to specific recognition sites on the protein which are involved in the interaction of that protein with other molecules. However, another proposal currently in vogue (at least for the O-linked glcNAc glycoproteins) is that the sidechains of regulatory proteins may affect their functional status by antigonizing the action of cellular kinases on that protein. This would be due to the

competition for the same amino acids i.e. serine and/or threonine which are potential sites for both O-linked glycosylation and phosphorylation (Holt et al., 1987a). This proposal is attractive in the sense that it has been shown that phosphorylation regulates the functional activity of many nuclear proteins (Jackson, 1992; Hunter et al., 1992).

II) Possible Role of Glycosylation in Targeting of Proteins to the Nucleus

Another possible function for the sugar modifications may be to target the proteins to the nucleus. A known example for sugars having a role in protein targeting in the cell is the phoshorylated mannose residues of N-linked oligosaccharides of lysosomal glycoproteins which are involved in the targeting process for the proteins to the lysosomes (von Figura and Hasilik, 1986). In terms of nucleoplasmic glycoproteins, Schmitt and Mann (1987) provided evidence that only the nucleoplasmic form of the SV-40 large T antigen was glycosylated and they suggested that glycosylation might be involved in the targeting of this protein to the nucleus. This idea was not given a lot of credence because it became well established that the nuclear targetting process is determined by the presence of a nuclear localization signal (NLS) which is encoded by the amino acid sequence of the protein (see reviews by Goldfarb, 1989, 1992). Recently, however, Jans et al. (1991) showed that phophorylation of a specific tyrosine residue (T^{124}) flanking the NLS inhibits the import of the SV-40 large T antigen into nuclei. Thus, it was shown that

factors outside the NLS could influence nuclear targeting. In light of this revelation and the data by Schmitt and Mann (1987), it is tempting to speculate that the glycosylation plays a role in the targeting of proteins to the nucleus. There was more evidence by Jarvis and Butel (1988), however, that indicated that a small portion of the cytoplasmic SV 40 T antigen is also glycosylated which would go against this theory but, as suggested by Schindler et al. (1987), such a targeting process might not be dependent upon the absolute presence or absence of glycosyl groups but on the number and/or location of the sites of glycosylation.

III) Role of Glycosylation in the Inhibition of Proteolysis

Another proposed role for glycosylation in some nuclear glycoproteins is that the carbohydrate might prevent or inhibit proteolysis of the proteins (Schindler et al., 1987). This suggestion was based on the finding that the O-linked glcNAc glycoproteins identified in their study showed a resistance to pronase digestion. Tomek et al. (1988) found that prosomes (small glycosylated cytosolic RNP particles) were highly resistant to proteinase K which also prompted the authors to suggest that the glycosyl groups might prevent prosome degradation. It is also noteworthy that a similar protective function of carbohydrate has been proposed for LAMP proteins, whose luminal portion faces the hydrolytic enzymes of lysosomes (Carlsson and Fukuda, 1992).

IV) Possible Role in the Formation or Stabilization of Protein Complexes

Lastly, as suggested by Holt et al. (1987a), glycosylation may be important to the formation and/or stabilization of multiprotein complexes. Applicable examples might be the nuclear pore complexes which have been shown to have multiple glycosylated proteins or the cytoskeletal interactions in erthrocytes of cytosolic Band 4.1 (a glycoprotein) with spectrin, actin, and glycophorin.

Similarly, the formation of a complex by a nucleoplasmic lectin with a substrate nucleoplasmic glycoprotein might be necessary for some functional event to occur as suggested by Hubert et al. (1989) and possibly applicable to the aforementioned CBP endogenous lectin.

E. Summary of Chapter 4

To summarize the results of chapter 4, these studies provide strong evidence that most if not all of the radioautographic reaction over the nucleoplasm in myeloma cells after exposure to ^{3}H-sugars was derived from molecules residing within the nucleoplasm with little contribution from extra-nucleoplasmic compartments such as the nuclear envelope or adjacent cytoplasm. This appeared to be true even for the reaction observed over the periphery of the nucleoplasm. In subfractionated stripped nuclei from cells labeled with ^{3}H-galactose, much of the label was retained in high salt-extracted residual nuclei providing evidence that the labeled molecules were constituents of the nuclear matrix. As in the

stripped nuclei, the labeled molecules were observed over both the peripheral and internal regions of the matrix including the nucleolus.

The results of biochemical experiments to determine the nature of the nucleoplasmic label showed that this label was almost entirely macromolecular, with strong indications that most if not all of it resided in protein. A protein of ~75 kd present in stripped nuclei fractions was shown to become significantly labeled after cells were exposed to any of three ^{3}H-sugars, indicating the presence of galactose, fucose, and mannose residues. A number of other less labeled glycoproteins with these sugars were also suggested to be present in these fractions.

Analysis of nuclear fractions derived from further sub-fractionating isolated nuclei from ^{3}H-gal labeled cells with different concentrations of salt indicated that the majority of the label resided in molecules of the nuclear matrix, thus supporting our radioautographic results. A number of radio-active peaks indicative of several labeled glycoproteins were observed in the nuclear matrix fraction with the most labeled one at 75 kd.

Lectin blot analysis of the nuclear matrix fractions indicated the presence of a variety of glycoproteins containing galactose, sialic acid and/or fucose residues, including some close to 75 kd. N-glycanase F treatment on this fraction before lectin blotting suggested that some but not all of the glycoproteins had N-linked sugars.

Table 5 - Macromolecular Label in Nuclear Fractions after Administration of ^{3}H-Sugars

Isotope	% of Total Cellular TCA-precipitable Label in Stripped Nuclei	DPM/10 μg of TCA-precipitable Protein			
		Stripped Nuclei	Low Salt Extract	High Salt Extract	Residual Nuclei
^{3}H-Gal	10.9	7200	3100	5700	12500
^{3}H-Fuc	5.9	2600			
^{3}H-Man	2.9	1100			

Fig. 89 EM radioautograph of a whole myeloma cell after incubation with ^{3}H-galactose for 18 hr.

This cell exhibits a reaction identical in silver grain distribution to that seen in Fig. 47, with the exception that very little Golgi apparatus (G) is observed in this section. The intensity of the reaction is less than that in Fig. 47, even though the radioautographic exposure was longer; this is due to the fact that in the present experiment, a much smaller dose of ^{3}H-galactose was administered. In the nucleus (N), grains occur both peripheral and internal regions, and many are associated with the nucleolus (n). In the cytoplasm, many grains occur over the plasma membrane (pm) and profiles of rough endoplasmic reticulum (rER).

Exposure: 7 wk (Chem. dev.) x 10,000

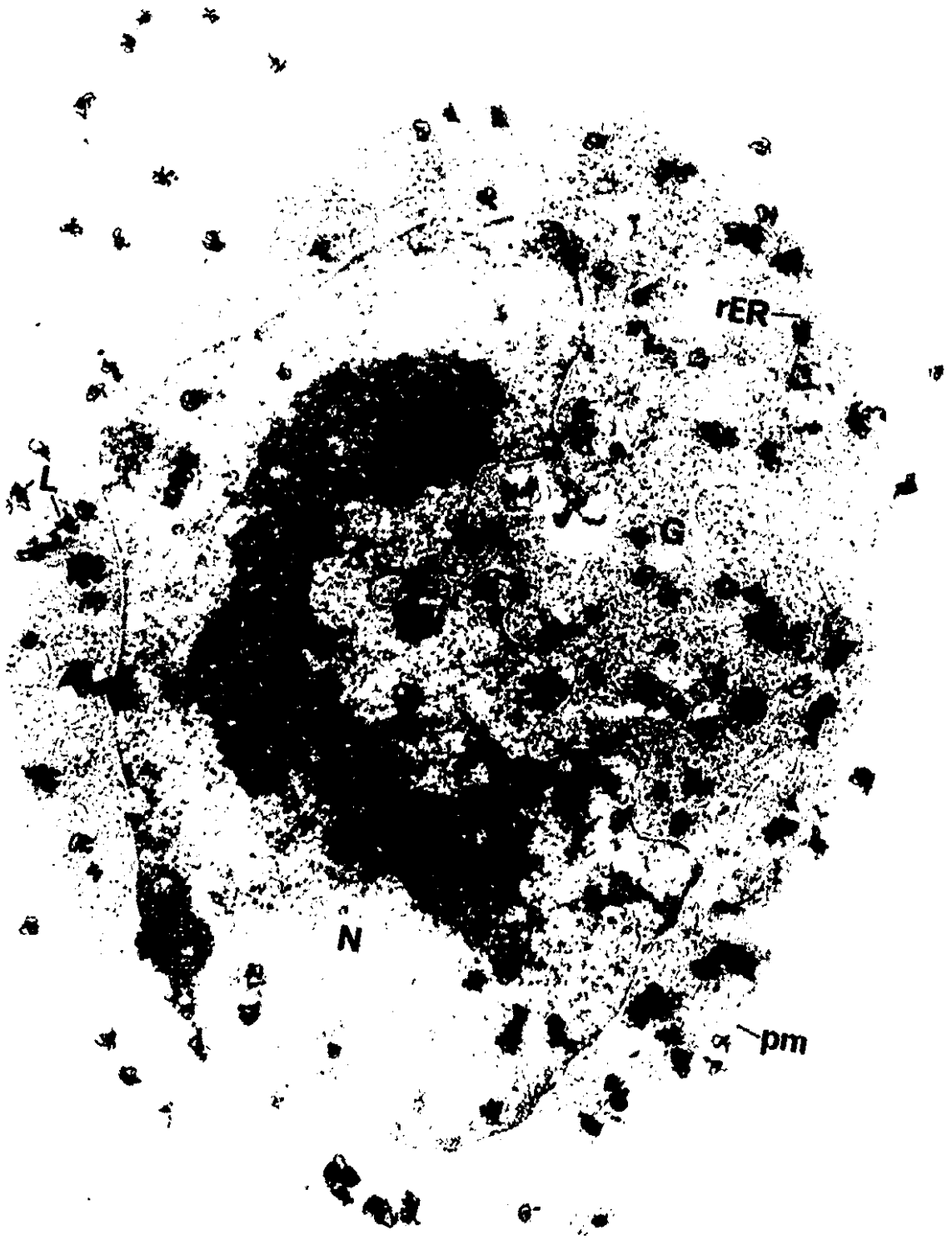

rER
L
G
N
pm

Fig. 90 EM radioautograph of intact nuclei isolated from myeloma cells which had been incubated with ^{3}H-galactose for 18 hr.

Almost all of the cytoplasm has been removed in this preparation. The nuclei are associated over most of their surface with the nuclear envelope (NE), which is still intact although somewhat ragged in appearance. The euchromatin areas of the nucleoplasm apprear somewhat washed out, but the heterochromatin and the nucleoli (n) appear normal. These nuclei exhibit a radioautographic reaction with an intensity at least as heavy as that seen over the nucleus of the intact cells in Fig. 89, and the silver grains have a similar distribution, i.e. over the nuclear periphery as well as over the internal euchromatin, and the nucleolus.

Exposure: 7 wks (Chem. dev.) x 10,000

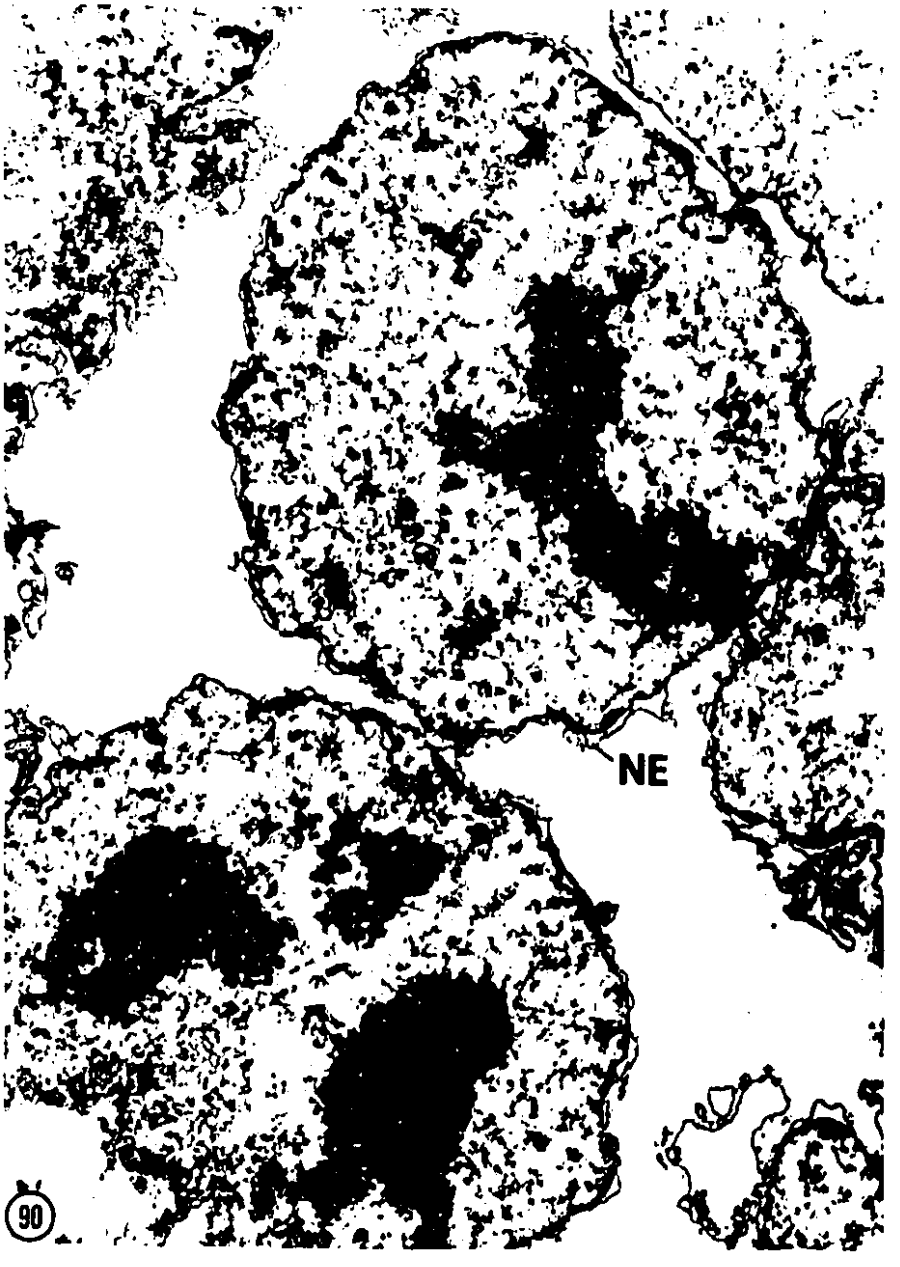

NE
90

Fig. 91 EM radioautograph of stripped nuclei isolated from myeloma cells which had been incubated with ^{3}H-galactose for 18 hr.

In these nuclei, the nuclear envelope has been stripped away, and there is little sign of membranous material at the nuclear surface. In other respects, the nuclei resemble the intact nuclei in Fig. 90, some flocculent material is occasionally observed between nuclei, and may represent sparce cytoplasmic remnants or material from disrupted nuclei. These nuclei exhibit a radioautographic reaction with intensity as least as heavy as that seen over intact nuclei, and again the grains are distributed over the nuclear periphery, euchromatin, and the nucleolus. Occasional grains overlie flocculent material between the nuclei, but this material is much less labeled than the nuclei.

Exposure: 7 wk (Chem. dev.) x 8,500

91

Fig. 92 EM radioautograph of stripped nuclei and isolated metaphase chromosomes from myeloma cells incubated for 18 hr with ^{3}H-galactose.

As in Fig. 91, the isolated nuclei show heavy labeling. Grains are again found at both the periphery and more centrally, sometimes in association with nucleolar complexes (n). In addition to this, isolated metaphase chromosomes (cc) from a disrupted dividing cell are also observed to be labeled.

