A STUDY OF THE ENVELOPE PROTEINS OF THE BHK-21 CELL NUCLEUS

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Virginia S. Wilson

Department of Biochemistry,
University of Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For Alan - whose idea

this was.

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Summary

Fractions of BHK cells were examined in both the normal C_{13} line and after transformation with Polyoma virus (PyY). Protein profiles of whole nuclei, nuclear envelope, pore complex lamina, nuclear membrane and rough endoplasmic reticulum were compared and the carbohydrates detected by their affinity for $\left({}^{125}\text{I} \right)$ - labelled lectins. Carbohydrates were found in all these fractions with minor differences in their relative proportions after transformation, and a loss of all affinity for RCA. Similarities in the affinities for WGA and lentil lectin were seen in nuclear membrane and rough endoplasmic reticulum suggesting that these membranes may have a common origin. A large proportion of the nuclear envelope carbohydrate was retained by the lamina after its separation from the membrane. A specific interaction between transmembrane glycoproteins and the lamina is proposed.

The interaction of lectins with whole nuclei was examined. This revealed some changes in the affinities and number of binding sites/nucleus after transformation.

The topography of the nuclear envelope was examined by comparison of the iodination profiles produced by soluble and immobilised lactoperoxidase. Nuclei were examined before and after removal of their membranes with Triton-X100. In the absence of the membrane, the lamina topography was examined by comparative iodinations after contraction and expansion in various concentrations of Ca^{2+}.

In whole nuclei the iodination profile of immobilised lactoperoxidase was very restricted, with heavy iodination in only one band of Mr 52,000. This was reduced by Triton-X100 extraction

which produced a concommitant increase in the labelling of a protein of Mr 63,000. This may indicate that it lies below the membrane on the lamina surface.

Contracted nuclei from which the membrane had been removed presented a similar range of proteins to both soluble and immobilised lactoperoxidase indicating a very close association between the proteins. After expansion, the iodination profiles of both agents were extended, suggesting that the proteins had undergone dissociation.

ABBREVIATIONS

5'-AMP	-	Adenosine-5'-phosphate
BSA	-	bovine serum albumen
BSS	-	balanced salts solution
ConA	-	Concanavalin-A
DMSO	-	dimethylsulphoxide
DOC	-	deoxycholate
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetraacetate
hn-RNA	-	heterogeneous nuclear RNA
INT	-	2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride
LL	-	lentil lectin
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	0.1M phosphate buffer, 0.1M NaCl, pH 7.3
PMSF	-	phenylmethylsulphonyl fluoride
PPO	-	2,5-diphenyloxazole
RCA	-	*Ricinus communis* agglutinin
SDS	-	sodium dodecyl sulphate
TCA	-	trichloracetic acid
TEMED	-	N,N,N',N',-tetramethyl-1, 2-diaminoethane
TKC	-	50mM Tris-HCl, 25mM KCl, 5mM $CaCl_2$, pH 6.9
"Tris"	-	tris (hydroxymethyl) aminomethane
WGA	-	wheat germ agglutinin

INTRODUCTION

I.1. Historical Aspects

During this century the organisation of the nuclear structure has been the subject of much investigation and controversy. Being the dominant feature of eukaryotic cells, the nucleus was easily observed by the early microscopists, most of whom assumed the existence of an envelope at the nuclear boundary. Alternative evidence for a nuclear envelope was provided by Kite (1913), and later by Chambers and Fell (1931), who reported that the nucleus offered more resistance to the passage of a needle than did the surrounding cytoplasm. Injury to the nucleus was followed by its slow collapse, indicating the importance of an intact envelope. There was some opposition to this view however. For example, Pischinger (1937), commented that observations of nuclei after fixation could not be applied to the situation *in vivo*, although many studies had been performed on unfixed nuclei. However most authors at that time appear to accept the existence of a nuclear envelope, although there was very little information as to its actual composition. Some isolated and apparently contradictory observations had been made. Schmidt (1938) showed by birefringence that protein fibres were present, tangential to the nuclear surface, whilst Chinn (1938) reported that there were fatty acid chains radial to it, as revealed by polarised light microscopy. A little later Dangeard (1943) found that nuclei suspended in distilled water developed large transparent blebs on their surface, but that the whole nucleus did not swell. He suggested that this indicated the presence of a nuclear membrane.

Investigation of the structure of isolated nuclear envelope was attendant upon the development of the electron microscope, during

the 1930s and 1940s. The first of these studies was performed by Callan et al.(1949, 1950). They isolated nuclei from Xenopus laevis oocytes by hand, washed them free of cytoplasm and opened the nuclei onto copper grids for electron microscopy. They described a porous (α) layer, and a structureless (β) layer, which they were later able to locate within the α-layer and identify as protein. They reported that the α-layer consisted of "lipoids" (as shown by its extraction with organic solvents and its affinity for Sudan black stain) and that it carried "annuli" on its surface which may have corresponded to pores. Debris of these "annuli" remained on the surface of the β-layer after 24 hours at room temperature, when the α-layer itself had disappeared. This suggested a supportive role for the β-layer and indicated the mechanical strength of the annuli. These observations confirmed the work of both Chinn and Schmidt and initiated the concept of a multi-layered, porous, nuclear envelope.

At that time the observation of a porous nuclear envelope opposed the contemporary theory that the eukaryotic genome was separated from the cytoplasm. This aspect of the nuclear envelope soon became highly controversial. Anderson et al.(1951a, 1951b) and his co-workers showed that nuclei do not behave osmotically, and that the reversible volume changes caused by certain solutions depended upon the condition of the DNA. He also reported that heparin could enter nuclei (after isolation, and in situ) and cause the release of intact DNA through the nuclear envelope. This implied that although large molecules could pass across the nuclear envelope in both directions, the pores were not necessarily open, because free diffusion across the envelope did not seem to occur. He

speculated that a specific control over passage across the envelope was exerted by the pores. He also suggested that enzymes, then thought to be the direct product of the genes, would pass across the nuclear envelope, to direct the activities of the cytoplasm. This concept, of informational macromolecules from the nucleus directing the activities of the cytoplasm, was a forerunner to the work of Jacob and Monod only finally accepted after 1961. Anderson's proposals were strongly disputed by Hogeboom & Schneider (1953), who pointed out that Anderson's experiments were performed on isolated nuclei and inadvertent damage to the envelope would explain their apparent porosity. They also referred to the finding that nucleotide-diphosphate synthesising enzymes were confined to the nucleus, indicating that as they could not leak out, the envelope was impervious to proteins. However, numerous subsequent observations of pores in the nuclear envelope provided convincing proof of its porosity.

In 1953 Anderson had proposed the name "nuclear envelope" for the structure in view of its complexity, and had discussed various aspects of nuclear activity in terms of its various components. In 1954 and 1955, however two papers appeared in which the double nuclear membrane was observed, perforated by pores (Bahr and Beermann, 1954; Afzelius,1955). The quality of the electron micrographs of these papers was far superior to those of Callan, and thin sections were shown, instead of whole mounts. It appears from subsequent papers that the double membrane, shown so clearly by Afzelius, was interpreted as the double layered structure reported by Callan because no reference is made thereafter to the non-porous protein

layer of the envelope. The concept of a double layered nuclear envelope gave way to that of a double nuclear membrane perforated by pores. Anderson himself accepted this, referring to the 'nuclear membrane' when publishing electron micrographs of large molecules passing through the nuclear periphery (Anderson and Beams, 1956).

The structure of the nuclear pore then became the subject of intensive investigation as it directly influenced the nature of nuclear-cytoplasmic exchange. There was a special emphasis on the question of whether or not the centre of the pore was open or filled with some structure for regulating the activity of the pore. Afzelius had reported pores with an internal diameter of 490Å. He said that the annulus consisted of a tube passing through both membranes penetrating both the nucleoplasm and cytoplasm, and that the membranes were continuous at the edge of the annulus. Gall (1954) reported that the annulus consisted of eight to ten spherical bodies and was closed by a "central structure". This was the first suggestion on the octagonal symmetry of the pore, which was confirmed by Wischnitzer (1958), and much later by Gall (1967). Dawson (1954) reported "nodes" embedded in the nuclear surface of the spiral ganglion cells. He observed that the pores were closed by material passing through them (cf. Anderson, 1956). Watson (1955) when describing pores for the first time in mammalian nuclear envelope, also reported that pores were "open but filled by material passing through". So it was difficult for these workers to distinguish between material in transit through the pore and a central structure belonging to the pore. Watson produced a comprehensive study of pores from various tissues in 1955 in which he established them as a universal feature of the nucleus. He reported a thin band across

the waist of the pore and speculated that this was due to the fusion of the two membranes prior to their dissolution to open the pore. This paper was the first to report the continuity between the outer nuclear membrane (ONM) and the endoplasmic reticulum (E.R). Watson mentioned biochemical evidence for the activity of the nucleus in resting cells and suggested the continuous cisternae of the nuclear membranes and the E.R. as an alternative route for nuclear cytoplasmic exchange. There was some evidence to substantiate this in later years (Leduc, 1968). During 1958 Wischnitzer reported that the nuclear pores of amphibian oocytes consisted of annuli made of eight to ten rods closed by a central granule and in the following year Watson made an intensive study of pore structure by taking thin sections at various depths (Wischnitzer, 1958; Watson, 1959). Watson again reported that pores were filled with material in transit and also described intranuclear channels leading into the depths of the nucleus from the pores. He said that there was no satisfactory evidence for a central structure or membrane across the pores, but that it would be invalid to assume free diffusion between the nucleus and cytoplasm. He concluded that there was enough structure present in the pore complex to control the passage of molecules across the nuclear envelope. In that year Barnes noted threads projecting from the edge of the pore into the cytoplasm, and a thick (300 Å) layer of phosphotungstic acid-positive material inside the inner nuclear membrane (INM) between the pores. He concluded pores were open, but structured, in agreement with Watson (Barnes and Davis, 1959). In the following year, Whaley et al.(1960) reported an alternative opening in the nuclear envelope of maize root meristem cells. Pores were noted with the usual structure, but discontinuities were also

described at which both nuclear membranes extended into the cytoplasm, leaving a structureless gap. This has not been found in other cells and is not regarded as a common feature of nuclear envelopes.

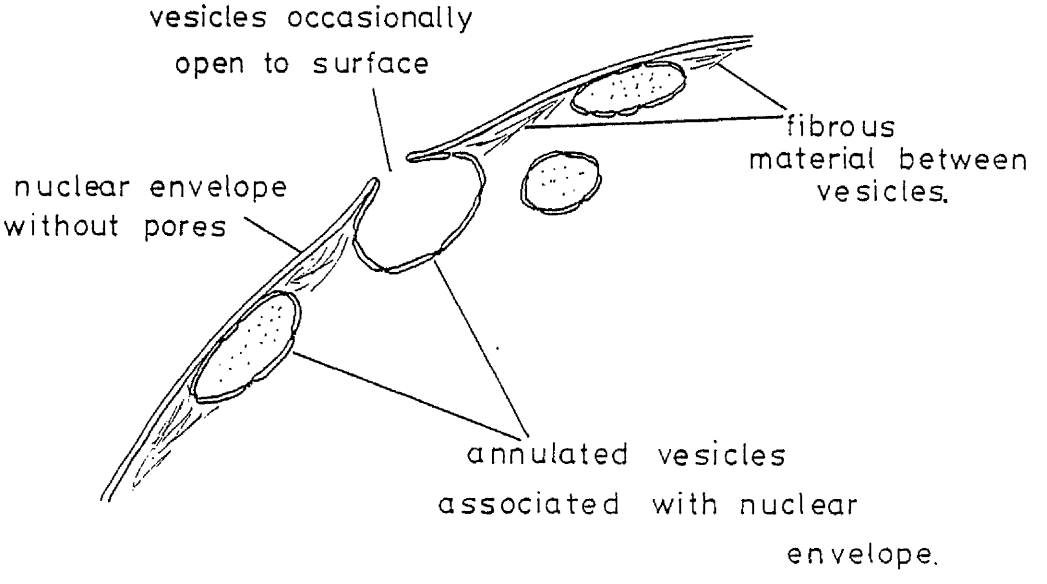
So, at the close of the 1950s the nuclear envelope was regarded as a double membrane perforated by pores, and which enclosed a cisternum continuous with that of E.R. The pores were structured by annuli composed of eight or ten units and were thought to be attached to the double membrane. The structure of the centre of the pore was still unclear, but many thought it able to control nuclear-cytoplasmic exchange. There were some isolated observations of material abutting the INM (Barnes and Davies, 1959; Watson, 1955; Pappas, 1956) but no significance had yet been attached to these.

During this period extensive work had been done to develope alternative methods for the isolation of nuclear envelopes. This work will be discussed later in section I.2.

In 1961 the messenger RNA hypothesis was published providing the answers to a number of outstanding questions surrounding the mechanism of gene expression (Jacob and Monod, 1961). Previously the ribosomal RNA itself was thought to direct protein synethesis, the problem being that although there is great variation in the amino acid compositions of different proteins, and in the DNA composition of different species, the size and composition of rRNA is almost universal. The discovery of an unstable species of RNA comprising 5% of the total, which hybridised specifically with the

DNA being expressed, provided the experimental evidence required for the rapid acceptance of the hypothesis. This, of necessity, meant that the nuclear envelope was porous and research was then directed towards finding the route and mechanism of translocation.

In 1963, Afzelius showed that the nuclear envelope of Noctiluca scintillans had large areas which were not porous or annulated, but which carried annulated vesicles just below their surfaces. Most of these were attached to the inside of the envelope although some were free in the nucleoplasm. These vesicles were densely annulated and occasionally seen to open to the cytoplasm (Afzelius,1963). Their appearance suggested that mRNA was secreted into them and they then opened to release the message to the cell. Afzelius suggested that this argued against the origin of nuclear



membrane in the E.R, but that the annulate lamella originates from the nuclear envelope. He also noted the presence of a fibrous layer of protein strands between the annulated vesicles inside the envelope. This paper seems to demonstrate the necessity of pores for nuclear-cytoplasmic exchange, even if they are not at the usual location.

The much discussed experiments of Feldherr in 1965 showed conclusively that there was not free diffusion across nuclear pores. Using colloidal gold particles, of various sizes, coated with polyvinylpyrrolidine, he showed that the nuclear envelope of Amoeba proteus could not be penetrated by particles greater than 110Å in diameter. Particles 89-106Å in diameter could enter the pores but not pass through them, whilst those less than 85Å in diameter could enter freely (Feldherr, 1965). The gold was seen to concentrate down the centre of the pore, as though passing through a tube (c f. Wischnitzer, 1958). However at that time the nuclear envelope of A. proteus was regarded as abnormal in its possession of a "honeycomb layer" (Pappas, 1956). So Feldherr's observations were not thought to be directly applicable to mammalian nuclei, in which the lamina had not yet been described. During 1965 Loewenstein and his co-workers also reported that the nuclear envelope did not permit free diffusion. They showed that the nuclear envelope of Dipteran gland cells had a higher electrical resistance, by several orders of magnitude, than would be expected if free diffusion between the nucleus and cytoplasm were possible (Weiner et al., 1965). However in 1963 they had shown that vertebrate nuclear envelope had no apparent resistance, (Loewenstein and Kanno, 1963) so there were large interspecies differences in this feature. In their later

paper they compared the ultrastructures of the pores of these various nuclear envelopes, but found no apparent morphological differences to account for the great difference in electrical resistance. They did suggest that differences in the electron dense material filling the pores could account for the observations. The electrical resistance of the nuclear envelope is apparently a dynamic feature: they also observed that it changed with the development of the cell and that changes could be induced by ecdysone (Ito and Loewenstein, 1965). The following year Kessel produced electron micrographs which showed Feulgen positive material passing through pores in close-packed granular accumulations, the diameter of which decreased in transit through the pore (Kessel, 1966). Stevens and Swift (1966) also showed, cytochemically, that RNA passes through the pores to the cytoplasm. The central granule was obscured in those pores through which material was passing but could be clearly seen in the others. This showed that the central granule was not an artifact as had been suggested previously. These investigations all tend to support the hypothesis that the pore is a structured gateway rather than a simple hole; and that it exerts control over the molecules that pass through it. It was not yet established whether the pore was the only route between the nucleus and cytoplasm.

Two papers appeared then which gave evidence for an alternative route to the cytoplasm. In 1968, Leduc showed that peroxidase could be detected immunologically in the perinuclear spaces of immature plasma cells. During the cells' development the enzyme invades the E.R. cisternae where it is found exclusively in mature cells (Leduc et al., 1968). This route was originally

suggested by Watson (1955) but was dismissed as being too rarely seen to be the usual mechanism. The following year Sharer, working with African lungfish oocytes, reported the massive enlargement of the nucleoli often seen during the development of amphibian occytes. She noted fine threads of rRNA passing from the nucleolar mass through the pores to form "nucleolar granules" in the cytoplasm. When the export of rRNA was at its height however, the pores appeared to be unable to cope because nucleolar granules then started to appear in the perinuclear space. These were exported to the cytoplasm by blebs in the ONM which pinched off to form cytoplasmic vesicles. Convincing electron micrographs were presented to substantiate the claim (Sharer and Wurzelmann, 1969). This work, together with that of Afzelius in 1963 and Whaley in 1960 show that there are other pathways of nuclear-cytoplasmic exchange besides the nuclear pore. However these are observations of isolated mechanisms in special tissues or conditions of development and do not form the main thoroughfare between the nucleus and the cytoplasm.

Further evidence for the dynamic control exerted by the pore complex was produced by Franke in 1970. He re-examined the nuclear envelope of the amphibian oocyte isolated by the method of Callan and Tomlin (1949) and reported that 61% of pores in larval eggs contained a central granule, whilst only 36% of those in the mature oocyte did (Franke and Scheer, 1970). This corroborates the earlier evidence of Ito, of Kessel, and of Stevens in support of the regulatory role of the pore complex in nuclear cytoplasmic exchange. Recently, indirect evidence for control exerted by the whole structure has been provided by Feldherr (1980). He found that

mechanical disruption of the nuclear envelope of defolliculated Xenopus laevis oocytes produced neither quantitative nor qualitative changes to the protein composition of the nucleoplasm. In later experiments he found, in the same circumstances, that the synthesis of 40s rRNA, its processing to the 28s and 18s species, and their distribution between the nucleus and cytoplasm were all unchanged. This implies that all the precursors and products in these processes are specifically bound to the nuclear structure. So the route of nuclear-cytoplasmic exchange is now seen as occurring chiefly through the pores, although alternatives appear to be available if necessary. The pores can exert a control over the size and the nature of the materials exchanged, and themselves change both in frequency and construction according to the state of the cell.

The next major development in our appreciation of the nuclear envelope was the discovery, or rediscovery of the lamina. This structure had been observed many times before it was actually discussed. It may have originally been observed by Schmidt, who described protein fibres tangential to the nuclear surface using birefringence (Schmidt, 1939). The lamina was almost certainly seen by Callan and Tomlin in 1949 and 1950, in amphibian oocyte nuclear envelope isolated by hand. They described the structureless (β) layer inside the porous lipid containing layer which closely fits our concept of the lamina. There were other isolated observations, namely : Barnes in 1959 noted phosphotungstic acid - positive material inside the INM; Watson noted that the inner membrane was thicker than the ONM (Watson, 1955) and Afzelius noted a fibrous protein layer inside the INM of Noctiluca (Afzelius, 1963). Thomas presented electron micrographs of human amnion epithelial cells in which a

lamina was very clearly seen below the INM, although no mention of this was made in her text. (Thomas, 1965). A protein structure below the inner membrane was first specifically described in A. proteus as the "honeycomb layer" by Pappas in 1956. Such a layer was also described in Gregarina melanopli (Beams et al 1957) and in the ventral neurones of Hirido medicinolis (Gray and Guillery, 1963).

The first description of the lamina in vertebrate cells was given by Fawcett in 1966 in which various tissues from different vertebrates were examined and the lamina was established as a universal feature of vertebrate nuclei (Fawcett, 1966). These observations were confirmed the following year by Mazenak in various human cells (Mazenak, 1967) and by Patrizi in chondrocytes of guinea-pig cartilage (Patrizi and Poger, 1967). Soon afterwards Patrizi studied the lamina in human plasma cells, before and after viral infection, finding no difference in its structure (Patrizi, 1968). He described the lamina as complete around the nuclear periphery below the INM, but absent just below the nuclear pores. As recently as 1979 a lamina was reported by Rifkin in the alveolar bone cells of monkey jaw (Rifkin and Heijl, 1979). Possibly the discovery of the lamina in vertebrates was delayed by its size as it appears to be thicker in invertebrates, the honeycomb layer of Amoeba being the thickest and most obvious. As previously suggested above it may have been overlooked because it was actually thought to be the inner membrane, after the double membrane structure had been established. Also, in vertebrates the lamina may vary in thickness and be more obvious at some times than at others. Ghadially and Fuller (1972) showed good evidence for the waxing and waning of the fibrous lamina in chondrocytes according to the state of the cell.

They examined the nuclei of guinea-pig cartilage during repair and reported that the lamina was 353Å thick two weeks after injury, rising to 725Å, three months after injury and falling again to 350Å six months after injury. Further work by this group in 1974 showed that mature chondrocytes had a much thinner lamina than did young cells (Oryschak et al., 1974). This suggests that the lamina is a dynamic feature of the nuclear envelope, which alters with the state of the cell, as apparently do pores.