Exposure: 7 wk (Chem. dev.) x 8,500

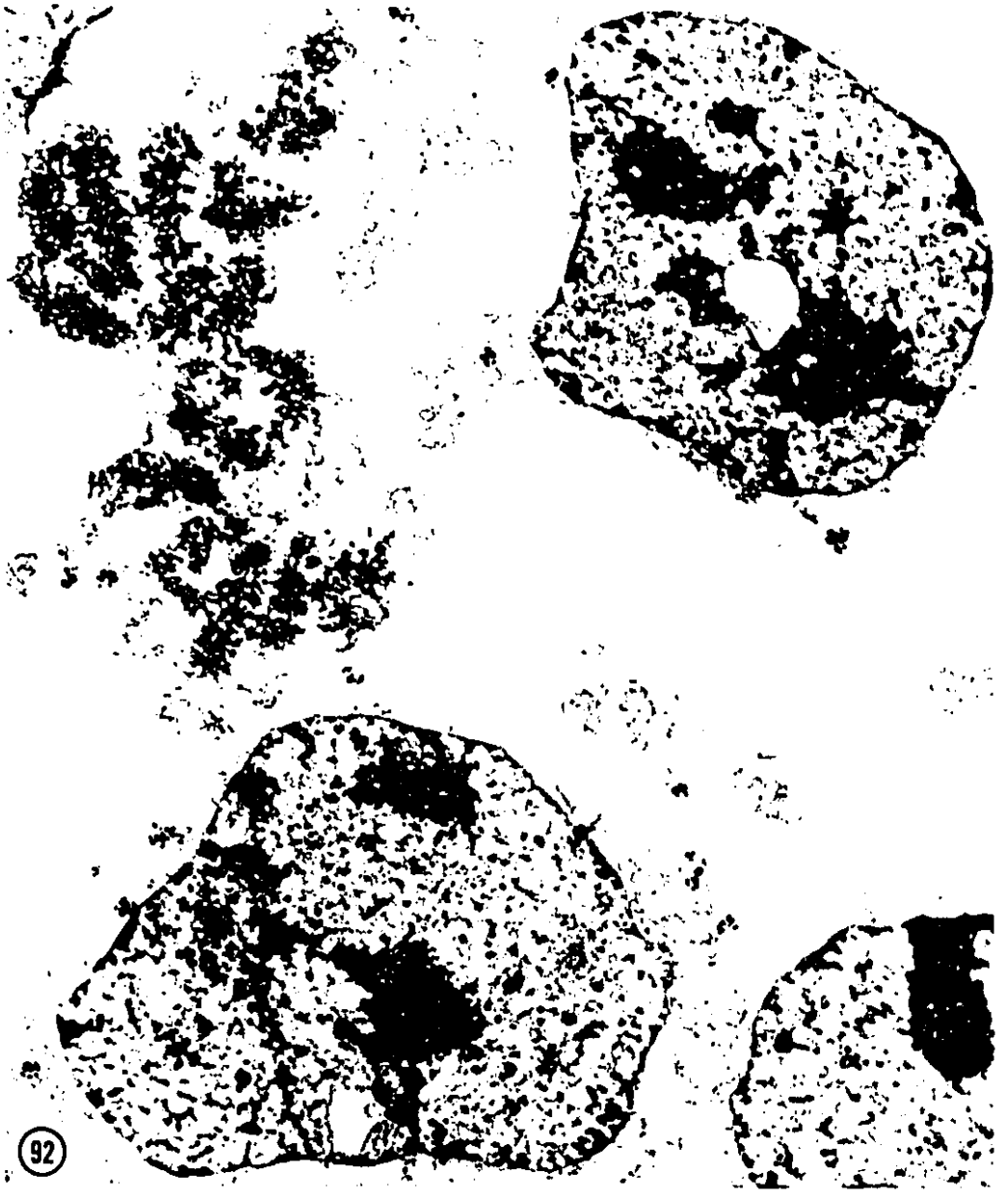

92

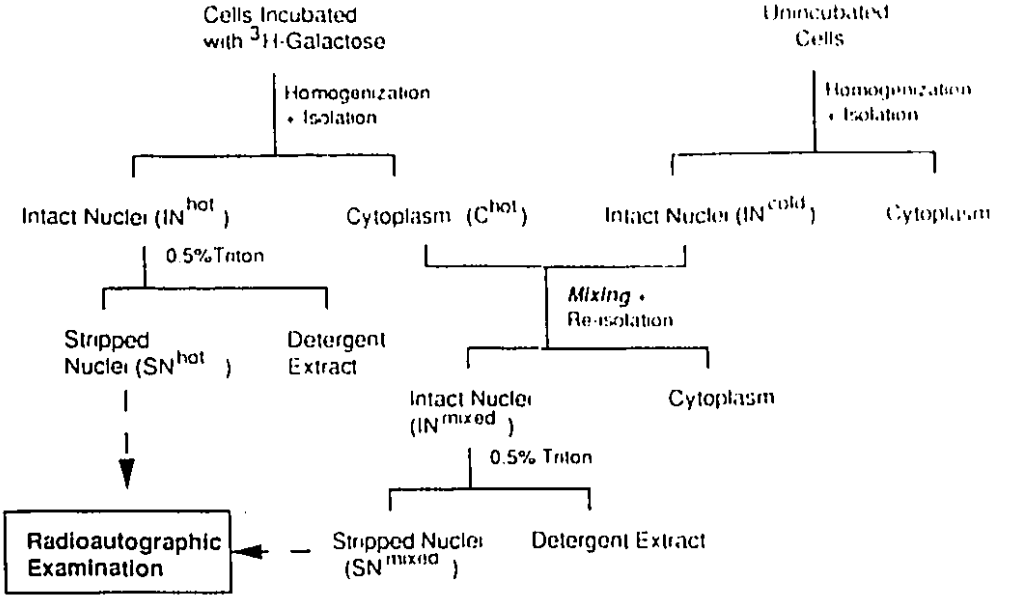
Fig. 93 Fractionation of myeloma cells incubated with ^{3}H-galactose: Mixing Experiment 1.

Carried out to test the possibility that some nucleoplasmic labeling observed in the stripped nuclei fraction was derived from cytoplasmic molecules which relocated to the nucleoplasm during the initial homogenization step.

Fig. 94 Fractionation of myeloma cells incubated with ^{3}H-galactose: Mixing Experiment 2.

Carried out to test the possibility that some nucleoplasmic labeling observed in the stripped nuclei was derived from molecules in the cytoplasmic tags or nuclear envelope which relocated to the nucleoplasm during the detergent treatment step of the isolation procedure.

Mixing Experiment 1



Mixing Experiment 2

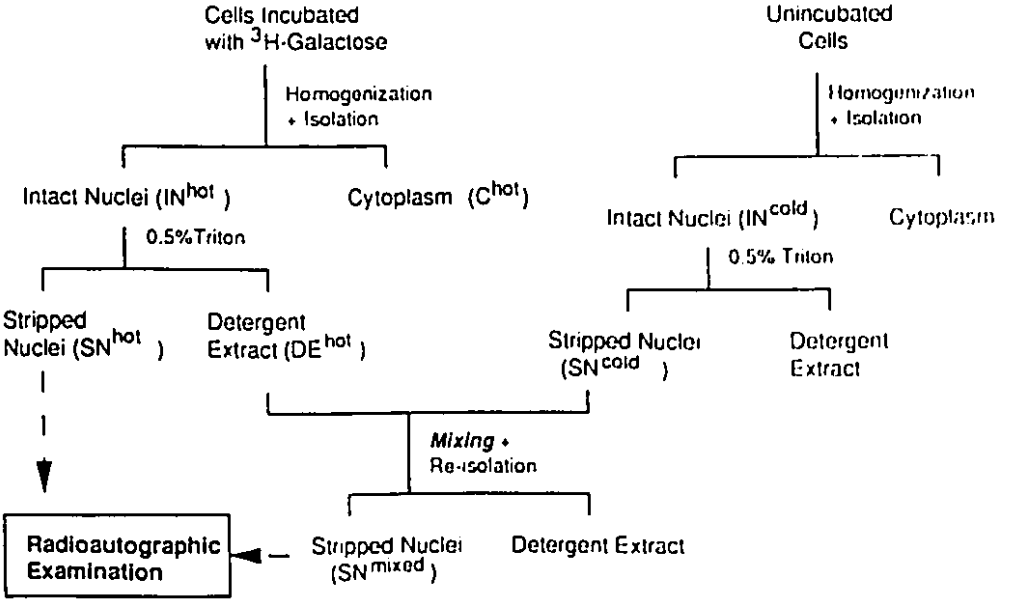


Fig. 95 LM radioautograph of a "mixed" stripped nuclei fraction from unincubated myeloma cells, derived according to Mixing Experiment 1 (outlined in Fig. 93).

There is no detectable radioautographic reaction over the nuclei in this fraction.

Exposure: 1 mo (Chem. dev.) x 1000

Fig. 96 LM radioautograph of a "hot" stripped nuclei fraction from myeloma cells incubated with ^{3}H-galactose, and derived according to Mixing Experiment 1 (outlined in Fig. 93).

There is a fairly heavy radioautographic reaction over the nuclei in this fraction.

Exposure: 1 mo (Chem. dev.) x 1000

Fig. 97 LM radioautograph of a "mixed" stripped nuclei fraction from unincubated myeloma cells, derived according to Mixing Experiment 2 (outlined in Fig. 94).

There is no detectable radioautographic reaction over the nuclei in this fraction.

Exposure: 1 mo (Chem. dev.) x 1000

Fig. 98 LM radioautograph of a "hot" stripped nuclei fraction from myeloma cells incubated with ^{3}H-galactose, and derived according to Mixing Experiment 2 (outlined in Fig. 95).

A fairly heavy radioautographic reaction is observed over the nuclei in this fraction.

Exposure: 1 mo (Chem. dev.) x 1000

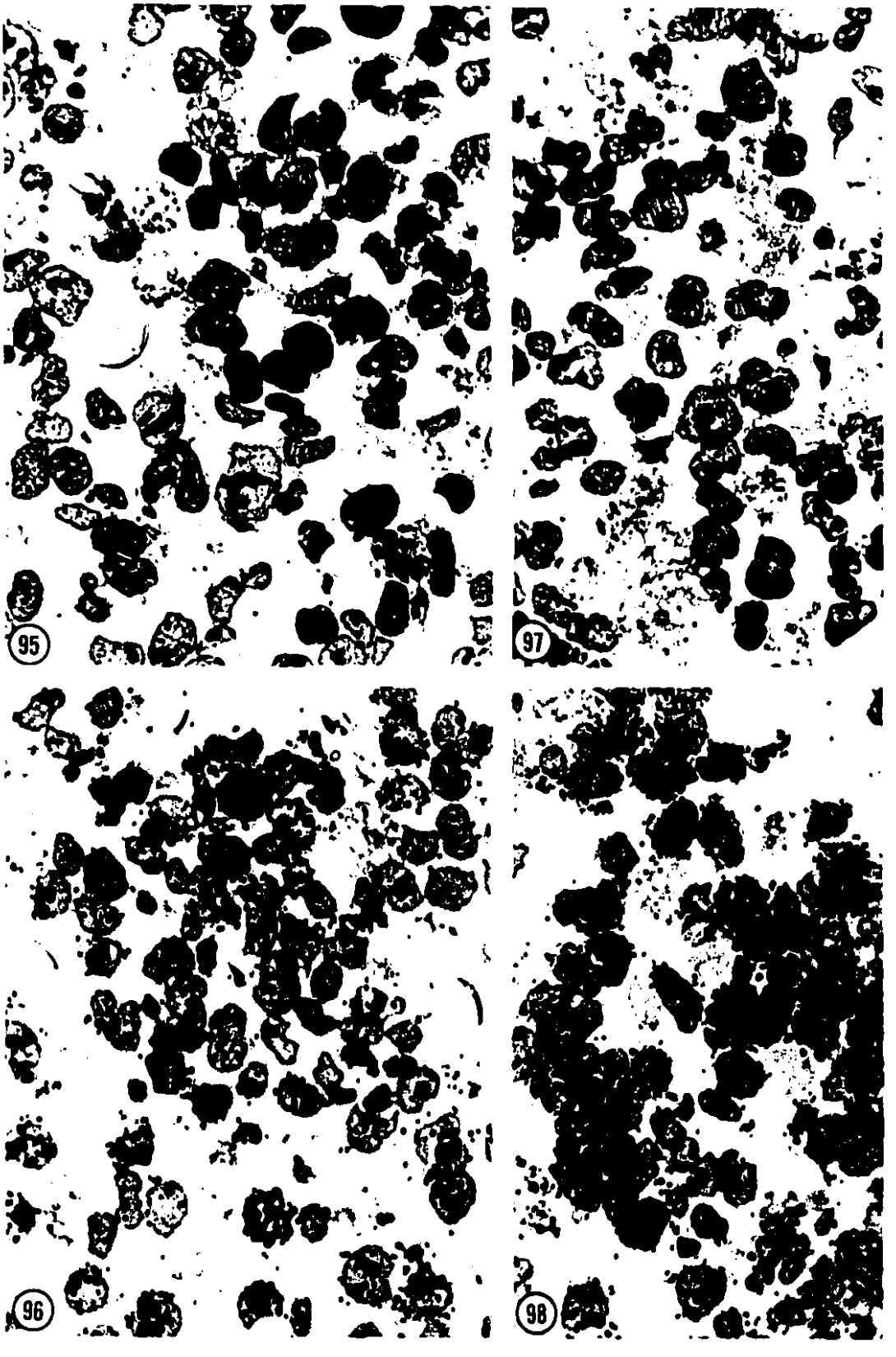

95
97
96
98

Fig. 99 LM radioautograph of whole myeloma cells incubated with ^{3}H-fucose for 18 hr.

These cells exhibit a fairly heavy reaction over their cytoplasm and cell surface, but also show a substantial labeling of their nuclei. Many of these latter grains are associated with nucleoli (arrows).

Exposure: 2 mo (Chem. Dev.) x 1000

Fig. 100 LM radioautograph of stripped nuclei isolated from myeloma cells which had been incubated with ^{3}H-fucose for 18 hr.

The nuclei exhibit a significant radioautographic reaction, in which some grains are at the periphery and others are more internal, often associated with nucleoli.

Exposure: 2 mo (Chem. Dev.) x 1000

Fig. 101 LM radioautograph of whole myeloma cells incubated with ^{3}H-mannose for 18 hr.

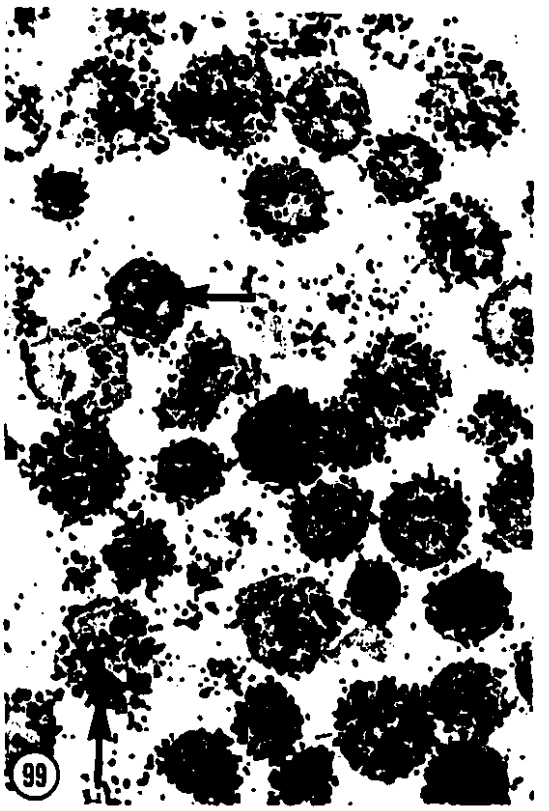
Some of these cells show very heavy reaction over their cytoplasm and cell surface while others are less labeled. In all cells there is a discrete nuclear reaction, with grains after associated with both the periphery (arrowhead) and the nucleolus (arrows).

Exposure: 2 mo (Chem. Dev.) x 1000

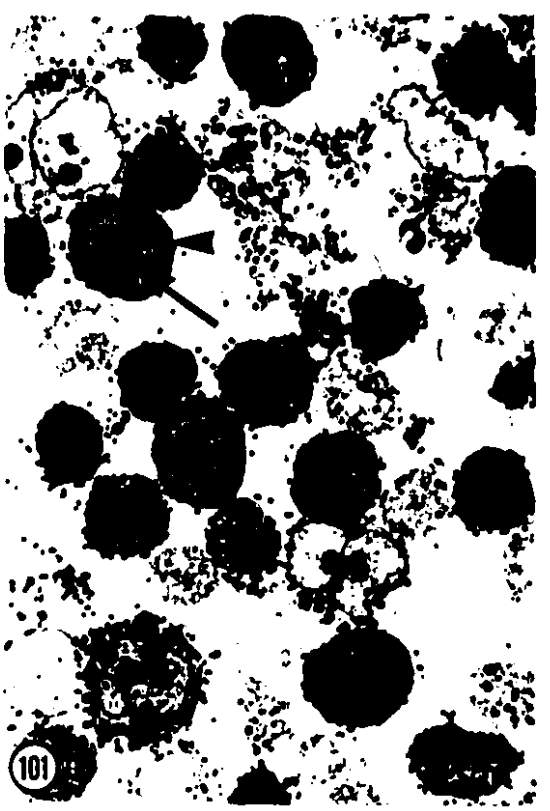
Fig. 102 LM radioautograph of stripped nuclei isolated from myeloma cells which had been incubated with ^{3}H-mannose for 18 hr.

The nuclei exhibit a light but significant radioautographic reaction. Some grains are located interiorly, but most are strongly localized over the periphery of the nuclei (arrow).

Exposure: 2mo (Chem. Dev.) x 1000


99


100


101


102

Fig. 103 Gel fluorographs of labeled stripped nuclei fraction proteins (SN) separated by SDS-PAGE from myeloma cells incubated for 18 hr. with either ^{3}H-mannose (Man), ^{3}H-fucose (Fuc), or ^{3}H-galactose (Gal). Lanes of ^{14}C-labeled molecular weights (MW) are indicated for each fluorograph.

A labeled band at ~75 kd appears to be common to stripped nuclei fractions of cells exposed to all three sugars (as indicated by the arrow and accompanying molecular weight). A band at 180 kd is occasionally observed in stripped nuclei preparations of cells exposed ^{3}H-gal (arrow, lane 3) as are at least two large bands of very low molecular weight (arrowhead, lane 3).

Exposures: Man (5 mo.); Fuc (3 mo.); Gal (1 mo.)