The relationship of the lamina to the rest of the nuclear envelope was clarified by Aaronsen in 1975, who prepared the "pore-complex-lamina" fraction. This was prepared by incubating nuclei with DNA-ase to remove chromatin, followed by Triton-X100 and 0.3M $MgCl_2$ to remove membranes and residual chromatin (Aaronsen and Blobel, 1975). This preparation demonstrated that the pore complexes were firmly attached to the lamina and not dependant upon the membrane for their distribution. This finding was confirmed by other workers (Scheer, 1976; Riley and Keller, 1975) and in 1976 Dwyer and Blobel modified the preparation (Dwyer and Blobel, 1976). The spectacular scanning electron micrographs of Kirschner and of Schatten also confirm the attachment of pores to lamina (Kirschner et al., 1977; Schatten and Thoman, 1978). In spite of these findings there may be some interaction between the edge of the pore and the surrounding membrane because the annulate lamella and the annulated vesicles of Noctiluca nuclei (Afzelius, 1963) do not have a lamina and yet do contain pores. Where a lamina is present though, its interaction with the pore appears to be the stronger. Kashnig and Kasper reported the presence of pores in their nuclear membrane preparation (Kashnig and Kasper, 1969). Were pores present lamina

protein may well have also been present. This may account for the high protein content reported for these nuclear membranes. The presence of lamina would not be surprising because the pore-complex-lamina preparation differed from most "nuclear membrane" preparations only in the addition of Triton-X100.

Therefore we arrived at the concept of a protein lamina, carrying the pore complexes, over which was laid a double membrane between the pores. The emphasis then appeared to shift from attempting to isolate a pore preparation of nuclear membrane to fractionating whole nuclei into various nuclear envelope components. For example in 1970 Franke reported a method for the isolation of pores from nuclear envelope by incubation with divalent cations followed by distilled water (Franke, 1970). Conversely, Bornens reported that pores could be removed from the lamina by incubation with EDTA to remove divalent cations (Bornens and Courvalin, 1978). Perhaps there is a critical concentration of cation needed for the attachment of pores to lamina. Krohne and his colleagues isolated pore-complexes from amphibian oocytes, a tissue enriched in the structures (Krohne et al., 1978). In 1976 Scheer disintegrated nuclei experimentally to reveal pore connecting fibrils distinct from those of the lamina (Scheer, 1976).

Many of the lamina preparations were investigated by polyacrylamide gel electrophoresis (PAGE), and invariably the protein profile is dominated by a triplet of proteins having Mr of 60,000 to 70,000. These have been given various labels, usually according to their apparent molecular weight by PAGE:- LP 71, 67 and 65 (Ely et al., 1978); P 70, 67 and 60 (Gerace et al., 1978); Mr 78, 74 and 68 (Lam and Kasper, 1979 a),

Before 1949.

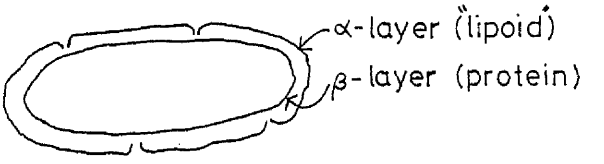
Nucleus observed.

? nuclear envelope.

protein & lipid detected.

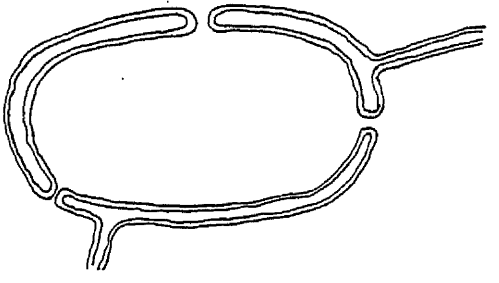
1949 - 1955.

Layered nuclear envelope.



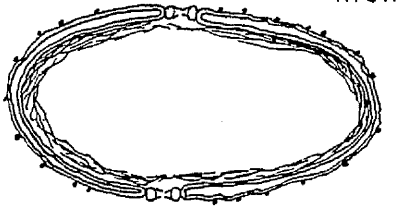
1955 – 1966

Porous double nuclear membrane continuous with E.R.

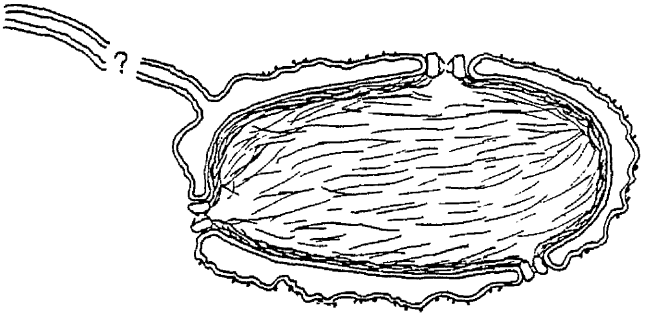


1966 – ~1974.

Nuclear lamina observed inside inner nuclear membrane.



~1974. →



Pore structures attached to lamina.

Matrix within interacts with DNA and hnRNA.

? nuclear membrane continuous with E.R.

Fig. 1. Development of our concept of the Nuclear Envelope

P75, 71 and 61 (Shelton, 1978); Lamins A, B and C (Gerace and Blobel, 1980) and N1, N2 and N3 (Richardson and Maddy, 1980 a,b). The biochemistry of these polypeptides is currently the object of much investigation.

The biochemistry of the pore complex itself has received rather scant attention, probably because of the difficulty of isolating pure proteins. This difficulty may reflect the strong affinity of the pore complex for the lamina. One paper in which such a separation was reported was that of Krohne et al.(1978). The nuclei of maturing oocytes of amphibia are characteristically enriched in pores, possibly as a result of the need for a massive export of rRNA during the cells' development (cf. Sharer and Wurzelmann(1969) working with African lungfish). Manual isolation of the nuclear envelope by the Callan and Tomlin(1949) method, followed by repeated extraction with high concentrations of salt (eg. 1.5M KCl) and 1% Triton-X100, gave a preparation very much enriched in pores as shown by their electron micrographs. Investigation by PAGE after treatment with sodium dodecyl sulphate (SDS) showed that these preparations are substantially enriched in proteins having apparent Mr of 150,000 and 73,000. It is possible that the latter protein corresponds to lamin B, the middle band of the characteristic pore-complex-lamina triplet. Some fibrous material is seen in the electron micrographs lying between the pores, and this may account for the minor protein bands seen in the profile (Krohne et al., 1978). The authors noted that isolation of rat liver nuclear envelope, followed by a similar extraction, produced the two bands described plus two additional bands with Mr of 78,000 and 66,000, forming the

characteristic triplet. They reported that further subfractionation reduced the intensity of the Mr 66,000 band, and tentatively ascribed this protein to the intranuclear matrix.

The appreciation of this matrix is closely related to the development of the concept of the lamina. As with the lamina,it was observed before it was described, and may have first been investigated by Mirsky in 1947, who noted residual non-histone protein associated with RNA after chromosomes had been extracted with 1M NaCl (Mirsky and Ris, 1947). A matrix within the nucleus has been noted by various workers since then (Zbarsky et al., 1962; Steele and Busch, 1966; Smetana et al., 1963; Narayan et al., 1967). The matrix was first described as such by Berezney in 1974. The protein profile showed a predominance of the triplet of proteins with molecular weights of 60,000 to 70,000 (Berezney and Coffey, 1974). This is to be expected because the isolation procedures for the pore-complex-lamina fraction (Dwyer and Blobel, 1976) are almost identical to those of the matrix fraction (Berezney and Coffey, 1977). Both employ DNAase to remove DNA, Triton-X100 (1-2%) to dissolve membrane components, and high concentrations of salt to remove residual chromatin proteins. The matrix preparation also involves the use of RNAase, and two treatments with low concentrations of magnesium initially. The enzymes are introduced at the end of the matrix preparation procedure, but are the first stage in the preparation of pore-complex-lamina. Both authors report the presence of pores attached to the fraction they are preparing and although there are differences in the molecular weights of the dominant proteins, these are only minor, namely: 69,000, 68,000 and 66,000 (Dwyer and Blobel, 1976) and 69,000,

66,000 and 62,000 (Berezney and Coffey, 1977). This work suggests that the demarcation between matrix and lamina is rather arbitrary, and it is possible that the lamina extends through the nucleus in a more diffuse form as the matrix. This is supported by the observation that the macronuclei of Tetrahymena do not have a lamina, but that the matrix has a peripheral layer (Wunderlich and Speth, 1972). However, recently two groups have used immunological techniques to locate the proteins of the dominant triplet in the nucleus. (Ely et al., 1978; Gerace et al., 1978). Using antibodies raised to SDS-denatured proteins (isolated by preparative electrophoresis) both groups found intense staining of the nuclear periphery, with very little in the internal regions. This suggests that the "triplet proteins" are exclusive to the lamina and that there is a distinction between the two fractions. Adequate penetration of the nucleus by immunoglobulins was demonstrated by Ely's group who showed intranuclear staining with antihistone antibodies. Berezney (1980) has reported the fractionation of the nuclear matrix into three matricin proteins (which apparently correspond to the three lamina polypeptides) and a residual ribonucleoprotein (RNP) fraction. He states that "the exact structural locations of the matricin and RNP fractions have not been defined ..... the contribution of the residual lamina layer and nucleoli to these fractions cannot be precisely evaluated". He also points out that the lack of immunological staining for these polypeptides within the nucleus, repeated by both Ely and Gerace, does not adequately demonstrate their absence. This is because the antibodies, raised to SDS-denatured proteins, may not react with the native proteins under certain conditions. This point has also been made in another recent

paper (Richardson and Maddy, 1980a). It is very probable that the so-called lamins and matricins are the same proteins. Possibly they are more densely packed in the lamina, and extend through the volume of the nucleus in a more diffuse way to form the matrix. If this were so the lack of immunological reaction in the matrix region could indicate either a change in the conformation of the proteins, or an interaction with another component, both of which could obscure the antigenic site.

Possible candidates for such an interaction are DNA and hn RNA. Numerous reports show an interaction between the structural component of the nucleus and both DNA and RNA. In 1971 Mizuno produced two papers which reported the association of a small percentage of the total DNA with the inner membrane of the nuclear envelope (Mizuno et al 1971). Using nuclear envelope prepared after Kashnig and Kasper (1969) Mizuno found that Triton-X100 removed what was assumed to be the outer nuclear membrane. DNA was found associated with the remaining fraction and after studying the specific activity of pulse labelled DNA in this fraction it was reported that DNA initiation sites were concentrated at the inner nuclear membrane. No mention was made of the lamina or matrix in this paper, but as we have seen their proteins were probably present in the preparation. The conclusions of Mizuno were denied in 1973 by three groups using pulse labelling techniques to identify newly synthesised DNA. Wise performed autoradiography to locate labelled DNA and concluded that the initiation sites were dispersed throughout the nucleus (Wise and Prescott, 1973). O'Brian reached the same conclusion through an examination of the specific activities of membrane-associated and pore DNA (O'Brian et al., 1973) and Comings found by auto-radiography that newly synthesised DNA was dispersed throughout the nucleus and nucleolus (Comings and Okada, 1973).