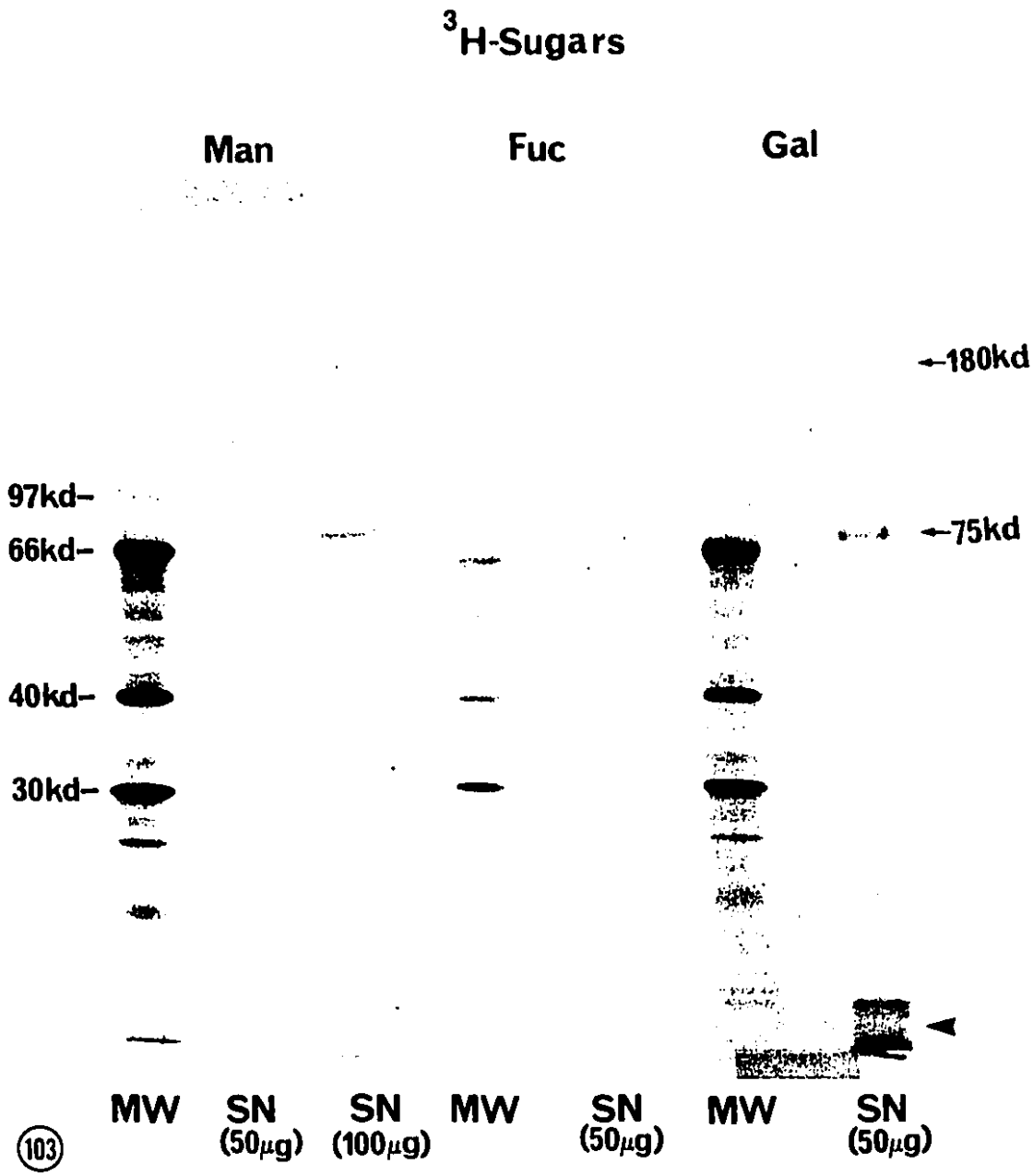

^{3}H-Sugars
Man
Fuc
Gal
180kd
75kd
97kd
66kd
40kd
30kd
MW
SN (50μg)
SN (100μg)
MW
SN (50μg)
MW
SN (50μg)
103

Fig 104 Distribution of radioactivity (DPM) in 2 mm gel slices of lanes of stripped nuclei fractions (50 μg protein/lane) separated by SDS-PAGE. The stripped nuclei were isolated from myeloma cells incubated for 18 hrs with 5 μCi/ml ^{3}H-galactose (Fig. 104a), ^{3}H-fucose (Fig.104b) or ^{3}H-mannose (104c) as described in Materials and Methods. Molecular weights as determined from standards are indicated by the arrowheads.

Numerous peaks of radioactivity were observed in gel slices of stripped nuclei proteins from ^{3}H-gal-labeled cells (Fig 104a). The most prominent high molecular weight peak corresponded to a protein of ~75 kd. Several other moderate peaks were evident in the molecular weight range between 30-100 kd. A small but distinct peak was observed at ~180 kd. A very large peak of radioactivity was detected at very low molecular weight.

The peaks of radioactivity observed in gel slices of stripped nuclei proteins ^{3}H-fuc-labeled cells (Fig. 104b) were much smaller, on the whole, and less numerous than those seen in Fig 104a. The largest high molecular weight peak was seen at ~75 kd. Smaller peaks are observed at ~95 kd and 180 kd. A small amount of radiactivity was observed to be scattered throughout low molecular weights between 30-70 kd. Again, the largest peak of radioactivity of the entire lane was again found at very low molecular weight.

In Fig. 104c, the radioactivity peaks observed in gel slices of stripped nuclei proteins from ^{3}H-man-labeled cells are very small but correspond roughly to the peaks in 104b. The most noticeable peak was again at ~75 kd with another peak at slightly higher molecule weight at ~80 kd and one at ~180 kd. At lower molecular weight, only one peak at ~30 kd was distinguishable in gel slices apart from the very end of the gel lane. As in the upper two figures, the largest peak of radioactivity in the lane was observed at very low molecular weight.

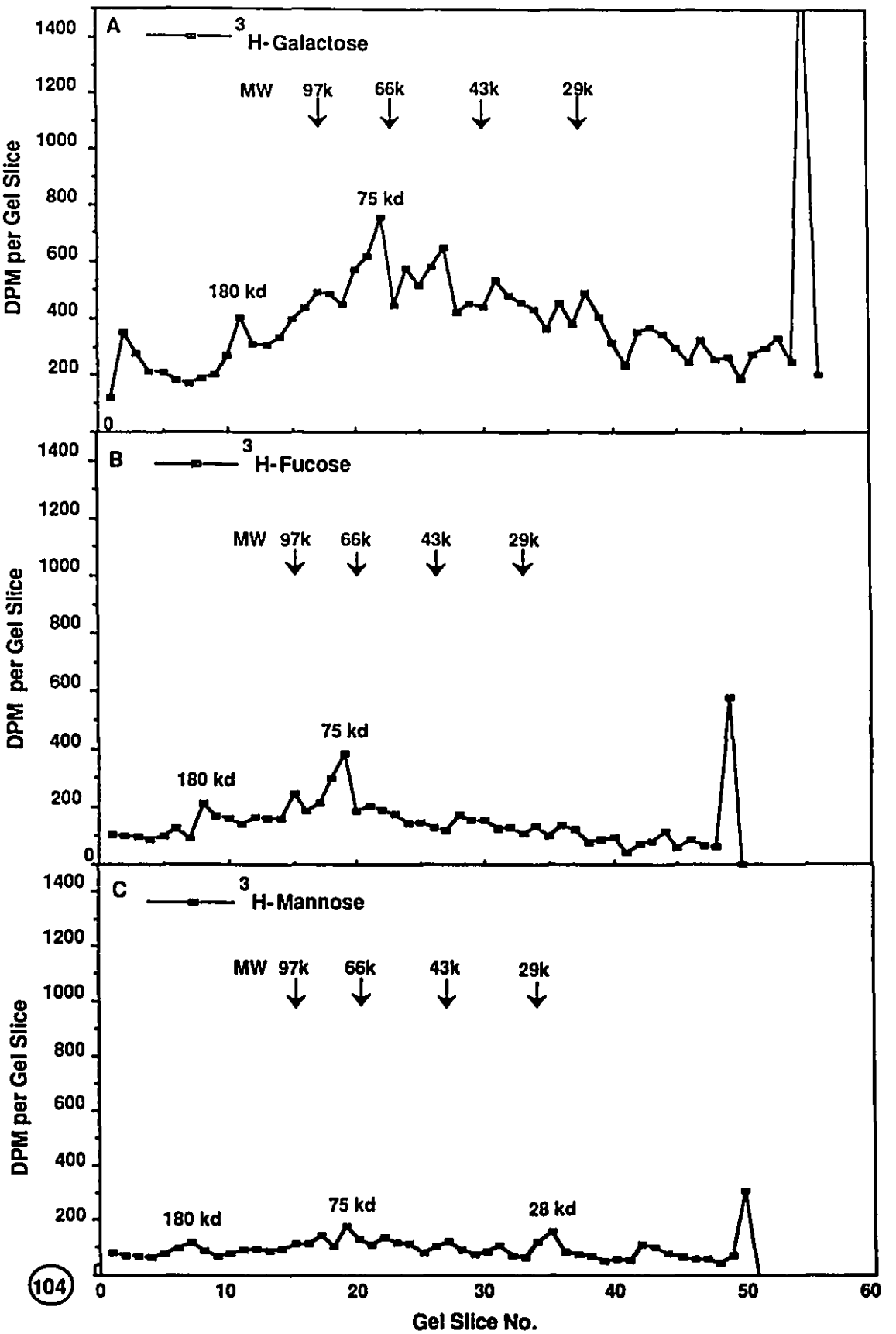
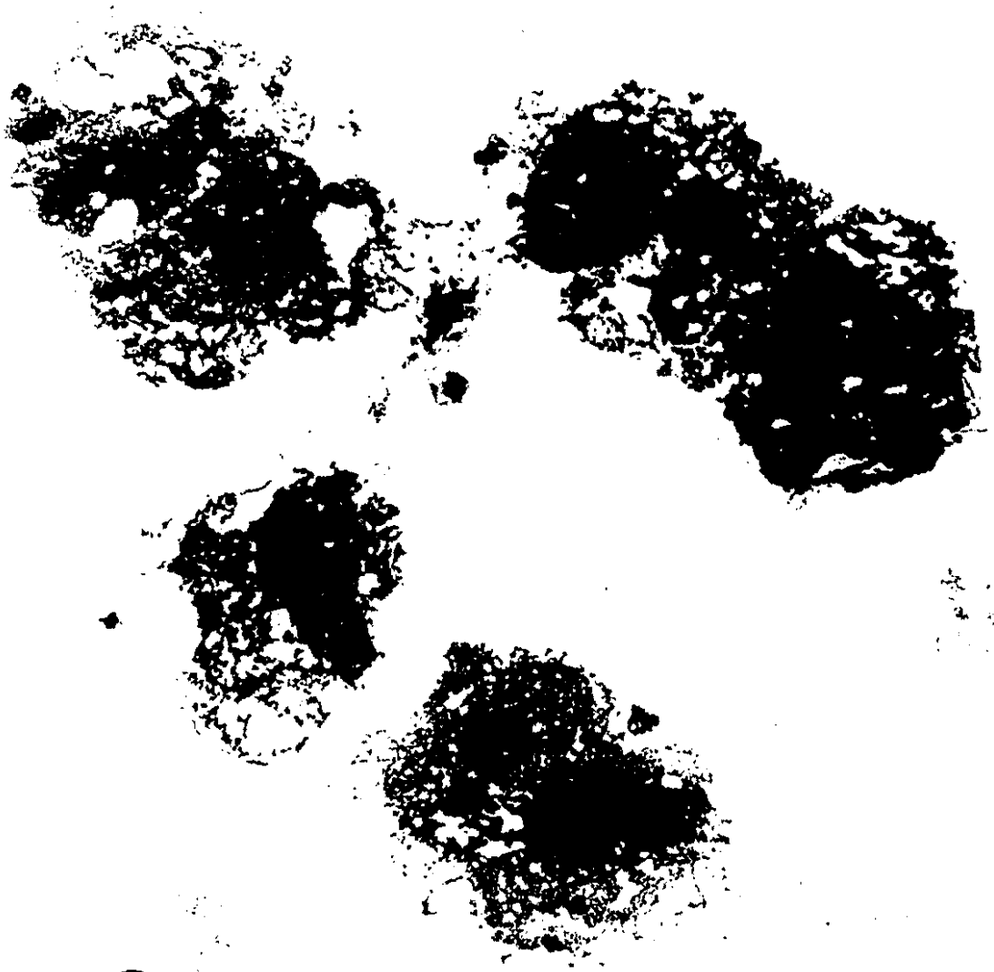

A
3H-Galactose
MW 97k 66k 43k 29k
75 kd
180 kd
B
3H-Fucose
MW 97k 66k 43k 29k
75 kd
180 kd
C
3H-Mannose
MW 97k 66k 43k 29k
180 kd
75 kd
28 kd
DPM per Gel Slice
0 200 400 600 800 1000 1200 1400
0 10 20 30 40 50 60
Gel Slice No.

Fig. 105 EM radioautograph of stripped nuclei which were subsequently treated with DNAse and extracted with low salt solution.

These nuclei appear much more condensed than the typical stripped nuclei seen in Figs. 91+92, appear-ing to consist mainly of heterochromatin and nucleoli (n). They remain substantially labeled, nonetheless, and several of the grains are associated with the nucleoli.

Exposure: 7 wks (Chem. dev.) x 8,500



105

Figs. 106-107 EM radioautograph of stripped nuclei which were subsequently treated with DNAse and extracted with high salt solution.

In this preparation, many nuclei have become disrupted, and those that remain recognizable (N) appear much more extracted than those nuclei in Fig. 105. The nuclei remain heavily labeled, however, and the silver grains are distributed throughout their structure (Fig 106). Grains are also localized over circular profiles of material which probably represent fibrous laminae (FL) of nuclei which have lost their content (Figs. 106 and 107).

Exposure: 7 wks (Chem. dev.) x 10,000


FL
n
RN
106
107

Fig. 108 Distribution of radioactivity (DPM) in 2 mm polyacrylamide gel slices of lanes of nuclear fractions (50 μg protein/lane) separated by SDS-PAGE. The nuclear fractions were derived from further fractionation of stripped nuclei isolated from myeloma cells incubated for 18 hrs with 5 μCi/ml ^{3}H-galactose as described in Materials and Methods. Molecular weights as determined from standards are indicated by the arrowheads.

Fig. 108a, showing distribution of radioactivity in gel slices of stripped nuclei fraction proteins, is identical to Fig. 104a but is included here for reference purposes for Figures 108b,c, and d. As earlier described, numerous peaks radioactivity were observed in gel slices of this fraction, the largest high molecular weight peak corresponding to a molecular weight of ~75 kd.

In 0.15 M NaCl nuclear extracts (low salt), only a few small peaks of radioactivity were observed (Fig 108b). The largest peak in the high molecular weight range did correspond to a molecular of ~75 kd but was much smaller than that observed in the stripped nuclei fractions. Other smaller peaks were scattered between 30-100 kd. A peak of radioactivity was also observed at very low weight.

In 2.0 M NaCl nuclear extracts (high salt), mostly small peaks of radioactivity were observed with two exceptions. In the high molecular weight range, a distinct peak of ~95 kd was observed which was much larger than any others in this region. Other small peaks were observed between 30-100 kd. An especially large peak of radioactivity was located in the very low molecular weight range of the gel.

In residual nuclei fractions representing the nuclear matrix, numerous large peaks of radioactivity were observed between 30-100 kd, the largest corresponding to a molecular weight of 75 kd. This peak was approximately double that observed at the same molecular weight in the stripped nuclei fraction. An enormous peak of radioactivity was again observed in the very low molecular weight region.

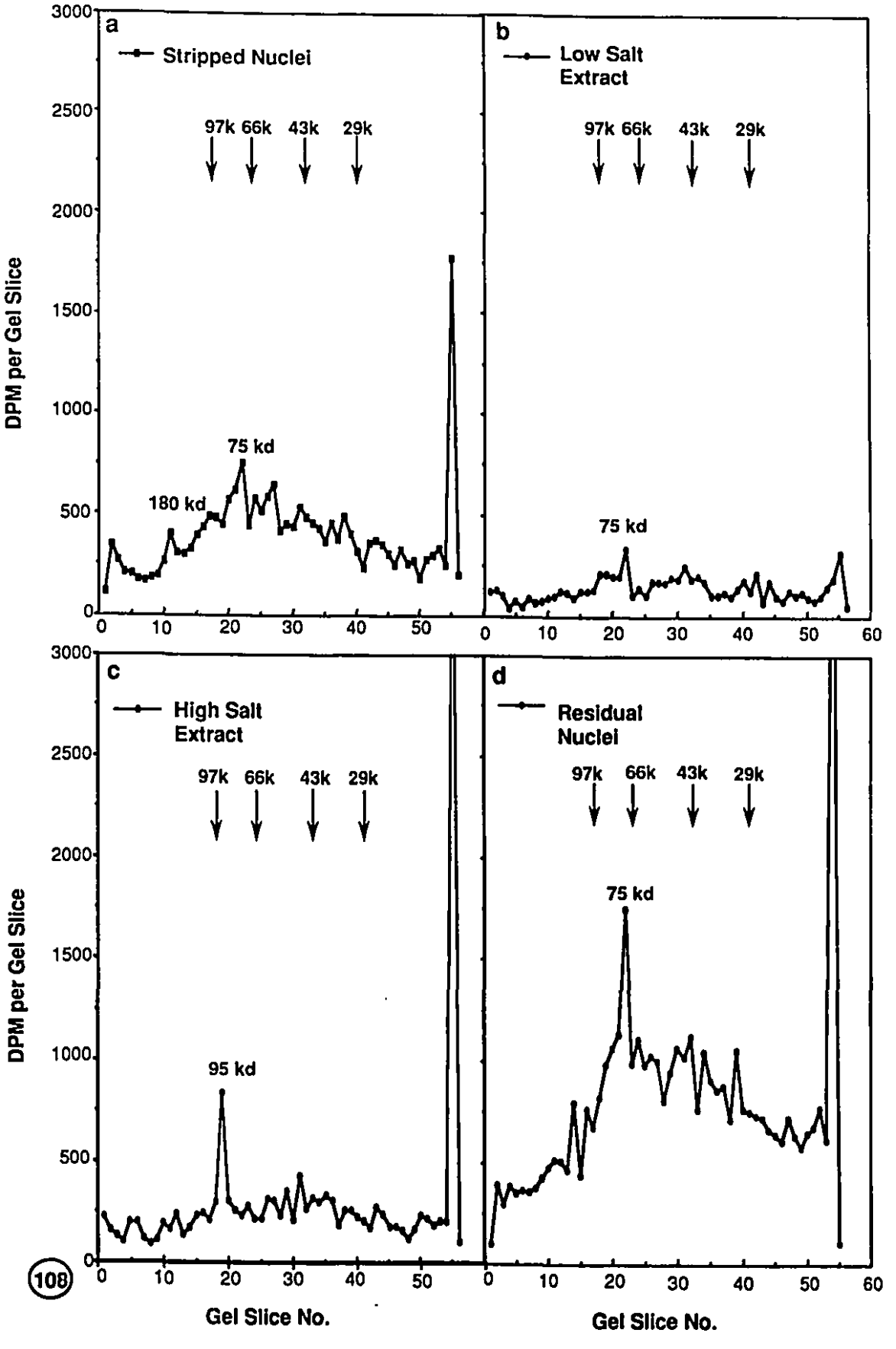

a
Stripped Nuclei
97k 66k 43k 29k
180 kd
75 kd
b
Low Salt Extract
97k 66k 43k 29k
75 kd
c
High Salt Extract
97k 66k 43k 29k
95 kd
d
Residual Nuclei
97k 66k 43k 29k
75 kd
DPM per Gel Slice
Gel Slice No.
Gel Slice No.