An association has been found more recently between the DNA and the matrix. For example, Comings reported that mouse nuclear matrix binds both homologous and heterologous DNA with high affinity. Filter-binding assays were used, and the matrix was isolated by a procedure similar to that of Berezney. There was preference for A-T-rich and single stranded DNA, but the highest affinity was between the matrix and the DNA removed from it during its isolation. This probably indicates a conformational change in the matrix proteins during their isolation or a missing factor in the filter binding assay (Comings and Wallack, 1978). A more recent paper confirms the earlier work of Wise and of O'Brian and Comings. DNA, pulse labelled in the presence of arabinosyl cytosine so that the label was confined to the Okasaki fragments, was quantitatively isolated with the nuclear matrix of mammalian interphase nuclei, showing that the initiation sites are associated with the matrix. After labelling all the DNA, i.e. without arabinosyl cytosine, the isolated matrix was treated with various DNAases specific for single or double stranded DNA. Single stranded DNA was preferentially released and was shown to consist of Okasaki fragments. After this treatment, double stranded DNA attached to the matrix was less accessible to the DNAase specific for it than before removal of the single stranded DNA. The authors concluded that DNA is attached to the matrix close behind the branch-point of the replicating fork (Dijkwel et al., 1979). Much recent work has supported this. If DNA is pulse-labelled for only 30 seconds, 90% of this label is subsequently isolated with the matrix (Pardoll et al., 1980). This figure falls to 5% if the pulse is extended to five minutes (Berezney, 1981).

Hybridisation kinetics have shown that no particular part of the DNA is matrix bound suggesting that all DNA is bound at some time (Berezney et al., 1981; Basler et al., 1981; Pardoll and Vogelstein, 1980). However, this is not confirmed by Matsumoto and Gerbi (1981) who found an enrichment of satellite DNA associated with the nuclear matrix of bovine cells. Eastment et al. (1981) also found changes in the DNA associated with matrix protein of granulocytes at different stages of their development. These two observations suggest that the role of the matrix in DNA replication is still uncertain.

Heterogeneous nuclear RNA was demonstrated as a structural component of the matrix in 1975 (Faiferman and Pogo, 1975) and this group later demonstrated that the nuclear matrix of rat liver was associated with a ribonucleo-protein RNP complex containing rapidly labelled RNA, possibly mRNA. Proteolysis destroyed this and made the RNA susceptible to RNAase, indicating the intimate nature of the association (Miller et al., 1978). In 1979 the same group investigated the interactions between hn RNA and the nuclear matrices of Friend erythroleukaemia cells in the undifferentiated and differentiated states, which have diffuse and condensed chromatin respectively. After removal of DNA, the matrices retained the characteristic diffuse or condensed morphology, indicating an intimate association with the structure of the chromatin. Heterogeneous nuclear RNA remained associated with the matrices through a variety of salt treatments and was only released by the disruption of hydrophobic and hydrogen bonds by chemical means. This produced lysis of the matrix, indicating a structural role for the RNA (Long et al., 1979). Such an intimate

association between the matrix and RNA was also demonstrated by Feldherr (1980), as previously mentioned. The findings of Berezney (1980) differ slightly from those just discussed, in that he reported the fractionation of the matrix into proteins and RNP particles which implies, perhaps, a looser relationship. However, Adolph (1980) has recently reported that the pretreatment of nuclei with ribonuclease A reduced their rate of sedimentation after removal of the histones and destroyed their internal structure. This may indicate again a structural role for RNA in the matrix, in agreement with the work of Pogo's group.

Recently the matrix has been implicated as controlling the transport and possibly the processing of RNA. Baglia and Maul (1982) treated nuclei with antibodies to matrix proteins and found that RNA transport was affected. Agutter and Suckling (1982) found that colchicine induced constriction of pore complexes, effected disordering of the nuclear membrane, and inhibited nucleo-cytoplasmic translocation of RNA. Finally Herlan et al. (1980) found that RNA was released from Tetrahymena nuclei when they were induced to expand in low concentrations of Ca^{2+}/Mg^{2+}. This is probably not physiologically significant, but does suggest that the matrix has some control over the RNA.

The role of the matrix in the processing of mRNA precursor molecules has been studied by three groups. Danchin and Slonimski (1981) speculated on the role of the matrix in generating mRNAs for immune specific proteins. A great variety of mRNAs are necessary and the authors suggest that the precursor mRNA molecules could be spliced together and processed on the matrix to generate this variety. Recently Ross et al. (1982) investigated the location

of globin nuclear RNA and globin genes in chicken erythroblasts and erythrocytes. No special affinity of globin genes for the matrix was found, but more than 90% of globin nuclear RNA precursors were matrix bound. As some of these precursor molecules were larger than the globin gene, the authors suggested that the matrix is a platform for the differential processing of nuclear RNA. Enzymes involved in the processing of nucleic acids have been co-purified with the matrix, which supports this suggestion. This will be discussed later.

The actual biochemistry of the lamina/matrix proteins (which I shall refer to as lamins A, B and C in accordance with the scheme of Gerace et al. 1978) has recently been investigated by Shelton's group. They have shown that there is considerable sequence homology between the three, but that while lamins A and C have similar features lamin B is different in some ways. Oxidation of the pore-complex-lamina with the o-phenanthroline-copper complex shows that lamin A forms oligomers and dimers, while B forms only dimers. Lamins A and C have similar sensitivity to acid cleavage whilst B is unusually stable. Further, two dimensional separation by isoelectric focussing and SDS-PAGE showed that lamin B was the most acidic (by approximately 0.7 pH units), and had only one isoelectric form. Lamins A and C have two similar isoelectric points, and several similar isoelectric variants. The conclusion is that Lamin C may be a cleavage product of A. Tryptic peptide mapping (Shelton et al., 1980) showed that the two proteins have identical peptides, apart from one extra peptide in A, whereas B has a different set of peptides, albeit containing some homologous sequences. The authors point

out that the immunological cross reaction between the three proteins, reported by both Ely and Gerace, could reflect their sequence homology. This may be in agreement with Krohne's work (Krohne et al., 1978) in which the middle protein of the triplet, lamin B, had fractionated differently from the other two, and was tentatively ascribed to the actual pore complex. Similar investigations by Lam, involving chemical and enzymic cleavage of lamina proteins, also led to the conclusion that lamins A and C had close sequence homology whilst B has some different polypeptide sequences (Lam and Kasper, 1979b). However, they concluded from their o-phenanthroline-copper oxidation studies that lamin B consists of two electrophoretically distinct species, probably existing as homo-trimers. This does not agree with the results of two dimensional separation produced by Shelton who has continued to publish evidence for a precursor-product relationship between lamins A and C. After the lamins were cross-linked, the results of mild proteolysis suggested that lamin B exists as a homotypic tetramer in vivo (Cochran and Shelton, 1981; Shelton et al., 1982). Recently these workers have identified subtypes of lamin B in chicken erythrocytes which are not seen in mammalian nuclei (Shelton et al., 1981). No subtypes of lamins A and C were found which suggests an inherent difference between these proteins and lamin B.

Interestingly, the search for lamins in non-mammalian species has not produced the familiar triplet of lamins. While investigating the germinal vesicle of Spisula solidissima as a source of pore complexes, Maul found only one protein (Mr 67,000) in the molecular weight range of the lamins, analogous

they suggest, to lamin B. Their preparation was enriched in pore complexes and they propose that lamin B is a constituent of the pore complex rather than the lamina, in agreement with Krohne's previous work (Maul and Avdalovic, 1980; Krohne et al., 1978). Krohne has recently studied the pore-complex-lamina from Xenopus laevis oocytes. This preparation also contains a high proportion of pore-complexes and a protein (Mr 68,000) which was correspondingly enriched. However, he points out that the isoelectric points of this protein are very different from that of lamin B which would preclude an analogy between them. He suggested that the 68,000 protein is a component of pore complexes but that in preparations containing a higher proportion of lamina (such as from rat liver or amphibian red cells) it is obscured by lamin B and is not detected (Krohne et al., 1981).

Lam and Kasper have also demonstrated that lamin C is specifically phosphorylated by endogenous protein kinases, when purified nuclear envelope is incubated with ^{32}P. This is reduced by treatment with Triton-X100, deoxycholate and sonication, all of which perturb the membrane (Lam and Kasper, 1979a), Phosphorylation of E.R. under the same conditions was much less specific and had different characteristics (such as pH optima and prepared ionic environment). This indicates that the nuclear reaction was not due to contamination by E.R. A specific topographic relationship between the kinase and the protein may be necessary for the reaction, as suggested by its sensitivity to the perturbation of the membrane. McDonald and Agutter (1980) have also examined the phosphorylation of nuclear envelope proteins under various

conditions. Kinetic studies suggested that the activity consists of an ATP-ase, a kinase and two phosphohydrolases. It was found that phosphorylation of the pore-complex-lamina proteins increased their affinity for RNA. It was also suggested that the phosphorylation state of the pore complex proteins may influence nucleo-cytoplasmic exchange of RNA. Another possible role for such a phosphorylation has recently been suggested by Gerace, namely that reversible enzymatic phosphorylations are involved in the mechanism of dissociation of the nuclear envelope, prior to mitosis and its reformation thereafter (Gerace and Blobel, 1980).

The dissociation of the nuclear envelope is clearly visible by light microscopy and must always have caused speculation, the more so as the complexity of the nuclear envelope structure became apparent.

The early observations of Porter showed the release of membrane vesicles from the nuclear envelope at the onset of mitosis. These vesicles then became indistinguishable from the E.R. pool (Porter and Machado, 1960). It was also reported in 1970 that during the development of the male pro-nucleus of the sea urchin Arabacia punctulata, membrane vesicles were lost from specific areas of the nucleus, but retained in those regions previously associated with the acrosome and centriole (Longo and Anderson, 1970). This is not strictly comparable with mitosis, but may give an indication of a mechanism of control over the movement of the membrane which is possibly exerted by the underlying structures, i.e. an interaction between the lamina and the membrane.

The first biochemical investigation of the mechanism was that of Sieber-Blum and Berger who investigated the biosynthesis of nuclear envelope proteins by labelling kinetics. The found no increase in de novo synthesis of proteins in conjunction with nuclear envelope reformation, there being continuous synthesis of nuclear envelope proteins throughout the cell cycle. This indicates that the nuclear envelope is reformed mostly from extant proteins, stored in the cytoplasm during mitosis (Sieber-Blum and Berger 1976). This result was confirmed in an extensive paper by Conner et al. (1980) who reported that in the nuclear envelope 60% of the protein and 50% of the lipid pre-exist mitosis, the remainder being supplied by de novo synthesis during the early G_1 phase. They concluded that there are no reproducible changes in the peptide composition of the nuclear envelope that can be correlated to specific changes in the cell cycle.