Fig. 109

Lectin blot of stripped nuclei fraction proteins probed with RCA I. Proteins were separated by SDS-PAGE on a 5-15% gel (100 µg/lane) and transferred to nitrocellulose membranes. Blots were incubated with 5 µg/ml biotinylated RCA I followed by incubation with Extravidin-peroxidase and then colorimetrically developed with chloronapthol as substrate as described in Materials and Methods.

In the stripped nuclei fractions (SN) a number of bands were clearly visible (Lane 3). These were observed as a single band at ~75 kd, a doublet at 67-68 kd and a single band at 53 kd. The positive control lactosyl-BSA (Lac-BSA) showed a strong reaction at ~80 kd (Lane 2). The labeling in negative control preparations (Control) with 0.2 M galactose added to the RCA-I was almost completely abolished (Lanes 4+5). Molecular weight markers (MW) are shown in lane 1.

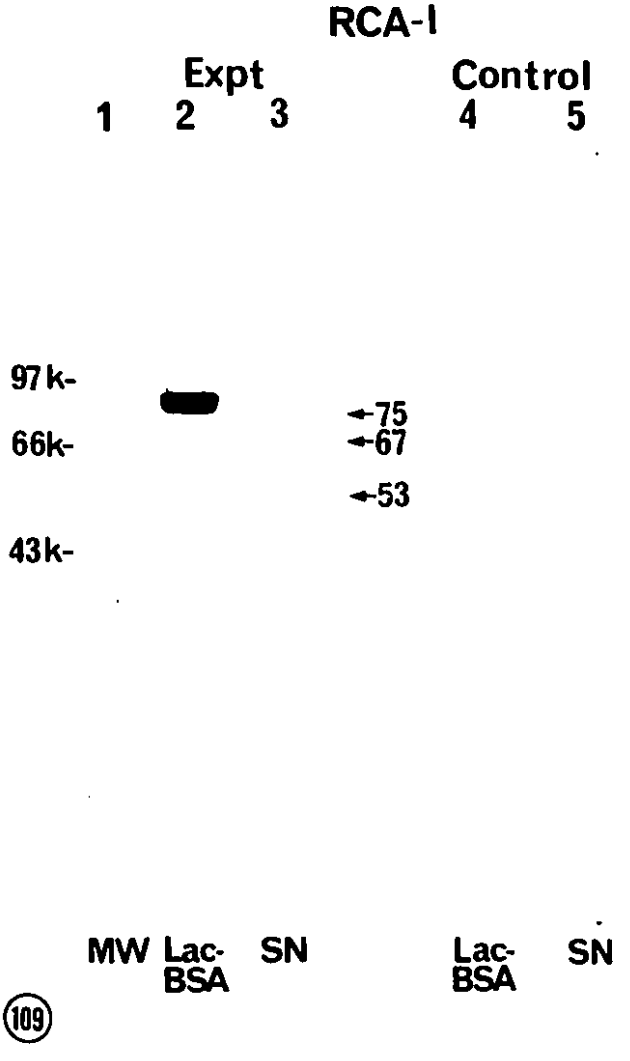

RCA-I
Expt
Control
1
2
3
4
5
97k-
66k-
43k-
75
67
53
MW
Lac-BSA
SN
Lac-BSA
SN

Fig. 110

Lectin blot of residual nuclei (RN) fraction proteins (representing the nuclear nuclear matrix) probed with RCA I lectin. Protein samples were separated by SDS-PAGE on an 8% slab gel (25 μg/lane) and transferred to nitrocellulose membranes. Blots were incubated with 5 μg/ml biotinylated RCA I followed by an incubation with Extravidin-peroxidase and colorimetrically developed using chloronapthol as substrate as described in Materials and Methods.

RCA I-binding proteins were observed as a number of light but identifiable bands in experimental preparation (Expt) as indicated by the arrowheads at ~31 kd, 48 kd, 60 kd, 67 kd, 75 kd, 92 kd and 120 kd (Lane 3). The Lac-BSA positive control again showed a strong reaction at 80 kd (Lane 2). In negative control experiments (Control), when 0.2 M galactose was added prior to incubation, labeling was totally abolished (Lanes 4+5). Lane 1 shows molecular weights (MW).

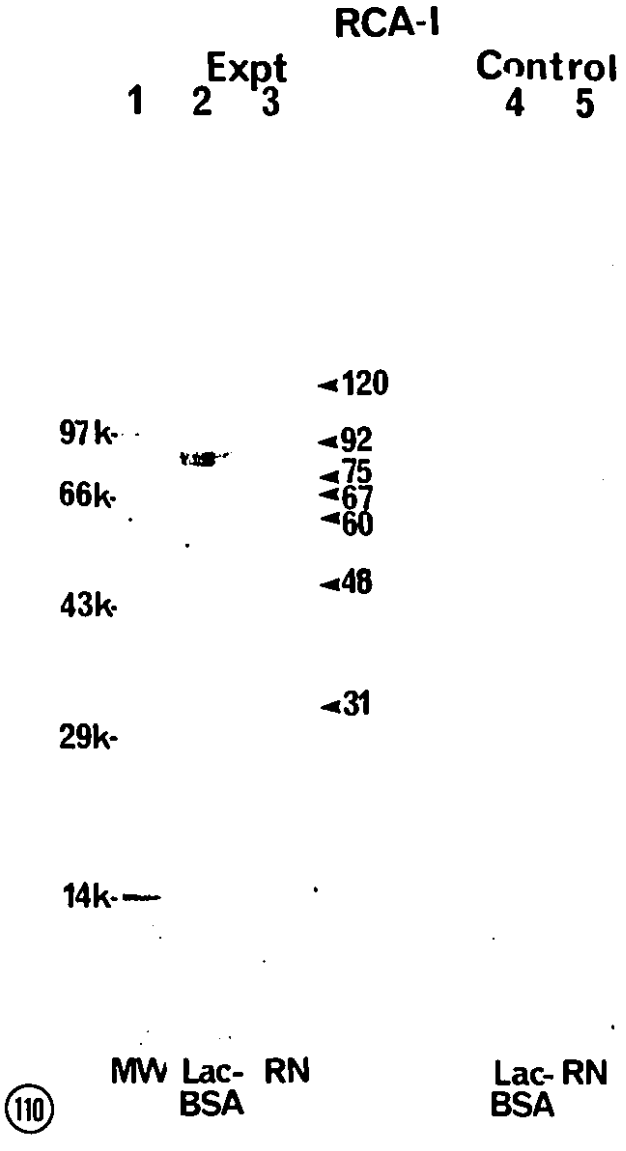

RCA-I
Expt
Control
1
2
3
4
5
120
97k
92
75
66k
67
60
46
43k
31
29k
14k
MW
Lac-BSA
RN
Lac-BSA
RN
110

Fig. 111(a)

Lectin blot of nuclear matrix proteins probed with RCA I lectin. Protein samples were either treated (+) or untreated (-) with N-glycanase F enzyme, separated by SDS-PAGE on 8% slab gels (25 μg/lane) and transferred to nitrocellulose paper. Blots were incubated with 5 μg/ml RCA I-digoxigenin followed antidigoxigenin-AP and colorimetrically developed using NBT and BCIP as substrates as described in Materials and Methods.

As in Fig 110, a number of RCA I reactive bands were observed in untreated nuclear matrix protein samples (Lane 6). Lane 5 shows RCA I reaction with an identical sample of the proteins treated with N-glycanase F. RCA I reactive bands which were affected by N-glycanase F treatment were observed at ~72 kd, 75 kd, 80 kd, and 94 kd as indicated by arrowheads to the right of Lane 6. Bands unaffected by N-glycanase F treatment were observed as a strong band at 68 kd, and a light band at 48 kd as indicated by arrowheads to the left of Lane 5.

Lane 1 shows a strong RCA I reaction at ~60 kd in untreated 1 μg sample of N-linked glycoprotein asialofetuin as indicated by the arrowhead to the left of this lane. Lane 2 shows a identical asialofetuin sample treated with N-glycanase F. The RCA I reactivity is oberved to be severely diminished.

In negative controls (Lanes 3+4 and 7+8), where 0.2 M lactose was added to the lectin immediately prior to incubation, reactivity was completely abolished.

(b)

Lectin blot of nuclear matrix proteins probed with MAA lectin. Protein samples were separated by SDS-PAGE on 8% slab gels (25 μg/lane) and transferred to nitrocellulose membranes as above. 10 μg/ml MAA-digoxigenin was used for the incubations followed by incubation with antidigoxigenin-AP and finally NBT and BCIP.

MAA-binding proteins were observed as a well-defined cluster of at least 3 bands between 72-76 kd (Lane 1). A light band is observed at ~48 kd. In a negative control where 200 μM fetuin was added to MAA prior to incubation, reaction was totally abolished (Lane 2).

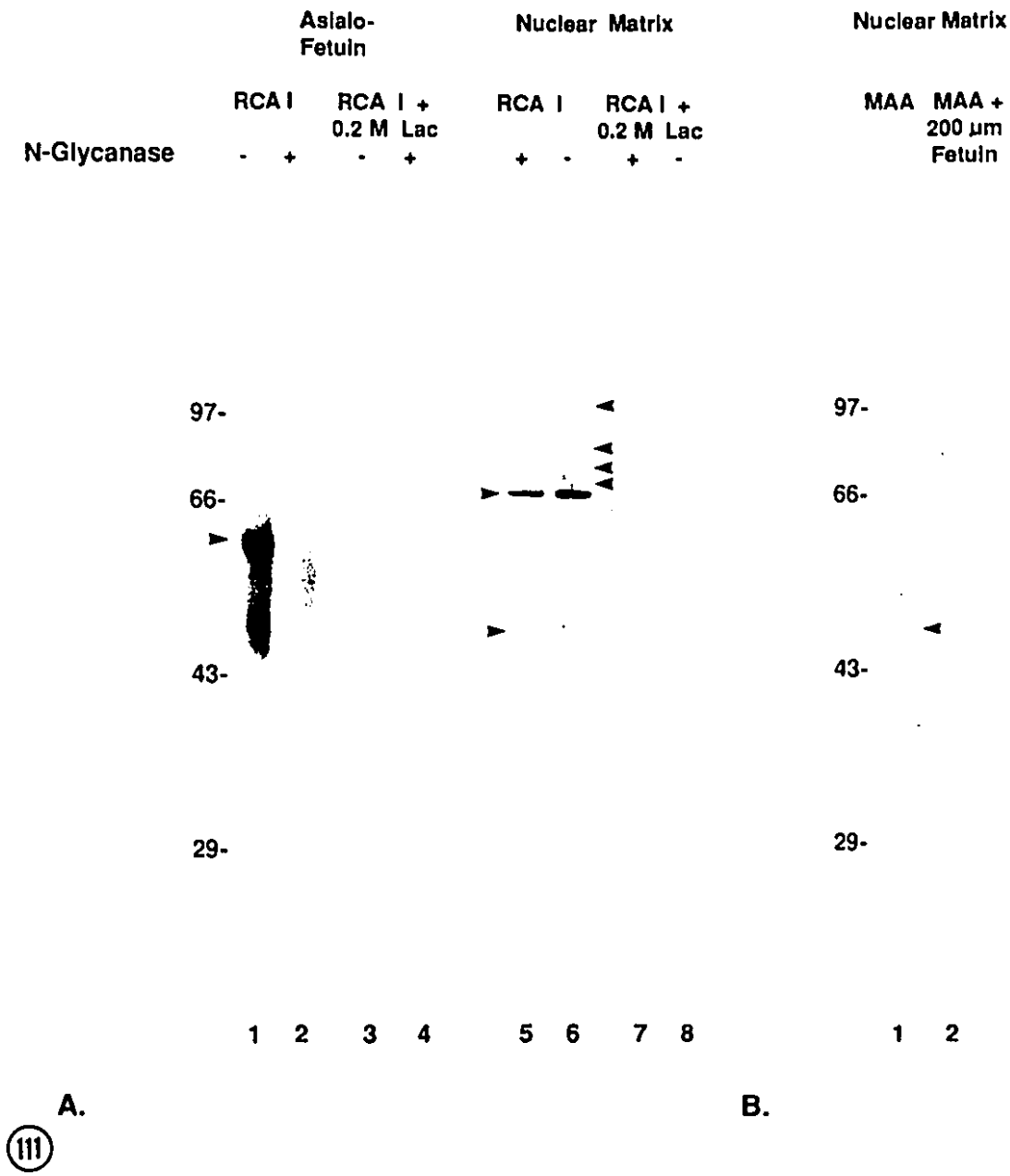

Asialo-
Fetuin
Nuclear Matrix
Nuclear Matrix
RCA I
RCA I +
0.2 M Lac
RCA I
RCA I +
0.2 M Lac
MAA
MAA +
200 µm
Fetuin
N-Glycanase
- + - + + - + -
97-
66-
43-
29-
1 2 3 4 5 6 7 8
1 2
A.
B.

Fig. 112(a)

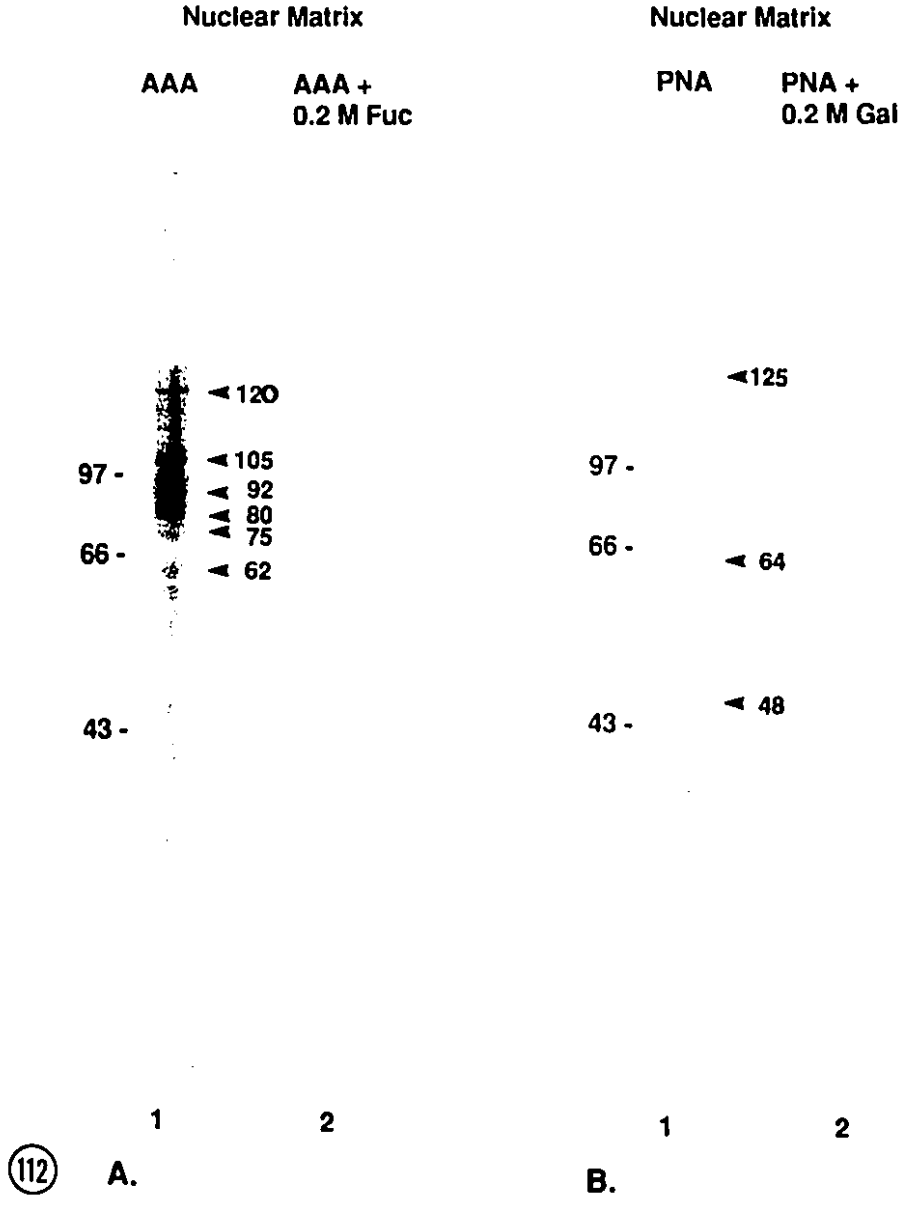
Lectin blot of nuclear matrix proteins probed with AAA lectin. Protein samples were separated by SDS-PAGE on 8% gels (40 μg/lane) and transferred to nitrocellulose membranes. 10 μg/ml AAA-digoxigenin was used for the incubations followed by incubation with antidigoxigenin-AP and then NBT and BCIP as previously described.

AAA-binding proteins are observed as a number of diffuse but identifiable bands at 62 kd, 75 kd, 80 kd, 92 kd, 95 kd, 105 kd and 120 kd as indicated by arrowheads (Lane 1). Lane 2 shows control reaction where 0.2 M L-fucose was added to the lectin before incubation. Labeling was completely abolished.

(b)

Lectin blot of nuclear matrix proteins probed with PNA lectin. Protein samples were separated by SDS PAGE on 8% slab gels (40 μg/lane), and transferred to nitrocellose membranes. 10 μg/ml PNA-digoxigenin was used for the incubations followed by incubation with antidigoxigenin-AP conjugate and NBT and BCIP as previously described.

PNA-binding proteins were observed as a light staining band at 48 kd, a doublet at 62 and 64 kd, and a single 125 kd band as indicated by arrowheads (Lane 1). Lane 2 shows control reaction where 0.2 M galactose was added to the lectin before incubation where labeling was abolished.


Nuclear Matrix
AAA
AAA +
0.2 M Fuc
120
105
92
80
75
62
97 -
66 -
43 -
1
2
A.
Nuclear Matrix
PNA
PNA +
0.2 M Gal
125
64
48
97 -
66 -
43 -
1
2
B.

(112)

I. Summary and General Conclusions

The work described in this thesis entailed the study of a number of aspects related to the presence of labeled molecules within nuclei of animal cells after exposure to ^{3}H-sugars or ^{35}S-sulphate, suggestive of the presence of newly synthesized nucleoplasmic glycoproteins.

Firstly, the tissue distribution of cells containing such labeled nucleoplasmic molecules after exposure to the above radiolabeled precursors was examined in a wide variety of animal cell types with the specific goal to try to determine whether or not nucleoplasmic glycoproteins might be common, or even ubiquitous, to animal cells. The radioautographic studies of Chapter 2 showed that after exposure to either ^{3}H-sugars or ^{35}S-sulphate, RAG reaction was present to some extent over nuclei of almost all cells examined. The known metabolic fates of the ^{3}H-sugars and ^{35}S-sulphate suggest that some or all of the nucleoplasmic label resided in glycoproteins containing a variety of sugar residues (e.g. fucose, galactose, mannose, glcNAc, and sialic acid), as well as sulfate residues. Thus, it was suggested that nucleoplasmic glycoproteins are indeed a common cellular feature. Further, variations in the pattern of nucleoplasmic labeling among different cell types also suggested a heterogeny in the type or level of expression of nucleoplasmic glycoproteins depending on the cell type.

A second aspect of the study focussed on the localization of the labeled molecules (or putative nucleoplasmic glyco-

proteins) within the nucleus. In quantitative radioautographic studies in which cultured cells were exposed to ^{3}H-sugars or ^{35}S-sulfate, label was localized to various subcompartments of the nucleus, including euchromatin and heterochromatin (and in some cases to the interface between these two compartments), nucleoli, and the nuclear envelope. Observations made on isolated nuclei from cells exposed to ^{3}H-sugars indicated no substantial changes in either the intensity or pattern of distribution of the nucleoplasmic label, providing confirmatory evidence that most, if not all, of the radioautographic reaction observed over nuclei of the undisrupted cells was actually derived from nucleoplasmic sources. This included reaction observed over the periphery of the nucleus.

The third and final aspect of the study focussed on the nature of the nucleoplasmic molecules incorporating label. The results of these studies indicated that the label in nuclei of myeloma cells exposed to ^{3}H-gal, ^{3}H-fuc, or ^{3}H-man was present in a variety of proteins of different molecular weights, most notably one of 75 kd. In cells exposed to ^{3}H-gal, further subfractionation of the nuclei showed that much of the label was present in nuclear matrix proteins. Lectin binding experiments on the matrix preparations provided evidence of the presence of proteins containing galactose, sialic acid, and fucose. Experiments using N-glycanase F in conjunction with the lectin binding indicated that some but not all of the sugar sidechains were N-linked to the proteins.

In conclusion, this work represents an addition to our knowledge of what is still an ill-defined group of glycoproteins. While no exact function(s) could be derived for the glycoproteins described in these studies, the indicated association of at least some of them with the functionally significant nuclear matrix compartment suggests that they could have important regulatory roles within the nucleus. The actual elucidation of the function(s) of these glycoproteins and the glycosyl groups themselves represents a worthy but formidable challenge for the future because of the various technical difficulties inherent to working with nuclear proteins (especially nuclear matrix proteins), but as investigators are now more aware that any given nuclear protein could realistically be glycosylated, progress along these lines in the future will likely be steady.

II. Original Contributions to the Literature

1) This study provides the first radioautographical evidence that nucleoplasmic glycoproteins are a common if not ubiquitous feature of animal cells. This is indicated by nucleoplasmic labeling of almost all cells exposed to ^{3}H-sugars combined with the evidence that in a model cell type most or all of the RAG reaction over the nucleoplasm of cells after exposure to the labeled sugars is truly derived from sources residing in the nucleoplasm.

2) This work provides the first quantitative radioautographical description of the distribution of labeled (newly

glycosylated) glycoproteins in nuclear subcompartments of intact cells after exposure to ^{3}H-sugars which include the euchromatin and heterochromatin (and in certain cases prominent localization to the H/E interface), the nucleolus and the nuclear envelope.

3) This work is the first to provide radioautographic evidence for the presence of galactosylated glycoproteins in the nuclear matrix which are located both in the peripheral and central regions of the matrix including the residual nucleolus.

4) This work provides biochemical evidence of a novel 75 kd glycoprotein present in stripped nuclei extracts which contains galactose, fucose, and mannose.

5) This work provides biochemical evidence of novel glycoproteins residing within the nuclear matrix, containing galactose and/or sialic acid. This is the first report to my knowledge sialylated glycoproteins to the nuclear matrix. This study also includes the first description of nuclear glycoproteins with fucose residues attached to core glcNAc of N-linked sugar sidechains.

6) This work also is the first to provide evidence of glycoproteins associated with the nuclear matrix that contain different glycosidic linkages as indicated by lectin binding studies in the presence or absence of N-glycanase F.

References

C. Abeijon and C. B. Hirschberg, Intrinsic membrane glycoproteins with cytosol-orientated sugars in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 85: 1010-1014 (1988).

C. W. Akey and D. S. Goldfarb, Protein import through the nuclear pore complex is a multistep process. J. Cell Biol. 109: 971-982 (1989).

D. A. Aquino, R. U. Margolis and R. K. Margolis, Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. I. Adult brain, retina, and peripheral nerve. J. Cell Biol. 99: 1117-1129 (1984a).

D. A. Aquino, R. U. Margolis and R. K. Margolis, Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. II. Studies in developing brain. J. Cell Biol. 99: 1130-1139 (1984b).

J. P. Aris and G. Blobel, Yeast nuclear envelope proteins cross react with an antibody against mammalian pore complex proteins. J. Cell Biol. 108: 2059-2067 (1989).

F. A. Baglia and G. G. Maul, Nuclear ribonucleoprotein release and nucleoside triphosphatase activity are inhibited by antibodies directed against one nuclear matrix glycoprotein. Proc. Natl. Acad. Sci. USA 80: 2285-2289 (1983).

J. R. Bartles, L. T. Braiterman, and A. L. Hubbard, Biochemical characterization of domain-specific glycoproteins of the rat hepatocyte plasma membrane. J. Biol. Chem. 260: 12792-12802 (1985).

J. G. Bekesi and R. J. Winzler, The metabolism of plasma glycoproteins. Studies on the incorporation of L-fucose ^{14}C- into tissue and serum in the normal rat. J. Biol. Chem. 242: 3873-3879 (1973).

M. Bendayan and N. Benhamou, Ultrastructural localization of glucoside residues on tissue sections by applying the enzyme-gold approach. J. Histochem. Cytochem. 35: 1149-1155 (1987).

D. M. Benko, R. S. Haltiwanger, G. W. Hart and W. Gibson, Virion basic phosphoprotein from human cytomegalovirus contains O-linked N-acetylglucosamine. Proc. Natl. Acad. Sci. USA 85: 2573-2577 (1988).

G. Bennett, and D. O'Shaughnessy, The site of incorporation of sialic acid residues into glycoproteins and the subsequent fate of these molecules in various rat and mouse cell types as shown by radioautography after injection of [^{3}H]N-acetylmannosamine. I. Observations in hepatocytes. J. Cell Biol. 88: 1-15 (1981).

G. Bennett and A. Haddad, Synthesis and migration of ^{3}H-fucose-labeled glycoproteins in the ciliary epithelium of the eye: Effects of microtubule-disrupting drugs. Am. J. Anat. 177: 441-455 (1986).

G. Bennett, R. Hemming and P. A. Lavoie, Presence of glycoproteins in the cell nucleus as shown by radioautographic studies after administration of ^{3}H-fucose and ^{3}H-galactose. Eur. J. Cell Biol. 42: 246-254 (1986).

G. Bennett, Radioautographic and cytochemical studies on the synthesis and intracellular transport of glycoproteins. Acta Histochem. Suppl. Band 36: 9-49 (1988).

G. Bennett and G. Wild, Traffic through the Golgi pparatus as studied by radioautography. J. Electron Microscopy Technique 17: 121-149 (1991).

V. Bennett, The membrane skeleton of human erythrocytes and its implications for more complex cells. Ann. Rev. Biochem. 54: 273-304 (1985).

K. G. Bensch, S. Tanaka, S-Z. Hu, T. S-F. Wang, and D. Korn, Intracellular localization of human DNA polymerase α with monoclonal antibodies. J. Biol. Chem. 257: 8391-8396 (1982).

R. Berezney and D. S. Coffey, Nuclear matrix: Isolation and characterization of a framework structure from rat liver nuclei. J. Cell Biol. 73: 616-637 (1977).

R. Berezney, The nuclear matrix: A heuristic model for investigating genomic organization and function in the cell nucleus. J. Cell. Biochem. 47: 109-123 (1991).

G. Berger, E. Buddecke, J. P. Kammerling, A. Kobata, and J. F. G. Vliegenthart, Structure, biosynthesis, and functions of glycoprotein glycans. Experientia 15: 1129-1258 (1982).

A. Bergman and G Dallner, Distribution of protein-bound sugar residues in microsomal subfractions and Golgi membranes. Biochim. Biophys. Acta 433: 496-508 (1976).

B. Bernard, M. C. Pugliarello, G. Sandri, G. L. Sottocasa

and F. Vittur, Glycoprotein components, sialic acid and hexosamines, bound to inner and outer mitochondrial membranes. FEBS Letters 12: 125-128 (1971).

G. Berthillier, J. P. Benedetto and R. Got, Présence de glycosyltransferases à accepteurs chromatiniens dans les membranes nucléaires d'hepatocytes de singe. Biochim. Biophys. Acta 603: 245-254 (1980).

G. Berthillier and R. Gott, Evidence for the mannosylation of a non-histone protein in monkey liver chromatin. Mol. Cell. Biochem. 44: 39-43 (1982).

E. C. Beyer and S. H. Barondes, Chicken tissue binding sites for a purified chicken lectin. J. Supramol. Struct. 13: 219-227 (1980).

P. Bhattacharya and S. Basu, Probable involvement of a glycoconjugate in IMR-32 DNA sunthesis: Decrease of DNA polymerase α2 activity after tunicamycin treatment. Proc. Natl. Acad. Sci. USA 79: 1488-1491 (1982).

P. Bhattacharya, I. Simet and S. Basu, Differential inhibition of multiple forms of DBA polymerase α from IMR-32 human neuroblastoma cells. Proc. Nat. Acad. Sci. USA 78: 2683-2687 (1981).

P. Bhattacharya, I. Simet and S. Basu, Inhibition of human neuroblastoma DNA polymerase activities by plant lectins and toxins. Proc. Batl. Acad. Sci. USA 76: 2218-2221 (1979).

V. P. Bhavanandan and E. A. Davidson, Mucopolysaccharides associated with nuclei of cultured mammalian cells. Proc. Natl. Acad. Sci. USA 72: 2032-2036 (1975).

J. Bischoff and R. Kornfeld, The soluble form of rat liver α-mannosidase is immunologically similar related to the ER membrane α-mannosidase. J. Biol. Chem. 261: 4758-4765 (1986).

A. M. Bolognani Fantin, G. Menghi, A. Franchini, A. M. Bondi, D. Accili, and A. M. Fuhrman Conti, Characterization of glycoconjugates in an embryonic human epithelial line and changes consequent to adaptation to a hyperosmotic medium. Histochem. J. 21: 79-88 (1989).

N. C. Bols, M. M. Roberson, P. L. Haywood-Reid, R. F. Cerra and S. H. Barondes, Secretion of a cytoplasmic lectin from Xenopus laevis skin. J. Cell Biol. 102: 492-499 (1986).

H. B. Bosmann and R. A. Winston, Syntheis of glycoprotein, glylipid, proteins and lipid in synchronized L5178Y Cells. J. Cell Biol. 45: 23-33 (1970).

C. A. Bourgeois, A. P. Sève, M. Monsigny and J. Hubert, Detection of sugar binding sites in the fibrillar and the granular components of the nucleolus: an experimental study in cultured mammalian cells. Exp. Cell Res. 172: 365-376 (1987).

M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254 (1976).

V. H. C. Bramwell, D. Crowther, J. Gallagher and R. W. Stoddart, Studies of lectin binding to normal and neoplastic lymphoid tissues. I. Normal nodes and Hodgkin's disease. Brit. J. Canc. 46: 568-581 (1982).

A. E. Brown, E. L. Schwartz, R. N. Dreyer and A. C. Sartorelli, Synthesis of sialoglycoconjugates during dimethylsulfoxide-induced erythrodifferentiation of Friend leukemia cells. Biochim. Biophys. Acta 714: 217-225 (1982).

M. Brown, F. J. Bollum and L. M. S. Chang, Intracellular localization of DNA polymerase α. Proc. Natl. Acad. Sci. 78: 3049-3052 (1981).

A. R. Buckley, D. W. Montgomery, M. J. C. Hendrix, C. F. Zukoski, and C. W. Putnam, Identification of prolactin receptors in hepatic nuclei. Arch. Biochem. Biophys. 296: 1-8 (1992).

G. R. Burrus, W. N. Schmidt, J. A. Briggs, L. S. Hnilica and R. C. Briggs, Lectin-binding proteins in nuclear preparations from rat liver and malignent tumors. Canc. Res. 48: 551-555 (1988).

S. R. Carding, S. J. Thorpe, R. Thorpe and T. Feizi, Multiple proteins related to the soluble galactose-binding animal lectin revealed by a monoclonal anti-lectin antibody. Biochem J. 220: 253-260 (1984).

S. R. Carding, S. J. Thorpe, R. Thorpe and T. Feizi, Transformation and growth related changes in levels of nuclear and cytoplasmic proteins antigenically related to mammalian ß-galactoside-binding lectin. Biochem. Biophy. Res. Comm. 127: 680-686 (1985).

S. R. Carlsson and M. Fukuda, The lysosomal membrane

glycoprotein Lamp-1 is transported to lysosomes by two alternative pathways. Arch. Biochem. Biophys. 296: 630-639 (1992).

M. Carmo-Fonseca, Androgen-dependent nuclear proteins in rat ventral prostate are glycoproteins associated with the nuclear matrix. Cell Biol. Int. Rep. 12: 607-620 (1988).

R. F. Cerra, P. L. Haywood-Reid and S. H. Barondes, Endogenous mammalian lectin localized extracellularly in lung elastic fibers. J. Cell Biol. 98: 1580-1589 (1984).

L. Cervoni, A. Ferraro, M. Eufemi and C. Turano, Fucose-carrying nuclear glycoproteins: distribution and tissue specificity. Italian J. Biochem. 39: 368-374 (1990).

N. Chaly, T. Bladon, G. Setterfield, J. E. Little, J. G. Kaplan, and D. L. Brown, Changes in distribution of nuclear matrix antigens during the mitotic cell cycle. J. Cell Biol. 99: 661-671 (1984).

N. Chaly, M. P. Sabour, J. C. Silver, W. A. Aitchison, J. E. Little and D. L. Brown. Monoclonal antibodies against nuclear matrix detect nuclear antigens in mammalian, insect and plant cells. Cell Biol. Int. Rep. 10: 421-428 (1986).

R. A. Childs and T. Feizi, ß-galactoside-binding lectin of human and bovine tissues. Cell Biol. Int. Rep. 4: 775 (1980).

L. Cocco, N. M. Maraldi, F. A. Manzoli, R. S. Gilmour and A. Land, Phospholipid interaction in rat liver nuclear matrix. Biochem. Biophys. Res. Comm. 96: 890 (1980).

B. M. Curtis, M. B. Widmer, P. deRoos, and E. E. Qwarnstrom. Il-1 and its receptor are translocated to the nucleus. J. Immunology 144: 1295-1303 (1990).

M. D'Erme and P. Caiafa, Localization of tightly bound non-histone glycoproteins in the third level of chromatin organization. Cell. Mol. Biol. 33: 755-765 (1987).

M. C. Dabauvalle, R. Benavente and N. Chaly, Monoclonal antibodies to a Mr 68,000 pore complex glycoprotein interfere with nuclear protein uptake in Xenopus oocytes. Chromosoma 97: 193-197 (1988a).

M. C. Dabauville, B. Schulz, U. Scheer and R. Peters, Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin Wheat Germ Agglutinin. Expt. Cell Res. 174: 291-296 (1988b).

L. Davis and G. Blobel, Identification and characterization of a nuclear pore complex protein. Cell 45: 699-709 (1986).

L. Davis and G. Blobel, Nuclear pore complex contains a family of glycoproteins that include p62: glycosylation through a previously unidentified cellular pathway. Proc. Natl. Acad. Sci. USA 84: 7552-7556 (1987).

S. Diaz and A. Varki, Metabolic labeling of sialic acids in tissue culture cell lines: Methods to identify substituted and modified radioactive neuraminic acids. Anal. Biochem. 150: 32-46 (1985).

C. Dingwall and R. A. Laskey, Protein import into the cell nucleus. Annu. Rev. Cell Biol. 2: 367-390 (1986).

G. H. Dixon, The HMG proteins of rainbow trout testis nuclei: Isolation, structure, and function. In "The HMG Chromosomal Proteins" (Johns E. W. ed., pp. 149-192 (1982).

H. Egami, W. Chaney, Y. Takiyama and P. Pour, Subcellular localization of blood group A substances produced bypancreatic adenocarcinoma induced in hamsters by N-nitrosobis (2-oxopropyl)amine (BOP) and by its cell line (PC-1). Carcinogenesis 12: 509-514 (1991).