In 1978, the immunological localization of the three lamins, reported by Ely and by Gerace, was used by both groups to follow the whereabouts of these proteins throughout the cell cycle. Both groups reported that during the dissociation of the nuclear envelope the peripheral nuclear staining decreases with a concomitant increase in overall cytoplasmic staining, until the whole cell is obscured. This is reversed as the nuclear envelope reforms (Ely et al., 1978; Gerace et al., 1978). The fact that the proteins do not still bind to their antibodies after their dissociation from the envelope does at least show that the proteins are not degraded during the process, and that they retain their antigenic specificity during mitosis. Recent criticism of these papers (Richardson and Maddy, 1980a; Berezney, 1980), suggesting

that antibodies to SDS-denatured proteins do not necessarily bind the native proteins *in vivo*, does not undermine this point. These groups also reported that antigens to each of the lamins cross-reacted with other lamins and with lamins from other species. This finding was supported by Stick and Hausen (1980) who suggested that the lamina really is constructed of antigenicly cross reactive proteins conserved during evolution. These may be generated through a precursor-product mechanism. Alternatively they propose that the lamina proteins are unique, but share a common prosthetic group which acts as the antigen in these studies.

Gerace and Blobel (1980) have recently extended this work using cell fractionation with immunoprecipitation on synchronised cell populations. They have shown that lamins A and C occur in a soluble, non-membrane-associated state during mitosis, whilst lamin B remains associated with the disassembled envelope fragments. This would be expected if lamin B is a pore complex protein, as suggested by Krohne *et al.* (1978). Gerace and Blobel also reported that sedimentation analysis revealed that all three lamins were monomeric during mitosis in contrast to their various oligomeric forms during interphase. They have shown that the mitotic monomers are phosphoproteins with more acid isoelectric points than their interphase counterparts, and suggest that the lamina is reversibly depolymerised as a result of specific phosphorylations, reflecting the work of Lam and Kasper (Lam and Kasper, 1979a).

Peterson and Berns (1978) reported that when mitotic chromosomes were treated with derivatives of the light sensitive

drug psoralen, using laser light to initiate the addition, cells without nuclear envelopes were produced. These had chromosome masses bearing disoriented pore complexes on their surfaces, no lamina, and excess amounts of E.R. vesicles. The quantity of reformed nuclear envelope was proportional to the quantity of psoralen attached to the chromosomes, a reduced laser treatment causing patches of reformed nuclear envelope. Transcription and translation had not been involved because cycloheximide and actinomycin D did not prevent nuclear envelope reformation. The cells were viable for up to 57 hours after the laser treatment, having normal nucleotide and amino acid uptake, but did not divide again. This might imply that the lamina reformation had been disrupted and incoming vesicles of E.R. could not spread around the chromosome mass without the intervening lamina. This suggests a specific interaction between the membrane and the lamina, as did the work of Longo and Anderson. The appearance of pore complexes on the chromosome surface suggests that their reformation is independent of both the membrane and the lamina. Maul (1977) has made a similar observation. He took serial sections of normal mitotic chromosomes and observed what were apparently remnants of pore complexes lying on their surfaces. These rapidly broke down as mitosis continued and he concluded that there was insufficient evidence for the retention of pore complex components during mitosis.

The possibility of an interaction between the matrix and the nuclear membrane has recently been investigated with a totally different approach. Anderson originally observed that within certain limits an increase in the concentration of calcium

and magnesium ions effected a reduction in nuclear volume, and that calcium had a more pronounced result than did magnesium (Anderson, 1953). Wunderlich's group, in 1978 reported that Tetrahymena macronuclei respond in a similar way to changes in the concentration of a Ca^{2+}/Mg^{2+} (3/2) mixture. At a concentration of 5mM this solution caused marked contraction of the nuclei as shown by a reduction in their diameter and an increase in the number of pores per μm^2. There was a converse expansion when the Ca^{2+}/Mg^{2+} solution was reduced to 1mM. Investigating the membrane by electron spin resonance spectroscopy with 5-doxylstearate as the probe, they have found a higher lipid fluidity in the contracted nuclear envelope than in the expanded state. Freeze-fracture studies revealed areas of lipid from which protein had been segregated, which are not seen in expanded nuclear envelope. Conversely extracted nuclear lipids shown an increase in rigidity in higher Ca^{2+}/Mg^{2+} concentrations (Wunderlich et al., 1978). In a subsequent report (Geise et al., 1979) they have shown that the lipids in the expanded state have a biphasic response to reduction in temperature. Fluidity changes slowly as the temperature is reduced, until a discontinuity shows a transition from the thermotropic fluid phase to an ordered phase, the temperature at which this occurs being dependent on the growth temperature (since this influences the extent of unsaturation in the lipids). Contracted nuclear membranes show no such transition, whatever their growth temperature, i.e. they have reduced thermotropic lipid clustering. The authors proposed that interactions between the matrix (these nuclei having no apparent lamina) and the nuclear membrane control the fluidity

and protein segregation of the membrane. Most recently they have attempted to remove the matrix from within the membrane by treatments with DNAase and RNA ase, followed by 1M NaCl. This, they claim, leaves the double membrane with pores, a preparation they call the nuclear ghosts. In comparison to untreated nuclei these ghosts show higher lipid fluidity, and have no thermotropic transition or response to changes in the concentration of divalent cations (Giese and Wunderlich, 1980). The authors suggest that removal of the matrix from below the nuclear membrane disrupts the protein - protein interactions between it and the matrix, in turn perturbing the lipid-protein interactions within the membrane, causing the differences in response to the environment. This, they say "supports the hypothesis that the Ca^{2+}/Mg^{2+} -sensitive nuclear matrix proteins can moderate nuclear membrane fluidity". This is reasonable and probably valid, except that one puzzling feature of this work is the preparation of their nuclear ghosts. This procedure differs only in the omission of Triton-X100 and a reduction in the salt concentration from that which they used for the preparation of a Ca^{2+}/Mg^{2+} -sensitive nuclear matrix from Tetrahymena macronuclei (Wunderlich and Herlan, 1977). It would seem likely therefore, that matrix proteins are still present in the nuclear ghosts. Indeed a comparison of the protein profiles of ghosts and matrix shows that all the matrix proteins are present in ghosts, although in slightly different proportions. If this is so, then the different responses of nuclear ghosts to temperature and divalent cation concentration could be due to the removal of nucleic acids, in turn causing changes in the conformation of the matrix proteins. The

authors noted that extracted nuclear lipids have even higher fluidity than the ghosts which may indicate a residual interaction between the matrix and nuclear membrane proteins in the ghosts. One recent observation also seems to suggest an interaction between the matrix and the nuclear membrane. Agutter and Suckling have shown that colchicine which binds to the nuclear envelope and causes a constriction of the pore complexes, also causes a disordering of the nuclear membrane lipids (Agutter and Suckling, 1982b).

Recently, work in this area has focused on the modification of nuclear membrane fatty acid composition, and the effect this has upon nuclear function. Agutter and his coworkers have shown that the cholesterol content of isolated nuclear envelopes can be modified by incubation with liposomes containing various concentrations of cholesterol. Nuclear envelope NTP-ase activity was significantly lowered (by 40%) when the cholesterol content was increased. However, the order parameters of lipids in isolated nuclear envelopes differ widely from those in intact nuclei, so these data are probably not physiologically significant (Agutter et al., 1979; Agutter and Suckling, 1981). Subsequently, manipulation of the fatty acid composition of the membrane of intact nuclei wasachieved by a dietary modification. According to Agutter and Suckling (1982a) this did not affect the rate of nuclear transport of RNA. However, others have reported that an increase in saturated fatty acids produced a decrease of 34% in the export of mRNA (measured by protein synthesis in a fortified cell free system) when compared to nuclei with a high content of unsaturated fatty acids (Yannarell and Awad, 1982). These obser-

vations suggest that phospholipids play a more important role in nuclear envelope function than was previously realized, and two other observations also support this. Cocco and Maraldi (1980) found that isolated nuclear matrix, treated with phospholipase C released the DNA that is normally retained during matrix isolation suggesting that phospholipid interactions may be involved in the association of DNA with the matrix. Another report suggests that sphingomyelin is involved in the interaction of DNA with the matrix (Alesenkov et al., 1982). Clearly, nuclear lipids have a much less passive role in the nucleus than has hitherto been supposed.

I.2. Isolation Techniques for Nuclei and Nuclear Envelope

The first observations of isolated nuclear envelopes were made on those obtained by hand from amphibian oocytes. These are large and easily manipulated and many subsequent investigations also used this tissue. However techniques for the bulk isolation of nuclei from other sources were being developed during this period. The nucleus is the most dense organelle in the cell, it can therefore, in theory, be separated from the other cellular constituents by differential centrifugation. However, the separation is never complete and various factors influence the extent of contamination. For example, whole cells will pellet with nuclei if the homogenisation stage has been ineffective, also proteins and larger fragments of other organelles can be adsorbed onto the nuclear surface, causing subsequent contamination of the nuclei. The continuity of the ONM with the E.R. has caused not only contamination by E.R. itself, but also mechanical trapping of other organelles between the cytoplasmic filaments on the nuclear surface. For these reasons contamination has always been a major problem in the bulk isolation of nuclei. A large number of methods have been described to circumvent these difficulties and these have been adequately reviewed elsewhere (Roodyn, 1963, Smuckler et al 1976). The discussion will therefore be restricted to the principles involved. Two basic techniques were developed during the 1950's and early 1960's, involving either organic or aqueous solutions. It was thought, at one time that "soluble" proteins would be removed by isolation in aqueous media so a variety of organic solutions were devised for the isolation of nuclei. The tissue was first lyophilised, then homogenised with organic solvents (Dounce et al., 1950; Allfrey

et al., 1951). Differential centrifugation of the homogenate was then used to separate the nuclei from the other organelles. Sometimes solvents of various different densities were used to facilitate the separation. This method is no longer used. Roodyn comments (Roodyn, 1963 p.24) that there was insufficient evidence to establish that soluble proteins were not lost. Nuclear membrane proteins would almost certainly be lost because of the extraction of membrane lipids. Another disadvantage is that proteins were denatured, so enzyme assays were not possible and the nuclear morphology was very much changed. This method also appears to have given inconsistent RNA/DNA and protein/DNA ratios (compare Dounce et al., 1950 with Naora and Takeda, 1954). However Georgiev stated that this method is the only way to establish unequivocably whether or not a protein was present in the nucleus (Georgiev, 1967 p.31).