D. C. Eischler, P. A. Fisher and D. Korn, Effect of calcium on the recovery and distribution of DNA polymaerase α from cultured human cells. J. Biol. Chem. 252: 4011-4014 (1977).

A. D. Elbein, Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annu. Rev. Biochem. 56: 497-534 (1987).

T. Elton and R. Reeves, Purification and postsynthetic modifications of Friend erythroleukemic cell high mobility group protein HMG-I. Anal. Biochem. 157: 53-62 (1986).

P. Engelhardt, U. Plagens, I. B. Zbarsky and L. S. Filatova, Granules 25-30 nm om diameter: Basic constituent of the nuclear matrix, chromosome scaffold, and nuclear envelope. Proc. Natl. Acad. Sci. USA 79: 6937-6940 (1982).

J. D. Esko, A. Elgavish, T. Prasthofer, W. H. Taylor, and J. L. Weinke, Sulfate transport-deficient mutants of Chinese Hamster Ovary cells. Sulfation of glycosaminoglycans dependent on cysteine. J. Biol. Chem. 261: 15725-15733 (1986).

J. D. Esko, J. L. Weinke, W. H. Taylor, G. Ekborg, L. Roden, G. Anantharamaiah, and A. Gawish, Inhibition of chondroitin sulphate biosynthesis in Chinese Hamster Ovary Cell mutants defective in galactosyltransferase. J. Biol. Chem. 262: 12189-12195 (1987).

M. Eufemi, F. Altieri, L. Cervoni, G. Spoto and A. Ferraro, Nuclear glycoproteins in higher vertebrates. Biochem. Int. 23: 35-42 (1991).

H. Evans and J. J. M. Bergeron, Nuclear receptors: a re-evaluation. Trends Biochem. Sci. 13: 7-8 (1988).

P. Facy, A. P. Seve, M. Hubert, M. Monsigny and J. Hubert, Analysis of nuclear sugar-binding components in undifferentiated and in vitro differentiated human promyelocytic leukemia cells (HL60). Expt. Cell Res. 190: 151-160 (1990).

S. Fakan and P. Nobis, Ultrastructural localization of transcription sites and of RNA distribution during the cell cycle of synchronized CHO cells. Exp Cell Res 113: 327-337 (1978).

D. R. Farr and M. Horisberger, Structure of a ß-galactan isolated from the nuclei of Physarum polycephalum. Biochim. Biophys. Acta 539: 37-40 (1978).

M. I. Fatehi, D. Z. Gerhart, T. G. Myers and L. R. Drewes, Characterization of the blood-brain barrier: glyco-conjugate receptors of 14 lectins in canine brain, cultured endothelial cells, and blotted membrane proteins. Brain Res. 415: 30-39 (1987).

Y. Fayet, S. Galland, A. Degiuli, R. Got and J. Frot-Coutaz, Glycoprotein mannosylation in rat liver nuclei. Biochem. Int. 16: 429-438 (1988).

C. Featherstone, M. Darby and L. Gerace, A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. J. Cell Biol. 107: 1289-1297 (1988).

N. S. Fedarko and H. E. Conrad, A unique heparin sulfate in the nuclei of hepatocytes: Structural changes with the growth state of the cells. J. Cell Biol. 102: 587-599 (1986).

M. E. Feigenson, H. P. Schnebli and M. Bagiolini, Demonstration of ricin-binding sites on the outer face of azurophil and specific granules of rabbit polymorpho-nuclear leukocytes. J. Cell Biol. 66: 183-188 (1975).

A. Ferraro, L. Antonilli, M. D'Erme, M. Eufemi, M. A. Rosei and G. Spoto, Glycoprotein distribution in non-histone chromatin proteins from pig liver. Ital. J. Biochem. 37: 213-218 (1988).

A. Ferraro, M. Eufemi, L. Cervoni, R. Marinetti and C. Turano, Glycosylated forms of nuclear lamins. FEBS Lett. 257: 241-246 (1989a).

A. Ferraro, M. Eufemi, F. Altieri and C. Turano, Tissue specificity of chromatin glycoproteins recognized by concanavalin A. Biochem. Int. 18: 405-414 (1989b).

A. Ferraro, P. Grandi, M. Eufemi, F. Altieri, L. Cervoni and C. Turano, The presence of N-glycosylated proteins in cell nuclei. Biochem. Biophy. Res. Comm. 178: 1365-1370 (1991).

D. Filpula, P. A. Fisher, and D. Korn, Polymerase-α. Common polypeptide core structure of three enzyme forms form human KB cells. J. Biol. Chem. 257: 2029-2040 (1982).

D. R. Finlay, D. D. Newmeyer, T. M. Price and D. J. Forbes, Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. 104: 189-200 (1987).

J. Finne, T. Krusius, R. K. Margolis and R. U. Margolis, Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain. J. Biol. Chem. 254: 10295-10300 (1979).

P. A. Fisher, M. Berrios and G. Blobel, Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of Drosophila melanogaster. J. Cell Biol. 92: 674-686 (1982).

H. G. Fromme, E. Buddecke, K. Von Figura and H. Kresse, Localization of sulfated glycosaminoglycans within cell nuclei by high-resolution autoradiography. Expt. Cell Res. 102: 445-449 (1976).

K. Furukawa and H. Terayama, Isolation and identification of glycosaminoglycans associated with purified nuclei from rat liver. Biochim. Biophys. Acta. 499: 278-289 (1977).

K. Furukawa and H. Terayama, Pattern of glycosaminoglycans and glycoproteins associated with nuclei of regenerating liver of rat. Biochim. Biophys. Acta. 585: 575-588 (1979).

H. E. Gabius, S. Bodanowitz and A. Schauer, Endogenous sugar-binding proteins in human breast tissue and benign and malignant breast lesions. Cancer 61: 1125-1131 (1988).

C. G. Gahmberg, Proteins and glycoproteins of hamster kidney fibroblast (BHK_{21}) plasma membranes and endoplasmic reticulum. Biochim. Biophys. Acta 249: 81-95 (1971).

S. Galland, A. Degiuli, J. Frot-Coutaz and R. Got, Transfer of N-acetylglucosamine to endogenous glycoproteins in the nucleus and in non-nuclear membranes of rat hepatocytes: electrophoretic analysis of the endogenous acceptors. Biochem. Int. 17: 59-67 (1988).

T. Gautier, C. Dauphin-Villemant, C. André, C. Masson, J. Arnault, and D. Hernandez-Verdun, Identification and characterization of a new set of nucleolar ribonucleoproteins which line the chromosomes during mitosis. Exp. Cell Res. 200: 5-16 (1992).

L. Gerace, Y. Ottaviano and C. Kondor-Koch, Identification of a major polypeptide of the nuclear pore complex. J. Cell Biol. 95: 826-837 (1982).

L. Gerace and B. Burke, Functional organization of the nuclear envelope. Annu. Rev. Cell Biol. 4: (1988).

L. A. Ginsel, J. J. M. Ondewater, W. T. Daems, Transport of radiolabeled glycoproteins to cell surface and lysosome-like bodies of adsorptive cells in cultured small intestinal tissue from normal subjects and patients with a lysosomal storage disease. Virchows Arch. Abt. B Zellpath 30: 245-273 (1979).

W. F. Glass, R. C. Briggs and L. S. Hnilica, Use of lectins for detection of electrophoretically-separated glycoproteins transferred onto nitrocellulose sheets. Anal. Biochem. 115: 219-224 (1981).

R. H. Glew and S. C. Kuhlen-Schmidt, M. S. Kayman, Studies on the binding of Concanavalin A to rat liver mitochondria. J. Biol. Chem. 248: 3137-3145 (1973).

A. H. Goldberg and L. C., Busch, H. Yeoman, Chromatin -associated glycoproteins of normal rat liver and Novikoff hepatoma ascites cells. Canc. Res. 38: 1052-1056 (1978).

D. S. Goldfarb, Nuclear transport. Current Option in Cell Biology 1: 441-446 (1989).

D. S. Goldfarb, Are the cytosolic components of the nuclear, ER, and mitochondrial import apparatus functionally related? Cell 70: 185-188 (1992).

I. J. Goldstein and C. E. Hayes, The lectins: carbohydrate-binding proteins of plants and animals. Advances in Carbohydrate Chem. and Biochem. 35: 127-312 (1978).

B. Gonzalez-Yanes, J. M. Cicero, R. D. Brown Jr., and C. M. West, Characterization of a cytosolic fucosylation pathway in Dictyostelium. J. Biol. Chem. 267: 9595-9605 (1992).

M. Gronow, F. A. Lewis and T. M. Thachrah, Studies on the degradation of HeLa non-histone proteins. Biochim. Biophys. Acta 606: 157-169 (1980).

A. Haddad, G. Bennett, and C. P. Leblond, Formation and turnover of plasma membranes glycoproteins in kidney tubules of young rats and adult mice, as shown by radioautography after an injection of ^{3}H-fucose. Am. J. Anat. 148: 241-248 (1977).

A. Haddad and G. Bennett, Synthesis of lens capsule and plasma membrane glycoproteins by lens epithelial cells and fibers in the rat. Am. J. Anat. 183: 212-225 (1988).

S. Hakamori, Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Ann. Rev. Biochem. 50: 733-764 (1981).

D. J. Hakes and R. Berezny, Molecular cloning of matrin F/G: A DNA binding protein of the nuclear matrix that contains putative zinc finger motifs. Proc. Natl. Acad. Sci. USA 88: 6186-6190 (1991).

R. S. Haltiwanger, G. D. Holt and G. W. Hart, Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-N-acetyl-glucosamine-peptidebeta-N-acetylglucosaminyltransferase. J. Biol. Chem. 265: 2563-2568 (1990).

R. S. Haltiwanger, M. A. Blomberg and G. W. Hart, Purification and characterization of a uridine diphospho-N-acetyl-glucosamine: polypeptide ß-N-acetylglucosaminyl-transferase. J. Biol. Chem. 267: 9005-9013 (1992).

A. R. Hand, Synthesis of secretory and plasma membrane glycoproteins by striated duct cells of rat salivary glands as visualized by radioautography after ^{3}H-fucose injection. Anat. Rec. 195: 317-340 (1979).

J. A. Hanover, C. K. Cohen, M. C. Willingham and M. K. Park, O-linked N-acetylglucosamine is attached to proteins of the nuclear pore. Evidence for cytoplasmic and nucleoplasmic glycoproteins. J. Biol. Chem. 262: 9887-9894 (1987).

G. W. Hart, R. S. Haltiwanger, G. D. Holt and W. G. Kelly, Glycosylation in the nucleus and cytoplasm. Annu. Rev. Biochem. 58: 841-874 (1989).

G. W. Hart, G. D. Holt and R. S. Haltiwanger, Nuclear and cytoplasmic glycosylation: novel saccharide linkages in unexpected places. Trends Biochem. Sci. 13: 380-384 (1988).

R. Hemming and G. Bennett, Radioautographic detection and localization of nucleoplasmic glycoproteins in various cell types after administration of labeled precursors of carbohydrate side chains. Proc. 4th Int. Cong. Cell Biol. 326a (1988).

A. Herscovics, Biosynthesis of thyroglobulin. Incorporation of [1-^{14}C]-galactose, [1-^{14}C]-mannose and [4,5-^{3}H]-leucine into soluble proteins by rat thyroids <u>in vitro</u>. Bioch. J. 112: 709 (1969).

A. Herscovics, B. Bugge, A. Quaroni, and K. Kirsch, Characterization of glycopeptides labeled from D-[2-^{3}H]mannose and L-[6-^{3}H]fucose in intestinal epithelial cell membranes during differentiation. Biochem. J. 192: 145-153 (1980).

S. O. Hoch and E. McVey, Purification and characterization of two major DNA-binding proteins in human serum. J. Biol. Chem. 252: 1881-1887 (1977).

G. D. Holt and G. W. Hart, The subcellular distribution of terminal N-Acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, O-linked GlcNAc. J. Biol. Chem. 261: 8049-8057 (1986).

G. D. Holt, R. S. Haltiwanger, C. Torres and G. W. Hart, Erythrocytes contain cytoplasmic glycoproteins. O-linked GlcNAc on band 4.1. J. Biol. Chem. 262: 14847-14850 (1987a).

G. D. Holt, C. M. Snow, A. Senior, R. S. Haltiwanger, L. Gerace and G. W. Hart, Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. J. Cell Biol. 104: 1157-1164 (1987b).

M. Horisberger, D. F. Farr and M. Vonlanthen, Ultrastructural localization of beta-D-galactan in the nuclei of the myxomycete Physarum polycephalum. Biochim. Biophy. Acta. 542: 308-314 (1978).

G. A. Howard and H. P. Schnebli, Eukaryote ribosomes possess a binding site for Concanavalin A. Proc. Natl. Acad. Sci. USA 74: 818-821 (1977).

J. Hozier and L. Furcht, Binding of lectins to mitotic chromosomes and interphase nuclear substructures. Cell Biol. Int. Rep. 4: 1091-1099 (1980).

K. L. Hsi, W. C. Copeland and T. S. F. Wang, Human DNA polymerase α catalytic polypeptide binds Con A and RCA I and contains a specific labile site in the N-terminus. Nuc. Acids Res. 18: 6231-6237 (1990).

J. Hubert, A. P. Sève, P. Facy and M. Monsigny, Are nuclear lectins and nuclear glycoproteins involved in the modulation of nuclear functions. Cell Diff. Devel. 27: 69-81 (1989).

J. Hubert, A. E. Seve, D. Bouvier, C. Masson, M. Bouteille and M. Monsigny, In situ ultrastructural localization of sugar-binding sites in lizard granulosa cell nuclei. Biol. Cell. 55: 15-20 (1985).

R. C. Hughes and G. Mills, Hybrid sialylated N-glycans are minor constituents of normal BHK-cell glycoproteins and a prominent feature in glycoproteins of some ricin-resistant cell lines. Biochem. J. 226: 487-498 (1985).

T. Hunter, and M. Karin, The regulation of transcription by phosphorylation. Cell 70: 375-387 (1992).

M. Ishihara, N. S. Fedarko and H. E. Conrad, Transport of heparan sulfate into the nuclei of hepatocytes. J. Biol. Chem. 261: 13575-13580 (1986).

R. C. Jackson, Polypeptides of the nuclear envelope. Biochemistry 15: 5641-5651 (1976).

S. P. Jackson and R. Tjian, O-glycosylation of eukaryotic transcription factors: Implication for mechanisms of transcriptional regulation. Cell 55: 125-133 (1988).

S. P. Jackson, Regulating transcription factor activity by phosphorylation. Trends Cell Biol. 2: 104-108 (1992).

L. Jacob, F. Tron, J-F. Bach, and D. Louvard, A monoclonal anti-DNA also binds to cell-surface protein(s). Proc.

Natl. Acad. Sci. USA 81: 3843-3845 (1984).

S. T. Jacob, and K. M. Rose, Phosphorylation and immunology of poly (A) polymerase. Adv. Enz. Regulation 22: 485-497 (1984).

D. A. Jans, M. J. Ackerman, J. R. Bischoff, D. H. Beach and R. Peters, p34 (cdc2)-mediated phosphorylation at T^{124} inhibits nuclear import of SV-40 T antigen proteins. J. Cell Biol. 115: 1203-1212 (1991).

D. L. Jarvis and J. S. Butel, Modification of Simian virus 40 large tumor antigen by glycosylation. Virology 141: 173-189 (1985).

D. L. Jarvis and J. S. Butel, Biochemical properties of SV 40 large tumor antigen as a glycosylated protein., J. Biol. Chem. 263: 15288-15296 (1988).

S. Jia and J. L. Wang, Carbohydrate binding protein 35; complimentary DNA sequence reveals homology with proteins for the heterogeneous nuclear RNP. J. Biol. Chem. 263: 6009-6011 (1988).

L. W. Jiang and M. Schindler, Chemical factors that influence nucleocytoplasmic transport: A fluorescence photobleaching study. J. Cell Biol. 102: 853-858 (1986).

F. W. K. Kan, M. Bendayan and E. Puvion, Lectin-gold cytochemistry of glycoconjugates in the nuclei of liver cells cultured under normal and experimental conditions. J. Cell Biol. 103: 48a (1986).

F. W. K. Kan and P. Pinto da Silva, Preferential association of glycoproteins to the euchromatin regions of cross-fractured nuclei is revealed by fracture-label. J. Cell Biol. 102: 576-586 (1986).

N. Kashihara, Y. Watanabe, H. Makino, E. J. Wallner, and Y. S. Kanwar, Selective decreased denovo synthesis of glomerular proteoglycans under the influence of a reactive oxygen species. Proc. Natl. Acad. Sci. (USA) 89: 6309-6319 (1992).

R. L. Kaufman and V. Ginsberg, The metabolism of L-fucose by HeLa cells. Exp. Cell Res. 50: 127-132 (1968).

S. H. Kaufmann, D. S. Coffey, and J. H. Shaper, Considerations in the isolation of rat liver nuclear matrix, nuclear envelope, and pore complex lamina. Exp. Cell Res. 132: 105-123 (1981).

K. P. Kearse and G. W. Hart, Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc. Natl. Acad. Sci. USA 88: 1701-1705 (1991).

W. G. Kelly and G. W. Hart, Glycosylation of chromosomal proteins: Localization of O-linked N-Acetylglucosamine in Drosophila chromatin. Cell 57: 243-251 (1989).

J. J. Kim and H. E. Conrad, Kinetics of mucopolysaccharide and glycoprotein synthesis by chick embryo chondrocytes. Effect of D-glucose concentration in the culture medium. J. Biol. Chem. 251: 6210-6217 (1976).

I. A. King and E. A. Hounsell, Cytokeratin 13 contains O-glycosidically N-aceylglucosamine residues. J. Biol. Chem. 264: 14022-14028 (1989).

S. Kinoshita, Heparin as a possible initiator of genomic RNA synthesis in early development of sea urchin embryos. Expt. Cell Res. 64: 403-411 (1971).

S. Kinoshita, Some observations on a protein-mucopolysaccharide complex found in sea urchin embryos. Expt. Cell Res. 85: 31-40 (1974).

S. Kinoshita and K. Yoshii, The role of proteoglycan synthesis in the development of sea urchins. II: The effect of administration of exogenous proteoglycan. Exp. Cell Res. 124: 361-369 (1979).

S. Kinoshita, K. Yoshii and Y. Tonegawa, Specific binding of lectins with the nucleus of the sea urchin embryo and changes in the lectin affinity of the embryonic chromatin during the course of development. Expt. Cell Res. 175: 148-157 (1988).

R. Kornfeld and S. Kornfeld, Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54: 631-634 (1985).

T. Krusius, J. Finne, R. K. Margolis and R. U. Margolis, Identification of a O-glycosidic mannose-linked sialylated tetrasaccharide and keratin sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain. J. Biol. Chem. 261: 8237-8242 (1986).

T. Krusius, V. N. Reinhold, R. K. Margolis and Margolis R. U. Structural studies on sialylated and sulphated O-glycosidic mannose-linked oligosaccharides in the chondroitin sulphate proteoglycan of brain. Biochem. J. 245: 229-234 (1987).

R. Kurl, S. C. Holmes, E. Verney and Herschel Sidransky,

Nuclear envelope glycoprotein with poly (A) polymerase activity of rat Liver: isolation, characterization and immunohistochemical location. Biochemistry 27: 8974-8980 (1988).

P. Kurth, M. Bustin, and E. Mondrianakis, Con A binds to puffs in polytene chromosomes. Nature 279: 448-450 (1979).

S. Kuzmich, L. A. Vanderveer, and K. Tew, Evidence for a glycoconjugate form of glutathione S-transferase pI, Int. J. Peptide Protein Res. 37: 565-571 (1991).

U.K. Laemmli, Cleavage of structural protein during the assembly of the head of a bacteriophage. Nature: 227:680 (1970).

B. Levy-Wilson, Glycosylation, ADP-ribosylation, and methylation of Tetrahymena histones. Biochemistry 22: 484-489 (1983).

J. G. Laing and J. L. Wang, Identification of carbohydrate binding protein 35 in heterogenous nuclear ribonucleoprotein complex. Biochemistry 27: 5329-5334 (1988).

S. Lichtsteiner and U. Schibler, A glycosylated liver-specific transcription factor stimulates transcription of the albumen gene. Cell 57: 1179-1187 (1989).

I. Londono and M. Bendayan, Ultrastructural localization of mannoside residues on tissue sections; comparative evaluation of the enzyme-gold and the lectin-gold approaches. Eur. J. Cell Biol. 45: 88-96 (1987).

O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951).

J. M. Lucocq, E. G. Berger and J. Roth, Detection of terminal N-linked N-acetylglucosamine residues in the Golgi apparatus using galactosyltransferase and endoglucosaminidase F/peptide N-glycosidase F; adaptation of a biochemical approach to electron microscopy. J. Histochem. Cytochem. 35: 67-74 (1987).

J. A. Magner, F. Wezeman, and J. N. Schluep, Autoradiographic demonstration that a Golgi-postranslational processing step (fucosylation) partially shifts to the rough endoplasmic reticulum in active mouse thyrotrophs: Implications for the physiologic modulation of TSH oligosaccharide structure. Proceedings of 73rd Annual Meeting of the Endocrine Society 882a: 267 (1992).

K. Mann and T. Hunter, Association of simian virus 40 T antigen with simian virus 40 nucleoprotein complexes. J. Virol. 29: 232-241 (1979).

R. K. Margolis, C. P. Crockett, W. L. Kiang and R. U. Margolis, Glycosaminoglycans and glycoproteins associated with rat brain nuclei. Biochim. Biophys. Acta. 451: 465-469 (1976).

R. K. Margolis, M. D. Thomas, C. P. Crockett and R. U. Margolis, Presence of chondroitin sulfate in the neuronal cytoplasm. Proc. Natl. Acad. Sci. USA 76: 1711-1715 (1979).

Y. Matau, N. Nishi, T. Negi, Y. Tanaka, and F. Wada, Isolation and characterization of an androgen-dependent nonhistone chromosomal protein from dorsolateral prostate of rats. Biochem. Biophys. Res. Com. 109: 334-340 (1982).

E. J. McGuire , G. W. Jourdian, D. M. Carlson, and S. Roseman, Incorporation of D-galactose into glycoproteins. J. Biol. Chem. 240: PC4112-PC4114 (1965).

W. Meikrantz, D. M. Smith, M. M. Sladicka and R. A. Schlegel, Nuclear localization of an O-glycosylated protein phosphotyrosine phosphatase from human cells. J. Cell Sci. 98: 303-307 (1991).

F. Melchers, Biosynthesis of the carbohydrate portion of immunoglobulins. Kinetics of synthesis and secretion of [^{3}H]leucine-, [^{3}H]galactose-, and [^{3}H]mannose-labeled myeloma protein by two plasma cell tumors. Biochem. J. 119: 765-772 (1970).

F. Melchers, Biosynthesis of the carbohydrate portion of immunoglobulin. Radiochemical and chemical analysis of the carbohydrate moieties of two myeloma proteins purified from different subcellular fractions of plasma cells. Biochemistry 10: 653-659 (1971).

I. Melon and J. Bhorjee, Isolation and characterization of nuclei and chromatin from differentiating cultures of rat skeletal muscle. Exp. Cell Res. 137: 147-154 (1982).

D. I. Meyer and M. M. Burger, The chromaffin granule surface. Localization of carbohydrate on the cytoplasmic surface of an intracellular organelle. Biochim. Biophys. Acta 443: 428-436 (1976).

B. L. A. Miki, J. W. Gurd and I. R. Brown, Characterization of mononucleosomes and associated glycoproteins from Ehrlich ascites tumour cells. Can. J. Biochem. 58:

1261-1269 (1980).

J. Mancilla, T. Ikejima, and Dinarello, Glycosylation of the interleukin-1 receptor type-I is required for optimal binding of interleukin-1. Lymphokine and Cytokine Research 11: 4 (1992).

N. M. Maraldi, N. Zini, S. Squarzoni, R. Del Coco, P. Sabatelli, and F. A. Manzoli, Intranuclear localization of phospholipids by ultrastructural cytochemistry. J. Histochem. Cytochem. 40: 1383-1392 (1992).

M. Mitranic and M. A. Moscarello, The conversion of mannose-^{3}H to protein bound sialic acid, fucose, galactose and hexosamines in rat serum. Biochim. Biophys. Acta 47: 74-80 (1972).

T. Miyagi and S. Tsuiki, Purification and characterization of cytosolic sialidase from rat liver. J. Biol. Chem. 260: 6710-6716 (1985).

F. Monaco and J. Robbins, Incorporation of N-acetylmannosamine and N-acetylglucosamine into thyroglobulin in rat thyroid in vitro. J Biol Cham 248: 2072-2077 (1973).

I. K. Moutsatsos, J. M. Davis and J. L. Wang, Endogenous lectins from cultured cells: subcellular localization of carbohydrate binding protein 35 in 3T3 fibroblasts. J. Cell Biol. 102: 477-483 (1986).

I. K. Moutsatsos, M. Wade, M. Schindler and J. L. Wang, Endogenous lectins from cultured cells: nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA 84: 6452-6456 (1987).

T. Muramatsu, P. H. Atkinson, and S. G. Nathenson, and C. Ceccarini, Cell-surface glycopeptides: growth-dependent changes in the carbohydrate-peptide linkage region. J. Mol. Biol. 80: 781-799 (1973).

T. Muramatsu, N. Koide, C. Ceccarini, and P. H. Atkinson, Characterization of mannose-labeled glycopeptides from human diploid cells land their growth-dependent alterations. J. Biol. Chem. 251: 4673-4679 (1976).

N. J. Nadler, The interpretation of grain counts in electron microscope radioautography. Appendix to Haddad et al., J. Cell Biol. 49: 877-882 (1971).

M. Nakajima, N. Ito, K. Nishi, Y. Okamura, S. Kawahara and T. Hiroa, Expression of blood group A and B antigens in

nuclear heterochromatin in mucous cells of human cervical glands. J. Histochem. Cytochem. 39, 469-478 (1991).

N. Nakayasu and R. Berezney, Nuclear matrins: Identification of the major nuclear matrix proteins. Proc. Natl. Acad. Sci. USA 88: 10312-10316 (1991).

D. M. Nelson, A. C. Enders, and B. F. King, Cytological events involved in glycoprotein synthesis in cellular and syncytial trophoblast of human placenta: An electron microscope autoradiographic study of [^{3}H]galactose. J. Cell Biol. 76, 418-429 (1978).

D. D. Newmeyer and D. J. Forbes, Nuclear import can be separated into distinct steps in vitro: Nuclear pore binding and translocation. Cell 52: 641-653 (1988).

G. Nicolson, M. Lacorbière and P. Delmonte, Outer membrane terminal saccharides of bovine liver nuclei and mitochondria. Expt. Cell Res. 71: 468-472 (1972).

E. A. Nigg, Nuclear function and organization: The potential of immunocytochemical approaches. Int. Rev. Cyto. 110: 27-92 (1988).

N. A. Nwokoro and H. Schachter, L-fucose metabolism in mammals. Purification of pork liver 2-keto-3-deoxy-L-fuconate: NAD^+ oxidoreductase by NAD^+-agarose affinity chromatography. J. Biol. Chem. 250: 6185-6190 (1975).

K. Nyame, R. D. Cummings, and R. D. Damian, Schistosoma mansoni synthesizes glycoproteins containing terminal O-linked N-acetylglucosamine residues. J. Biol. Chem. 262: 7990-7995 (1987).

A. Oldberg, L. Kjellen and M. Hook, Cell-surface heparin sulfate. Isolation and characterization of a proteoglycan from rat liver membranes. J. Biol. Chem. 254: 8505-8510 (1979).

D. E. Olins, A. L. Olins, A. P. Seve, C. A. Bourgeois, J. Hubert and M. Monsigny, Lectin-like components in the macronuclear replication bands of Euploites eurystomus. Biol. Cell 62: 95-98 (1988).

M. K. Park, M. D'Onofrio, M. C. Willingham and J. A. Hanover, A monoclonal antibody against a family of nuclear pore proteins (nucleoporins) O-linked N-acetylglucosamine is part of the immunodeterminant. Proc. Natl. Acad. Sci. USA 84, 6462-6466 (1987).

C. H. Pedemonte, G. Sachs, and J. H. Kaplan, An intrinic membrane glycoprotein with cytosolically-oriented N-linked sugars. Proc. Natl. Acad. Sci. 87: 9789-9793 (1990).

G. Pelletier, and R. Puviani, Detection of glycoproteins and autoradiographic localization of [^{3}H]-fucose in the thyroidectomy cells of rat anterior pituitary gland. J. Cell Biol. 56: 600-612 (1973).

G. Pelletier, Autoradioautographic studies of synthesis and intracellular migration of glycoproteins in the rat anterior pituitary gland. J. Cell. Biol. 62: 185-195 (1974).

J. Pitcher, C. Smythe and P. Cohen, Glycogenin is the priming glucosyltransferase required for the initiation of glycogen biogenesis in rabbit skeletal muscle. Eur. J. Biochem. 176: 391-395 (1988).

J. Pitcher, C. Smythe, D. G. Campbell and P. Cohen, Identification of the 38-kDa subunit of rabbit skeletal muscle glycogen synthase as glycogenin. Eur. J. Biochem. 169: 497-502 (1987).

H. Polet and J. Molnar, Demonstration that some of the nonhistone proteins, inducible to translocate into the nucleus, are glycosylated. J. Cell Physiol. 135: 47-54 (1988).

E. M. Rakowicz-Szulczynska and H. Koprowski, Nuclear uptake of monoclonal antibody to a surface glycoprotein and its effect on transcription. Arch. Biochem. Biophys. 271: 366-379 (1989).

R. Reeves, D. Chang and S. Chung, Carbohydrate modifications of the high mobility group proteins. Proc. Natl. Acad. Sci. USA 78: 6704-6708 (1981).

R. Reeves and D. Chang, Investigations of the possible functions for glycosylation in the high mobility group proteins. Evidence for a role in nuclear matrix association. J. Biol. Chem. 258: 679-687 (1983).

I. Reisert, Autoradiographischer hinweis auf das vorkommen sialinsaurehaltiger glykokonjugate im zellkern. Acta. Histochem. Suppl.-Band XX: 113-117 (1978).

I. Reisert and C. Pilgrim, Metabolism of glycoconjugates in hypothalamic neurons and glial cells; Comparison ofincorporation of ^{3}H-fucose and ^{3}H-N-acetylmannosamine by electron microscopic autoradiography. Cell Tissue Res. 196: 135-145 (1978).

M. Richard, A. Martin and P. Louisot, Evidence for glycosyltransferases in rat liver nuclei. Biochem. Biophys. Res. Comm. 64: 108-114 (1975).

J. A. Ripellino, M. Bailo, R. U. Margolis and R. K. Margolis, Light and electron microscopic studies on the localization of hyaluronic acid in developing rat cerebellum. J. Cell Biol. 106: 845-855 (1988).

J. A. Ripellino, R. U. Margolis and R. K. Margolis, Immunoelectron microscopic localization of hyaluronic acid-binding region and link protein epitopes in brain. J. Cell Biol. 108: 1899-1907 (1989).

W. B. Rizzo and M. Bustin, Lectins as probes of chromatin structure. Binding of Concanavalin A to purified rat liver chromatin. J. Biol. Chem. 252: 7062-7067 (1977).

I. Rodriguez and W. J. Whelan, A novel glycosyl-amino acid linkage: rabbit-muscle glycogen is covalently linked to a protein via tyrosine. Biochem. Biophys. Res. Comm. 132: 829-836 (1985).

J. Roth, Application of lectin-gold complexes for electron microscopic localization of glycoconjugates on thin sections. J. Histochem. Cytochem. 31: 987-999 (1983).

J. Roth, Light and electron microscopic localization of glycoconjugates with gold-labeled reagents. Scanning Microsc. 1: 695-704 (1987).

K. Scherrer, Prosomes, subcomplexes of untranslated mRNP. Mol. Biol. Rep. 14: 1-9 (1990).

M. Schindler, and M. Hogan, Carbohydrate moieties of nuclear glycoproteins are predominantly N-acetyl-D-glucosamine. J. Cell Biol. 99: 99a (1984).

M. Schindler, M. Hogan, R. Miller and D. DeGaetano, A nuclear specific glycoprotein representative of a unique pattern of glycosylation. J. Biol. Chem. 262: 1254-1260 (1987).

M. Schliephacke, A. Kremp, H-P. Scmid, K. Kohler, and U. Kull, Prosomes (proteasomes) of higher plants. Eur. J. Cell Biol. 5: 114-121 (1991).

W. N. Schmidt, K. B. McKusick, C. A. Schmidt, L. H. Hoffman and L. S. Hnilica, Nuclear matrix antigens in azo dye-induced primary rat hepatoma. Canc. Res. 44: 5291-5304 (1984).

M. K. Schmitt and K. Mann, Glycosylation of Simian virus 40 T antigen and localization of glycosylated T antigen in the nuclear matrix. Virology 156: 268-281 (1987).

H. Schroter, C. G. F. Mueller, K. Meese and A. Nordheim, Synergism in ternary complex formation between the dimeric glycoprotein $p67^{SRF}$, polypeptide $p62^{TCF}$ and the c-fos serum response element. EMBO 9: 1123-1130 (1990).

A. G. L. Senior, Integral membrane proteins specific to the inner nuclear membrane and associated with the nuclear lamina. J. Cell Biol. 107: 2029-2036 (1988).

L. Sevaljevic and K. Krtolica, Demonstration of glycoproteins which are associated with chromatin nonhistone proteins. Int. J. Biochem. 4: 345-348 (1973).

L. Sevaljevic, M. Konstantinovic, M. Tomovic and Z. Pavlovic, Lectin activity of chromatin non-histone proteins. Mol. Biol. Rep. 3: 265-267 (1977).

L. Sevaljevic, S. L. Petrovic and M. Petrovic, Binding of Lens culinaris lectin to sea urchin embryo chromatin. Experientia 35: 193-194 (1979).

L. Sevaljevic, M. Petrovic and K. Ktrolica, Protein kinase activity and lectin binding ability of the nuclear matrix. Biochem. Int. 2: 77-84 (1981).

A. P. Sève, J. Hubert, D. Bouvier, C. Masson, G. Geraud and M. Bouteille, In situ distribution in different cell types of nuclear glycoconjugates detected by two lectins. J. Submicrosc. Cytol. 16: 631-641 (1984).

A. P. Sève, J. Hubert, D. Bouvier, M. Bouteille, C. Maintier and M. Monsigny, Detection of sugar-binding protiens in membrane-depleted nuclei. Expt. Cell Res. 157: 533-538 (1985).

A. P. Sève, J. Hubert, D. Bouvier, C. Bourgeois, P. Midoux and A. C. Roche. Analysis of sugar-binding sites in mammalian cell nuclei by quantitative flow microfluorometry. Proc. Natl. Acad. Sci. USA 83: 5997-6001 (1986).

A. P. Sève, P. Codogno, C. Bauvy, E. Ogier-Denis, M. Aubery and M. Monsigny, Fucose is a component of glycoproteins in membrane-depleted nuclei. Proc. 4th Int. Cong. Cell Biol. 324a (1988).

A. P. Sève, J. Hubert, C. A. Bourgeois, P. Midoux, A. C. Roche and M. Monsigny, Analysis of nuclear sugar-binding

sites in two cell types by quantitative flow cytofluorometry. Lectins - Biology, Biochemistry, Clinical Biochemistry (Sigma) 6: 439-443 (1988).

N. L. Shaper, G. F. Hollis, J. G. Douglas, I. R. Kirsch and J. H. Shaper, Characterization of the full length cDNA for murine beta-1,4-galactosyltransferase. Novel features at the 5'-end predict two translational start sites at two in-frame AUG'S. J. Biol. Chem. 263: 10420-10428 (1988).