The alternative procedure employed a wide variety of aqueous solutions, many including sucrose or glycerol, to prevent loss of proteins (Hogeboom et al., 1948). The sucrose concentration could be adjusted to provide a variety of densities for differential centrifugation. For example, Chauveau in 1956 centrifuged nuclei through 2.2M sucrose to prevent fragments and organelles less dense than nuclei from pelleting and to remove E.R. attachments. This decreased the yield but gave a relatively good preparation. A more recent modification of this, on which my own isolation procedure is based, is that of Blobel and Potter (1966) in which the density of the solution above the dense sucrose cushion is increased. This has the effect of floating the cytoplasmic debris away from the interface towards which the nuclei are pelleting.

Many modern preparations still employ this technique.

A wide variety of buffers and salts in the sucrose solutions have also been employed in order to improve the yield and appearance of the nuclei. For example citrate was used because it improved the appearance of the nuclear surface, but was abandoned later as it was found to remove large amounts of protein. It was subsequently used for nuclear envelope isolation. Calcium ions are also said to improve the quality of isolated nuclei (Schneider and Peterman, 1950). Anderson showed later that this cation reduced nuclear volume (Anderson and Wilber, 1951), which may have increased their density, so facilitating their separation by differential centrifigation.

Preparations have been assessed by phase contrast and electron microscopy, chemical composition, enzyme activities and biological viability. The great variety in the morphological appearances and enzyme profiles of nuclei prepared in different ways, or from different tissues, makes the assessment of a published procedure rather difficult. Similarly, the interpretation of the chemical composition of nuclei was confused, in the early days, by ignorance of the <u>in vivo</u> situation. Enzymology is often used to assess the purity and viability of other organelles, but the enzyme profile of the nucleus was, and is, controversial. Roodyn, 1963, gave a "depressing catalogue of contamination" (p.28), possibly because it was then assumed that so-called 'mitochondrial enzymes' were confined to the mitochondria. For example, cytochrome oxidase activity in nuclei was shown in 1952 to be unequivocally due to mitochondrial contamination (Hogeboom <u>et al.</u>, 1952). However in 1978 Zbarsky reported that "appreciable activity of cytochrome oxidase.....is usually found in liver nuclear envelopes ..... the activity of the enzyme exceeds that of isolated nuclei five to

eight times, and amounts to 25-50% of mitochondrial activity" (Zbarsky, 1978, p.321). The biological activity of a preparation was assessed by inserting a nucleus into an enucleated cell from the same tissue, but this was not possible, for example, in liver. The incorporation of labelled nucleotides is now used to assess nuclear viability. Modern isolation techniques often employ "Tris-HCl" as a buffer, (usually at a concentration of 50mM) adjusted to a pH around neutrality, containing isotonic sucrose (approximately 0.25M), divalent cations (usually 3-5mM) and some KCl (10-20mM). Differential centrifugation, using a series of various sucrose solutions, is often though not always employed to separate and purify the nuclei. Preparations are often assessed by several techniques, phase contrast and electron micrographs being given alongside chemical and enzyme estimations. No marker enzyme exclusive to the nucleus has yet been found, although the recent papers of Stubbs and Harris (1978) and Baird et al.,(1980) report peroxidase activities which may be developed as a marker because their characteristics differ from cytoplasmic peroxidase. The use of immunological markers for nuclear envelope may soon be developed.

Before continuing to trace the development of the concept of nuclear envelope, its isolation from nuclei will be briefly discussed. This has often been reviewed (e.g. Kasper, 1974; Harris and Agutter, 1976) so again the discussion will be confined to the principles involved. The isolation of the nuclear envelope involves: disruption of the nucleus, removal of the chromatin and associated proteins, and finally, separation from the dissociated components. The most commonly used methods of disruption are sonication, freezing and thawing, and disruption

by low ionic strength. Chromatin and associated proteins have most often been removed by DNAase (Berezney, 1970; Kay et al., 1972), low ionic strength (Agutter, 1972), and high ionic strength to disrupt salt bridges (Zentgraf, 1971 and Monneron, 1974). Polyanions have also been used to remove histones and so dissolve DNA (Anderson and Wilber, 1951; Bornens, 1973; Cook and Aikawa, 1973; Demidenko and Tsvetkova, 1978; Hildebrand and Okinata, 1976). In 1972 Price reported that nuclei incubated with 1mM $NaHCO_3$ lost their DNA, providing nuclear ghosts after zonal and isopycnic centrifugation (Price et al., 1972). Another ingenious way of removing nuclear contents was by incubation with shear-degraded DNA which removed the histones by competition, causing dissolution of the chromatin (Bach, 1967). This has not been repeated.

The purification of nuclear envelopes is usually achieved by sucrose density gradient centrifugation, or repeated resuspension and pelleting. The density of the nuclear envelope preparations is usually between 1.16 and 1.2 g/cm^3, and there has been some speculation as to the possibility of separating various components on such gradients, although this has not been developed (Albert and Davies, 1973).

I.3. Biochemistry and Enzymology of the Nuclear Envelope

In 1969 Kashnig and Kasper produced one of the first extensive investigations of the nuclear envelope, although they actually refer to it as the "nuclear membrane". They reported its composition as 67.4% RNA; with densities of 1.16-1.18 and 1.18-1.20 g/cm^3. (Kashnig and Kasper, 1969). Subsequent nuclear envelope preparations vary somewhat from these values especially in their DNA and RNA contents. These values depend on the extent of nuclease treatment in the preparation, and there are many contradictory reports of the "correct" content of DNA. For example 8% DNA was reported as essential to the structural integrity of the pore (Agutter, 1972) whereas Kashnig shows electron micrographs of pores in a nuclear envelope preparation containing, apparently, no DNA. Likewise the protein content varies from 64% to 77% and the phospholipid content from 13% to 26%. Protein/lipid ratios in all nuclear envelope preparations are consistently much higher than that of e.g. the red cell membrane (protein/lipid = 1.3) (Dodge et al., 1963). This indicates that the 'nuclear membrane' preparation did not have as simple a structure as most authors apparently believed. The phospholipid composition of rat liver nuclear envelope was published by several authors and did not vary a great deal, e.g. phosphatidylcholine 57% - 61%; phosphatidylethanolamine 18%-24%; phosphatidylinositol plus phosphatidylserine 8%-14% and sphingomyelin 3%-6%. Other phospholipids were present in very low concentrations, or not detectable. Other tissues had a rather different composition e.g. onion root tip contained phosphatidylcholine 25%; phosphatidyl-ethanolamine 17%; phosphatidylinositol plus phosphatidylserine 9%;

phosphotidic acid 34%. Cardiolipin was detected in the nuclear envelope of this tissue (2%). As this is not detected in preparations from other sources, Harris (1978, p. 79) speculated that the unusual profile of phospholipids was due to mitochondrial contamination. The nuclear envelope phospholipids from rat liver are, however, very similar to those of E.R., reflecting their continuity and morphological relationship. Both types of membrane have a very low cholesterol content compared to that of plasma membrane and Golgi vesicles. This indicates that the lipids are not highly ordered by the influence of cholesterol although there may be other ways in which the fluidity of the nuclear membrane lipids are regulated. The phospholipid profile of nuclear envelope can vary with the growth temperature, in common with other membranes. For example in Tetrahymena macronuclei, growth at 18°C gave nuclear envelopes with a higher proportion of unsaturated lipids than those grown at 28°C. This would compensate for the reduced fluidity of the membrane at the lower temperature and indicates the importance of fluidity to the nuclear membrane, possibly for the binding and subsequent action of steroid hormone receptors. (Giese et al., 1979).

In recent times emphasis has been placed on investigating the role of nuclear enzymes in the various functions of the nucleus. Both 5'-nucleotidase and DNA polymerase have been found to have a strong affinity for the nuclear matrix, such that they were purified with that structure. (Smith and Berezney, 1980; Bukhalov et al., 1981). This supports the suggestion that the nuclear matrix is actively involved in processing nucleic acids. A nuclear envelope

NTP-ase activity has been shown to be involved in nuclear transport of RNA. It was stimulated by poly-G and by insulin which may suggest that it has a regulatory function in RNA transport (Agutter et al., 1979a, 1979b; Agutter, 1980; Agutter and MacKenzie, 1980; Purello et al., 1982).

Several workers have reported that the nuclear matrix contains glycosylated proteins (Guinivan et al., 1980; Reeves et al., 1981). At least one glycosyl transferase has been reported (Berthillier and Got, 1982), although it is highly probable that others are present. One very interesting result was reported by Sevaljevic et al., (1981) who found that the lectin-binding capacity of the nuclear matrix of sea urchin embryos passed through stage-specific changes with the development of the cell. This suggests that the pattern of glycosylation may be important in the regulation of gene expression during development.

The enzymology of the nuclear envelope has also been extensively investigated by many workers and was well reviewed by Zbarsky in 1978. A wide variety of enzymes have been found, e.g. ATPase (Yasuzumi and Tsubo, 1966; Chardonnet and Dales, 1972), phosphatase (Goldfisher et al., 1964), glucose-6-phosphatase (Kartenbeck et al., 1973; Kashnig and Kasper, 1969; Kanamura et al. 1979; Sikstrom et al., 1976, endonucleases (Hewish and Burgoyne, 1973), protein kinases (Steer et al., 1980; McDonald and Agutter, 1980; Maul, 1980; Lam and Kasper, 1979), and 5'-nucleotidase (Sikstrom et al., 1976). Enzymes usually attributed to the mitochondria are found, e.g. succinate dehydrogenase (Berezney and Crane, 1972), monoamine oxidase (Gorkin, 1971), cytochromes and cytochrome oxidases (Rupec et al., 1971; Sikstrom et al., 1976;

Ueda, 1969). Some of these have been attributed to mitochondrial contamination, as previously mentioned, and the authentic enzyme profile of the nuclear envelope has been the centre of no little controversy. However, the concensus now seems to be that some enzymes, previously though attributed exclusively to the mitochondrion, are present in the nuclear membrane. This implies the existence of an electron transport chain in the nucleus. It lacks the succinate dehydrogenase branch however, (Zbarsky, 1978) so that this enzyme is useful for assessing mitochondrial contamination of nuclei.

Section I.4. Introduction to the work presented

In this introduction I have attempted to trace the development of our understanding of the nuclear envelope over the past 30 years. During this period there has been a continuous refinement of the techniques involved and, consequently, of the results. Initial observations by light microscopy were performed on whole nuclei. This was followed by the examination of the gross morphology of the nuclear envelope by electron microscopy, the resolution of which improved over the years as better techniques of fixation and sectioning were developed. This led to an appreciation of the double membrane structure, pore complexes, and, finally, the lamina.

Thereafter biochemical techniques took over somewhat, nuclear fractionation and analysis supplying information on a molecular level about the relationships of the lamina to the matrix, pore complexes, and membranes. Finally the individual polypeptides have been examined by immunology and protein chemistry to discover the relationships between them.