V. A. Shoup and O. Touster, Purification and characterization of the α-D-mannosidase of rat liver cytosol. J. Biol. Chem. 251: 3845-52 (1976).

M. Sikorska, L. M. Brewer, T. Youdale, R. Richarads, and J. F. Whitfield, Evidence that mammalian ribonucleotide polymerase is a nuclear membrane associated glycoprotein. Biochem. Cell. Biol. 68: 880-888 (1990).

P. J. Smith, G. P. Sabbatini, K. I. Grant and C. Von Holt, Identification of nuclear envelope proteins and glycoproteins which co-isolate with the nuclear protein matrix. Biochim. Biophys. Acta. 904: 365-372 (1987).

C. Smythe, F. B. Caudwell, M. Ferguson and P. Cohen, Isolation and structural analysis of a peptide containing the novel tyrosyl-glucose linkage in glycogenin. EMBO J. 7: 2681-2686 (1988).

C. M. Snow, A. Senior and L. Gerace, Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. 104: 1143-1156 (1987).

M. Soulard, J-P. Barque, V. Della Valle, D. Hernandez-Verdun, C. Masson, F. Danon, and C-J. Larsen, A novel 43-kDa glycoprotein is detected in the nucleus of mammalian cells by autoantibodies from dogs with autoimmune disorders. Exp. Cell Res. 193: 59-71 (1991).

M. Spangler, M. L. Coetzee, S. L. Katyal, H. P. Morris and P. Ove, Some biochemical characteristics of rat liver and Morris hepatoma nuclei and nuclear membranes. Cancer Res. 35: 3131-3145 (1975).

A. M. Spence, P. C. Sheppard, J. R. Davie, Y. Matuo, N. Nishi, W. L. McKeehan, J. G. Dodd, and R. J. Matusik. Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin. Proc. Natl. Acad. Sci. USA 86: 7843-7847 (1989).

C. Srisomsap, K. L. Richardson, J. C. Jay and R. B. Marchase, Localization of the glucose phospho-transferase to a

cytoplasmically accessible site on intracellular membranes. J. Biol. Chem. 263: 17792-17797 (1988).

C. Srisomsap, K. L. Richardson, J. C. Jay and R. B. Marchase, An α-glucose-1-phosphate phosphodiesterase is present in rat liver cytosol. J. Biol. Chem. 264: 20540-20546 (1989).

C. M. Starr and J. A. Hanover, Glycosylation of nuclear pore protein p62. Reticulocyte lysate catalyzes O-linked N-acetylglucosamine addition in vitro. J. Biol. Chem. 265: 6868-6877 (1990).

M. Staufenbiel and W. Deppert, Intermediate filament systems are collapsed onto the nuclear surface of nuclei after isolation from tissue culture. Exp. Cell Res. 138: 207-214 (1983).

G. S. Stein, R. M. Roberts, J. L. Davis, W. J. Head, J. L. Stein and C. L. Thrall. Are glycoproteins and glycosaminoglycans components of the eukaryotic genome? Nature 258: 639-641 (1975).

G. S. Stein, R. M. Roberts, J. L. Stein and J. L. Davis, Nuclear glycoproteins and glycosaminoglycans. In: The cell nucleus. Vol. 10: 341-357 (1981).

M. Stryjecka-Zimmer, W. N. Schmidt, R. C. Briggs, and L. S. Hnilica, Immunological specificity of Novikoff Hepatoma chromatin: isolation of three antigenic proteins. Int. J. Biochem. 14: 591-692 (1982).

G. Tischendorf, W. Liebrich, P. Trapitz, G. Wood and M. Schwochau, A group of non-Y encoded Drosophila hydei primary spermatocyte nuclear glycoproteins exhibits epitopes depending on formation of Y chromosomal giant lampbrush loops. Chromosoma 98: 144-152 (1989)

W. Tomek, G. Adam, and H-P. Schmid, Prosomes, small cytoplasmic RNP particles, contain glycoproteins. FEBS Letters 239: 155-158 (1988).

C. R. Torres and G. W. Hart, Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked glcNAc. J. Biol. Chem. 259: 3308-3317 (1984).

H. Towbin, T. Staehlin, and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354 (1979).

D. Tuan, S. Smith, J. Folkman and E. Merler, Isolation of the nonhistone proteins of rat Walker carcinoma 256. Their association with tumor angiogenesis. Biochemistry 12: 3159-3165 (1973).

D. R. Tulsiani and R. Carubelli, Studies on the soluble and lysosomal neuraminidases of rat liver. J. Biol. Chem. 245: 1821-1827 (1970).

D. R. Tulsiani and O. Touster, Substrate specificities of rat kidney lysosomal and cytosolic α-D-mannosidase and effects of swainsonine suggest a role of the cytosolic enzyme in glycoprotein catabolism. J. Biol Chem 262: 6506-6514 (1987).

J. R. Turner, A. M. Tartakoff and N. S. Greenspan, Cytologic assessment of nuclear and cytoplasmic O-linked N-acetylglucosamine distribution by using anti-streptococcal monoclonal antibodies. Proc. Natl. Acad. Sci. USA 87: 5608-5612 (1990).

M. A. Vannier-Santos, E. M. B. Saraiva and De Sousa. W., Nuclear and cytoplasmic lectin binding sites in promastigotes of Leishmania. J. Histochem. Cytochem. 39: 793-800 (1991).

P. Vischer and W. Reutter, Different turnover of fucose residues in plasma membranes of rat liver and Morris Hepatoma 7777. Biochem. J. 190: 51-55, (1980).

K. von Figura and A. Hasilik, Lysosomal enzymes and their receptors. Ann. Rev. Biochem. 55: 167-193 (1986).

W. J. Welch, J. I. Garrels, G. P. Thomas, J. J. C. Lin and J. R. Feramisco, Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose- and Ca2+-ionophore-regulating proteins. J. Biol. Chem. 258: 7102-7111 (1983).

W. J. Welch and J. Feramisco, Purification of the major mammalian heat shock proteins. J. Biol. Chem. 257: 14949-14959 (1982).

B. Wolff, M. C. Willingham and J. A. Hanover, Nuclear import: Specificity for transport across nuclear pore. Exp. Cell Res. 178: 318-334 (1988).

A. H. Wyllie, J. F. R. Kerr, and A. R. Currie, Cell death: the significance of apoptosis. Int. Rev. Cytol. 68: 251-305 (1980).

K. Yamashita, N. Kochibe, T. Ohkura, I. Ueda, and A. Kobata.

Fractionation of L-fucose-containing oligosaccharides on immobilized Aleuria aurantia lectin. J. Biol. Chem. 260: 4688-4693, 1985.

M. Yanagishita and V. C. Hascall, Characterization of heparan sulfate proteoglycans synthesized by rat ovarian granulosa cells in culture. J. Biol. Chem. 258: 12857-12864 (1983).

L. C. Yeoman, J. J. Jordan, R. K. Busch, C. W. Taylor, H. E. Savage and H. Busch, A fetal protein in chromatin of Novikoff hepatoma and Walker 256 carcinosarcoma tumors that is absent from normal and regenerating rat liver. Proc. Natl. Acad. Sci. USA 73: 3258-3262 (1976).

Y. Yoneda, N. Imamoto-Sonobe, M. Yamaizumi and T. Uchida, Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Expt. Cell Res. 173: 586-595 (1987).

K. Yoshida, The presence of ribosomal glycoproteins. Agglutination of free and membrane-bound ribosomes from wheat germ by Concanavalin A. J. Biochem. 83: 1609-1614 (1978).

S. Yotsumoto, S. Tsuyama, M. Tashiro and Fusayoshi Murata, Ultrastructural immunocytochemical studies of blood group substances in human eccrine glands. J. Histochem. Cytochem. 38: 1815-1821 (1990).

P. D. Yurchenko, C. Ceccarini, and P. H. Atkinson, Labeling complex carbohydrates of animal cells with monosaccharides. Methods in Enzymology 50: 175-204 (1978).

D. Zagury, J. Uhr, J. D. Jamieson and G. E. Palade, Immunoglobulin synthesis and secretion. II. Radioautographic studies of sites of addition of carbohydrate moieties and intracellular transport. J. Biol. Chem. 46: 52-53 (1970).

L. Zardi, A. Siri, B. Carnemolla, L. Santi, W. D. Gardner and S. O. Hoch, Fibronectin: a chromatin-associated protein? Cell 18: 649-657 (1979).

Appendix 1

Biochemical Studies on the Fate of ^{14}C- or ^{3}H-Sugars and ^{35}S-Sulphate Administered to Various Cell Types

A number of biochemical studies have investigated the fate of ^{14}C, ^{3}H and ^{35}S label after administration of ^{14}C- or ^{3}H-sugars and ^{35}S-sulfate to whole animals or to cells in culture. Several studies are mentioned below for each radiolabeled sugar used in our studies as well as sulphate pertaining to the ultimate fate of the label in different cells.

I) Fucose

Radiolabeled fucose (either ^{3}H- or ^{14}C-labeled) has been found in almost all biochemical studies to be a highly specific precursor for fucose residues of glycoproteins or glycolipids. Coffey et al. (1964) showed that after the administration ^{14}C-fucose to rats, the label in intestinal tissue was exclusively localized to fucose residues of glycoproteins.

Bekisi and Winzler (1967) similarly showed that the incorporated label after administration of ^{14}C-fucose lay almost exclusively in fucose residues in glycoproteins of rat liver and intestine.

In cultured HeLa cells, Kaufman and Ginsburg (1968) found that after administration of ^{14}C-fucose the label was exclusively incorporated into fucose residues of macromolecules, with no detectable labeling of other sugar residues.

In cultured lymphoma cells, Bosmann and Winston (1970)

administered ^{3}H-fucose and examined the distribution of label in intracellular and secreted proteins. All of the label in secreted proteins was in fucose residues. In the case of intracellular proteins, on the other hand, 80% of the label ended up in fucose residues with 20% in galactose and mannose residues.

Gahmberg (1971) showed that all of the macromolecular label recovered in cultured hamster fibroblasts (BHK cells) exposed to ^{3}H-fucose was in fucose residues. Similarly, Hughes and Mills (1985) found that when BHK cells were exposed to ^{3}H-fucose, virtually all of the label in certain glycoproteins (containing N-linked side chains) was present in fucose residues.

Vischer and Reutter (1980) found that more than 90% of protein-bound radioactivity in isolated plasma membranes of both Morris hepatoma and normal rat liver of cells exposed to ^{3}H-fucose was detected in fucose residues.

Among the only evidence that fucose might be metabolized to other products is a study which showed free fucose can be oxidized to 2-keto-3-deoxy-L-fuconate in some animals (Nwokoro and Schachter, 1975). Little or no such oxidation was observed in rats, however (Bekesi and Winzler, 1967).

II) Galactose

Labeled ^{14}C- or ^{3}H-galactose has been shown to be a fairly specific precursor for galactose residues of glycoconjugates although there has been some variance in the specificity

reported in the literature depending on the cell type. McGuire et al. (1965) showed that 95% of the incorporated radioactivity released from particulate fractions of liver tissues exposed to ^{14}C-galactose was in galactose residues. Herscovics (1969) found that after exposure of slices of rat thyroid to ^{14}C-galactose, 78% of the label incorporated into non-iodinated thyroglobulin was was recovered as galactose residues while 13% was found in mannose residues.

In a study particularly relavent to the present radioautographical work, Melchers (1970) showed that over 90% of incorporated ^{3}H-galactose label in murine myeloma cells was present in galactose residues of immunoglobulin glycoproteins.

Muramatsu et al. (1973) found that all of the label recovered from human diploid cell fibroblasts exposed to ^{3}H-galactose was in galactose residues of glycoproteins. Similarly, in the study mentioned above by Hughes and Mills (1985), when BHK cells were exposed to ^{3}H-galactose virtually all of the label in the glycoproteins examined (containing N-linked side chains) was present in galactose residues.

In terms of nuclear proteins, Jarvis and Butel (1985) provided evidence that the label in incorporated into the nucleoplasmic form of the SV 40 antigen after administration of ^{3}H-galactose to TC7 cells resided in galactose residues.

III) Mannose

Biochemical studies characterizing the labeled molecules

in cells exposed to ^{14}C- or ^{3}H-mannose have shown that much of the label ends up in mannose residues of glycoproteins. Muramatsu et al. (1976) found that ^{14}C- or ^{3}H-mannose was incorporated into glycoproteins of human diploid fibroblasts without significant conversion into other sugars. 5% of the label was found in fucose residues. Melchers (1971) found that over 60% of the incorporated label of cultured murine myeloma cells exposed to ^{3}H-mannose was present in mannose residues of glycoproteins with 35% in glucosamine residues. Herscovics (1969) found that after exposure of slices of rat thyroid to ^{14}C-mannose, between 45-64% of the label incorporated into non-iodinated thyroglobulin was was recovered as mannose residues while 7-25% was found in galactose residues.

In some other studies, however, a more extensive incorporation of the label into other sugar residues was reported. Though Herscovics et al. (1980) found after intravenous injection of rats with ^{3}H-mannose that certain intestinal epithelial cell membrane glycoproteins (Fraction 3) contained over 96% of their label in mannose residues, other intestinal epithelial cell membrane glycoproteins (Fraction 1) contained over 82% of their label in fucose residues. Mitranic and Moscarello (1972) reported that when the distribution of radioactivity in blood serum glycoporteins was examined five minutes after an intravenous injection of ^{3}H-mannose to rats, 83% was recovered as sialic acid, 8.0% as fucose, 3.1% as hexosamine, 1.8% as galactose, and only 3.2% as mannose

residues.

IV) Glucosamine and N-acetylglucosamine (GlcNAc)

Radiolabeled glucosamine and GlcNAc have been shown to be generally good precursors for glucosamine residues of glycoproteins (including proteoglycans). However, a small amount of conversion of the labeled sugar to other sugars has been shown to occur, with label found in sialic acid or galactosamine residues.

Bosmann and Winston (1970) showed that after exposure of cultured mouse lymphoma cells to ^{14}C-glucosamine, 85% of label incorporated into cellular proteins was found in glucosamine residues. The remaining label was found to be in sialic acid (10%) and galactosamine residues (5%).

Gahmberg (1971) found that 70% of the label recovered from hamster fibroblast plasma membranes after the cells were exposed to ^{14}C-glucosamine was present in glucosamine residues, while the remaining label was found in galactosamine (20%) and sialic acid residues (10%).

In murine myeloma cells (Melchers, 1971), over 90% of the label incorporated into myeloma immunogloblulin proteins after exposure ^{3}H-glucosamine was found in glucosamine residues.

Lastly, in another study relevant to ours, Stein et al. (1975) showed that the label incorporated into chromosomal glycoproteins from HeLa cells radiolabeled with ^{3}H-glucosamine was found almost exclusively in glucosamine and galactosamine residues.

V) N-Acetylmannosamine

Biochemical studies of labeled molecules in cells exposed to ^{3}H-manNAc have shown that this sugar is often highly specific for sialic acid residues of glycoproteins and glycolipids.

Monaco and Robbins (1973) provided evidence that all label incorporated into thyroglobulin in rat thyroid slices incubated with ^{3}H-manNAc was present in sialic acid residues.

Bennett and O'Shaughnessy (1981) found that over 90% of the label incorporated into glycoproteins of rat liver and duodenal tissue after injection of ^{3}H-manNAc was present in sialic acid.

In cultured cells, Brown et al. (1982) found that over 90% of the label recovered from erythroleukemia cells after administration of ^{3}H-manMAc was in form of sialic acid. Diaz et al (1985), on the other hand, reported that when different lines of cultured cells were exposed to ^{3}H-manNAc, the percentage of incorporated label present in sialic acid residues of cell membrane glycoproteins was highly variable, ranging from 30-80%. One of the cultured cell lines used to derive these conclusions was the principal one used in our studies i.e. the murine myeloma (P3X63AG8.653 line) where these investigators found that 72% of the ^{3}H-manNAc-labeled molecules in cellular membranes were in sialic acid residues.

VI) Sulphate

Lastly, biochemical studies have indicated that ^{35}S-

sulphate is a very good to excellent precursor for sulfate residues of glycoprotein side chains (the glycosaminoglyan side chains of proteoglycans). As early as 1956, Kent et al. showed that administered ^{35}S-sulfate was incorporated into sulfated mucin molecules. When cultured chick embryo chondrocytes were incubated with ^{35}S-sulfate, the label was incorporated into chondroitin sulfate molecules (Kim and Conrad, 1976). After injection of ^{35}S-sulfate into rats, Oldberg et al. (1979) showed that over 95% of the macromolecular label in a liver plasma membrane fraction resided in heparin sulfate molecules. Yanagishita and Hascall (1983) showed that when cultured rat ovarian granulosa cells were incubated with ^{35}S-sulfate, virtually all of the label was incorporated into glycos-aminoglycan molecules. Esko et al. (1986, 1987) showed that mutant CHO cells, which were incapable of synthesizing glycosaminoglycans, incorporated only small amounts of ^{35}S-sulfate in comparison to normal CHO cells. This suggests that most of the ^{35}S-sulfate incorporated by normal cells resided in sulfated glycosaminoglycan molecules. Kashihara et al. (1992) showed that ~95% of the total incorporated ^{35}S-sulphate in isolated rat renal glomeruli was in the proteoglycan/GAGs fraction.

Some studies have documented the specificity of administered ^{35}S-sulfate for nucleoplasmic proteoglycan-like glycoproteins. Fromme et al. (1976) provided biochemical evidence that the ^{35}S-label responsible for radioautographic

reaction observed over the nuclei of human fibroblast cells was located in sulphated glycosaminoglycans. Finally, Fedarko et al. (1986) showed that ^{35}S-sulphate label was incorporated into sulfate residues of heparin sulphate molecules residing in both the nucleus and cytoplasm of cultured rat hepatocytes. The nuclear heparin sulfate molecules were shown to be structurally different than those contained in the cytoplasm.