Although the protein profiles of nuclei and their fractions have frequently been examined, our knowledge of the disposition and topography of the individual proteins within the nuclear envelope is far from complete. This is a difficult structure to isolate, being in physical continuity with other components on both its faces. Contamination has always been a major problem with this system, especially as there has been considerable equivocation over its enzyme profile. This is, I think, the main reason for our ignorance. Part of the work presented here has been to try to locate some of the nuclear envelope proteins by fractionating nuclei

in various ways, and by exposing the nuclear surface to various conditions of iodination. Fractions and iodinated proteins were then compared by polyacrylamide gel electrophoresis.

An additional aim of the project was to compare the carbohydrate complement of nuclear glycoproteins before and after viral transformation. Consequently, the BHK cell line was chosen, as it provided normal cells, together with their transformed isogenic counterpart. There is an extensive body of evidence for the existence of glycoproteins in the nucleus, drawn from many different types of experiment. Biochemical analyses (Kashnig and Kasper, 1969), nuclear agglutination by lectins (Nicolson et al., 1972), studies with radiolabelled metabolites (Wu et al., 1969), and the visualisation of carbohydrates by fluorescent-labelled lectins (Virtanen and Waatiovaara, 1976), have all contributed to the conclusion that the nucleus contains glycoproteins. These are almost entirely confined to the nuclear membranes, with their carbohydrates in the perinuclear space. After the onset of malignancy, there is usually a dramatic change in the social behaviour of the cell (Abercrombie, 1979). This is accompanied by changes both in the distribution and the carbohydrate content of the plasmalemma proteins. (Hynes, 1976; Tumanova, 1978; Vaheri and Mosher, 1978). Consequently the changes which occur in a cell membranes after transformation have been extensively studied, and nuclear membranes have been included in this work (Meezan et al., 1969; Buck et al., 1970; Sheinin and Onodera, 1972; Sakiyama and Burge, 1972). Other studies have been more specifically directed toward the nucleus (Phillips, 1973; Kawasaki and Yamashina, 1972 a,b)

and some changes in the relative amounts of the various carbohydrates have been detected in whole nuclei after transformation (Wu et al., 1969). My own work has been to compare the carbohydrates of the various nuclear envelope fractions isolated from normal and transformed cells. The glycoproteins were identified by their different affinities for various [^{125}I-]-lectins after separation by gel electrophoresis. This was done in order to localise any changes found to a particular part of the envelope.

MATERIALS and METHODS

II.1. Materials

a) Chemicals and Substrates

"Tris" buffer, triethanolamine buffer, heparin, PMSF, deoxycholate, dithiothreitol, Triton-X100, and chloramine-T were all purchased from Sigma Chemical Co., of Poole. Amino acids for mixing specialised media were purchased from Gibco Biocult, Paisley. Glutaraldehyde was obtained from Taab Ltd., Reading. Saccharides for the inhibition of lectin affinities were purchased from Koch Light of Colnbrook. The enzyme substrates glucose-6-phosphate, ρ-nitrophenol phosphate and 5'-AMP were purchased from the Sigma Chemical Co. INT for use in the assay of succinate dehydrogenase was obtained from Aldrich Chemical Co., of Gillingham. All other chemicals and reagents were of the Analar grade.

II.1.b. Enzymes and Lectins

DNAase-I (Type B) and lactoperoxidase were both purchased from the Sigma Chemical Co., ConA, WGA and lentil lectin were all obtained from Vector Laboratories of Burlingame, California. RCA was obtained from the Sigma Chemical Co.

II.1.c. Radioisotopes

^{125}I was obtained from the Regional Radionuclide Dispensary of the Western Infirmary, Glasgow. All other isotopes were purchased from the Radiochemical Centre, Amersham.

II.2 Methods

II.2.a. Cells and harvesting procedures

Nuclei were isolated from baby hamster kidney cells (BHK 21) obtained from the Wellcome Cell Culture Unit, Glasgow, either untransformed (C13/406), or transformed with Polyoma virus (PyY) (Stoker & Macpherson, 1964). The cells were grown to confluence on the inner surface of glass roller bottles (2.5 litre capacity) in 180 ml of Eagles minimum essential medium (Paul, 1979) (Flow Laboratories) supplemented with 10% ($^{v}/v$) newborn calf serum (Gibco, Biocult). Cell lines were screened fortnightly for mycoplasma infection. The bottles were rotated at 0.5 r.p.m. and maintained at 37^{o}C. The atmosphere above the cells contained 4.0% CO_2.

After removal of the medium the cells were washed, in situ, with "Earle's balanced salts solution" (BSS) (Paul, 1970) and harvested using a plastic "policeman" into 20ml of that solution.

II.2.b. Isolation of Nuclei

This procedure was based on that of Blobel and Potter (1966) designed for the isolation of hepatocyte nuclei. Cells were pelletted from BSS and washed in 0.25M sucrose in an aqueous solution of "Tris" (5mM) adjusted to pH 7.4 with HCl (Isolation buffer"). They were then resuspended for 8 minutes at 4^{o}C in Tris-HCl (5mM, pH 7.4) at a density of 3.5 x 10^{8} cells/ml. After the cells had swollen, they were homogenised in a "Teflon"-glass homogeniser (Camlab Glass Ltd.) clearance 0.02mm, 8-10 strokes, driven by an Anderman Multispeed Stirrer, setting 80) surrounded by ice. The concentration of sucrose was rapidly raised to 0.25M by the

For each preparation three burlers (3×10^8 cells each) were used. This typically yielded 80 mg protein and 10 mg of DNA.

Mitochondria were prepared from post-nuclear supernatants by centrifugation (gmax 7,500, 7 min, MSE high speed 18 centrifuge). Endoplasmic reticulum was obtained from post mitochondrial supernatants by centrifugation (260,000 gmax, 2 hr, MSE Prepspin 50, SW40TI rotor).

addition of one volume of 0.5M sucrose/5mM Tris-HCl (pH 7.4) and the suspension was then checked for at least 95% cell lysis by phase contrast microscopy (Leitz Orthoplan). Nuclei were pelletted from this homogenate (4,500 gmax, 5 min) and washed twice in isolation buffer, using a syringe and 18G needle for resuspension. The sucrose concentration of the final suspension was raised to 1.4M by adding two volumes of 2M sucrose in 5Mm Tris, 5mM $CaCl_2$ adjusted to pH 7.4 with HCl. Portions of this suspension (3 ml) were transferred to six polycarbonate centrifuge tubes (capacity 14 ml), underlaid with 1 ml of 2M sucrose in 5mM Tris, 5mM $CaCl_2$ adjusted to pH 7.4 with HCl and overlaid with isolation buffer to fill the tube. Centrifugation (260,000 gmax, 2hr, MSE Prepspin 50, SW40TI rotor) produced a cream pellet of purified nuclei, with contamined nuclei trapped above the 2M sucrose cushion and cytoplasmic debris at the upper (1.1M-0.25M sucrose) interface. Purified nuclei were washed once in isolation buffer, pelletted and stored at $-18^{o}C$.

This fractionation method typically produced a total of approximately 80 mg of protein and 10 mg of DNA. The nuclear fraction contained 30-40mg of protein and the mitochondria approximately 5mg.

The purity of the nuclei was assessed by phase contrast microscopy, DNA/protein ratios, and by the activities of succinate dehydrogenase, 5'-nucleotidase, acid phosphatase, and glucose-6-phosphatase. Nuclear size was determined from phase contrast photographs. Three diameters of 50 nuclei were measured for each determination and their volumes and surface areas calculated from

the average diameter, assuming them to be spherical.

II.2.c. Preparation of Nuclear Envelope

Nuclei were resuspended ($\sim$ 2 x 10^6 nuclei/ml) in 20 mM Tris-HCl, pH 7.4 containing freshly dissolved PMSF in DMSO (200mM) to a final concentration of 0.2mM, and frozen/thawed three times. Sodium phosphate (Bornens, 1973, 1977) and sucrose were added to final concentrations of 0.8mM and 0.25M respectively. Heparin was added, such that the DNA/heparin ratio was 1.0 or slightly less (Hildebrand and Okinata, 1976) and the suspension was vortexed and inclubated for 8 minutes at 4^{o}C. Two volumes of isolation buffer were added, the nuclear envelope fragments were pelletted, (120,000 gmax, 20 minutes) and then washed twice in isolation buffer. The resulting suspension was centrifuged down a discontinuous sucrose gradient (37% - 54% (w/w) in 50mM Tris-HCl, pH 6.9, 16 hours, 120,000 gmax) and the purified envelope fragments harvested from the gradient at densities of 1.166 - 1.197 and 1.197 - 1.236. These were pooled, washed and stored at -18^{o}C. Microsomal fragments were trapped at densities of 1.032 - 1.166 and there was usually a pellet of material containing histones.

Density Gradient Analysis of Nuclear Envelope Preparations

After various nuclear envelope preparations the densities of nuclear envelope fragments and the distribution of nuclear DNA and lipid between these densities were analysed on sucrose gradients as follows. Cells were labelled with $\left[1\text{-}^{3}H\right]$-ethan-1-ol-2-amine or

[methyl-^{3}H]-thymidine (The Radiochemical Centre, Amersham) using 5μCi/bottle 48 hours before harvest. Gradients were fractionated by upwards aspiration with a long needle inserted to the bottom of the gradient, using a Perpex peristaltic pump (type 10200, gear ratio S125 ^{8}/120) and divided into 0.5ml portions. Samples (0.1ml) were counted on a Philips 2 Liquid Scintillation Analyser using a scintillant of Triton : toluene (30:70 vol:vol) containing 1% (w/v) of 2,5-diphenyloxazole.

II.2.d. Preparation of the Pore-Complex-Lamina fraction

Pellets containing 10^8 nuclei in 50ml centrifuge tubes were resuspended in 3ml 0.1mM $MgCl_2$ and DNAase I was added, to a final concentration of 1μg/ml. Sucrose solution (10%, w/v) in 10mM triethanol-amine-HCl; 0.1mM $MgCl_2$, pH 8.5 ("STM" buffer) (20ml) was rapidly added and the mixture incubated for 15 minutes at 22°C. A layer of "STM"-buffer (pH 8.5) containing 30% (w/v) sucrose was placed beneath the mixture which was then centrifuged at 20,000 g.av. for 10 minutes (8 x 50ml angle rotor, MSE High Speed 18 centrifuge). The supernantant was discarded and the pellet was re-incubated (15 min. 22°C) with DNAase I (5μg/ml in "STM" buffer pH 7.5) then underlaid with 30% (w/v) sucrose in "STM" buffer pH 7.5 and centrifuged as before. The pellet was briefly vortexed with 5ml of Triton-X100 (2% w/v in "STM" buffer, pH 7.5, i.e. 100mg of Triton X100) containing 0.2mM PMSF, and incubated (10 min 4°C). Twenty ml of "STM" buffer containing 10% (w/v) sucrose was added and the mixture then centrifuged as before but without the underlying layer. The supernatant from this extraction was retained for the isolation of membrane proteins which are removed with the lipids. The pellet was finally incubated with NaCl (2.0M) in "STM" buffer

pH 7.5 (4ml) for 10 minutes (4°C). After the addition of 10ml "STM" buffer and the cushion of 30% sucrose in "STM" buffer, the mixture was centrifuged as before. The pellet containing pore complex lamina was washed twice with 10% (w/v) sucrose in "STM" buffer and stored at -18°C. This method is adapted from that of Dwyer and Blobel (1976) with minor adjustments to the volumes used.

II.2.e. Isolation of Nuclear Membrane Proteins

These were isolated from whole or DNAase I treated nuclei by extraction (10min. 4°C) with Triton-X100 (2% w/w) in isotonic sucrose containing 0.2mM PMSF. The solubilised membrane proteins were either precipitated with ethanol (3 volumes, -18°C, 16hr) for polyacrylamide gel electrophoresis, or dialysed (2 days, 2 changes per day) against 20mM Tris-HCl pH 7.4; 0.1% SDS containing SM2 Biobeads (Biorad Laboratories Ltd.,) to remove the Triton-X100 (Yu and Branton, 1976).

II.2.f. Isolation of Endoplasmic Reticulum

Endoplasmic reticulum (E.R) was isolated with a solution containing 50mM Tris-HCl pH 7.6; 25mM KCl, and 10mM $MgCl_2$ ("TKM" buffer). Washed BHK-21 cells were suspended ($\sim$ 3 x 10^8 cells/ml) in 0.35M sucrose/"TKM" buffer and homogenised in a "Teflon"-glass homogeniser (Camlab Glass Ltd., 0.15mm clearance) driven by an Anderman Multispeed Stirrer, setting 90 for one minute. Nuclei and mitrochondria were pelletted (8 x 50 angle rotor, MSE Prepspin Ultracentrifuge) at 2.5 g.max. for 10 minutes, and the supernatant re-centrifuged using the same conditions. The supernatant was then transferred to capped polycarbonate centrifuge tubes and underlaid with 3ml of 1.3M sucrose/"TKM". After

centrifugation (160,000 gmax, 2½ hours) smooth E.R.was collected from the interface above the sucrose cushion and rough E.R.formed brownish pellets. These fractions were washed once in 0.35M sucrose/TKM and stored at -18°C.

II.2.g. Polyacrylamide Gel Electrophoresis

a) Column gels

Fractions were initially analysed on column gels (10 x 0.35cm) containing 5.6% acrylamide; 10% SDS; and a 0.025% TEMED (Fairbanks et al.,1971). Samples containing 25-200µg protein were boiled for 2 minutes in a solution containing 1.0% SDS; 5% sucrose; 10mM Tris-HCl, pH 8.0; 1.0mM EDTA; 40mM DTT and bromophenol blue (trace). Gels were electrophoresed at a constant current of 8mA per gel (3-4 hr) using a reservoir buffer containing 0.04M Tris adjusted to pH 7.4 with acetic acid; 0.02M sodium acetate; 2mM EDTA and 1.0% SDS. Gels were then fixed and stained in 0.05% Coomassie Blue (Gurr) in 25% isopropanol and 10% acetic acid and destained with 10% acetic acid. Densitometric scans of the gels were performed at 550nm on a Gilford 240 spectrophotometer carrying a 2410 linear transport.

(b) Slab gels

The method was based on that of Laemmli (1970). Fractions were analysed on 8% polyacrylamide slab gels (20 x 20 x 0.15cm) containing 0.1% SDS and 0.032% TEMED. Stacking gels contained 3.96% acrylamide and 0.1% SDS. Samples (5-30µg) were boiled for 2 minutes in a solution of 15% sucrose; 5% SDS; 5mM DTT in 20mM Tris-HCl pH 8.8 with 2mM EDTA and a trace of bromophenol blue. Electrophoresis was performed with a Shandon SAE 2761 power pack (15mA constant current, 16hr) using a reservoir buffer which

contained 27mM Tris-HCl, pH 8.8; 190mM glycine and 0.1% SDS.

After electrophoresis gels were either fixed and stained in the same way as column gels or, if the gel was to be overlaid with lectin, the following regime of Tanner and Anstie (1976) was used. Gels were first incubated in 100ml of 50% (v/v) methanol for 30 min. after which glutaraldehyde (Taab) was added to a final concentration of 0.05% (v/v). After 1.5 hr, gels were transferred to 150ml of 0.5M phosphate buffer containing 0.1M NaCl (PBS) and 0.02mg/ml $NaBH_4$ for 2 hr. and this treatment was repeated overnight. Gels were then washed in PBS without $NaBH_4$ (3 x 2 hrs) before the application of iodinated lectin. This procedure was compared with various others as discussed in Section III.

Proteins used as molecular weight markers were as follows: crosslinked oligomers of pyruvate dehydrogenase, Mr 100,000 and 83,000; pyruvate kinase, Mr 56,000 and lactate dehydrogenase, Mr 36,000. These were a generous gift of Dr. J. Coggins.

II.2.h. Iodination of Lectins

These were iodinated either by the chloramine-T reaction (Hunter and Greenwood, 1962), or by the use of "Enzymobeads" (Biorad Laboratories Ltd) which consist of immobilised lactoperoxidase and glucose oxidase. When the chloramine-T reaction was used, 1-2mg of lectin, dissolved in 200µl PBS pH 7.3 containing the specific monosaccharide (0.1M), was mixed with 300-500µCi Na ^{125}I. Chloramine-T (1mg in 100µl) was added and the reaction was stopped after 1 minute (room temperature) by the addition of 1mg of freshly dissolved sodium metabisulphite.

When Enzymobeads wer used, 1mg of each lectin in 200µl PBS containing the inhibitor was mixed with 100µl of rehydrated

Enzymobeads, 100µl of 1% α-D-glucose and up to 500µCi of Na ^{125}I. The suspension was then incubated (1 hour room temperature, shaken gently) and the Enzymobeads removed by centrifugation (40,000 gmax, 10 min).

Iodinated lectins were dialysed overnight to remove the iodide and monosaccharides, counted, and their activity estimated by their ability to agglutinate trypsinised erythrocytes. They were then stored at -18°C in aliquots containing approximately 10^8 c.p.m. Concanavalin-A was particularly sensitive to denaturation by chloramine-T and after dialysis inactive ConA was removed by affinity chromatography on a column (1 x 10cm) of "Sephadex" G50 (Pharmacia). The lectin was incubated on the column for one hour to allow specific binding and inactive lectin was then removed by extensive washing with PBS. Specifically bound lectin was then eluted with 0.2M α-methyl-D-glucoside in PBS and stored as above. Iodination in these conditions typically produced lectins with a specific activity of 1-3 x 10^5 cpm/µg.

II.2.i. Identification of glycoproteins by lectin overlay.

After fixation by the method of Tanner and Anstie (1976) and washing in PBS, polyacrylamide slab gels were placed in 50ml PBS containing 0.02% (w/v) sodium azide and bovine haemoglobin (~ 1mg/ml). Lectin (~ 10^7 c.p.m) was added and the gels incubated for three days (room temperature, occasional shaking). Gels were then washed for three days in PBS containing 0.02% (w/v) azide, and either dried onto filter paper and autoradiographed by exposure to "Kodirex" or X-Omat film (Kodak) at room temerpature, or prepared for fluorography according to the method of Bonner and Laskey (1974). The gels were first dehydrated by repeated washing

in DMSO, then impregnated by soaking in 20% (w/v) PPO in DMSO (40 min). The PPO was then precipitated in the gel with distilled water and the gels dried and exposed to Kodirex or X-Omat film (Kodak) at -70°C.

II.2.j. Estimation of agglutination activity of iodinated lectins

Erythrocytes from whole fresh blood (human, A.Rh+) were washed three times with PBS (4°C). A portion was diluted in PBS to 4% (v/v) red cells and incubated (37°C, 1hr) with 0.25% (w/v) trypsin. The cells were then pelleted, washed three times in PBS, and resuspended to 1.5% (v/v) red cells for use in agglutination assays.

Assays were performed in round bottomed "Microtitre" trays (Gibco) in a final volume of 100µl. Lectins were added at concentrations of 1 - 12.5 µg/ml in the first well, with serial dilution thereafter. After addition of the red cells the trays were shaken gently, in order to mix the contents of the wells, then left at room temperature to settle.

II.2.k. Iodination of nuclei

Nuclei were iodinated with either soluble lactoperoxidase or with the "Enzymobeads" (Biorad Laboratories Ltd) described previously. When soluble lactoperoxide was used, nuclei ($\sim 10^7$) were suspended in a total volume of 250µl of 20mM Tris-HCl pH 7.8 containing 50µg of lactoperoxidase (Sigma) and 500µCi Na ^{125}I at room temperature. Aliquots of hydrogen peroxide (20µl of a freshly prepared 2mM solution) were added every two minutes for 20 minutes, so that the final total volume was 450µl. Alternatively, 50µg of glucose oxidase (Sigma) was added to the above mixture with

D-glucose to a final concentration of 1% (w/v) to provide a constant supply of peroxide. This mixture was incubated at room temerpature for 1 hour with gentle agitation.

Nuclei were then pelletted (4,500g, 10 min) and washed three times in 20mM Tris-HCl pH 7.8 containing 20mM KI, and twice in .25M sucrose/5mM Tris-HCl pH 7.4.

Alternatively nuclei ($\sim$1 x 10^7) were suspended in a total of 300µl of 20mM Tris-HCl pH 7.8 which contained 100µl of rehydrated enzymobeads, D-glucose (1%, w/v) and 500µCi of the Na ^{125}I. The suspension was incubated (1 hr, room temperature, gently shaken) and nuclei and beads were then pelletted and washed together exactly as were nuclei iodinated with lactoperoxidase.

II.2.l. Chemical Investigations

DNA was estimated by the diphenylamine assay of Burton (1956). Protein was estimated by the method of Lowry et al.(1951) using the modification of Campbell and Sargent (1967) for membrane proteins. This involves performing the assay in 0.1M NaOH containing DOC (0.5% w/v). Phospholipids were extracted by the method of Folch (1957) and after hydrolysis in 72% (v/v) perchloric acid (140°C 2 hr), phosphorus was estimated with 1-amino-2-hydroxy-naphthalene-6-sulphonic acid according to Fiske and Subba-Row (1925) and converted to the quantity of phospholipid by multiplication by 25 (Wittcoff 1951). RNA was estimated by the orcinol reaction of Mejbaum (1939).

II.2.m. Estimation of Enzyme Activities

Glucose-6-phosphatase: nuclei ($\sim$1 x 10^7) were incubated with glucose-6-Phosphate (30mM) in maleic acid (0.1M) pH 6.5 for 20 minutes at 37°C.

Acid phosphatase: nuclei ($\sim 1 \times 10^7$) were incubated (20 minutes, 37°C) with p-nitrophenol-phosphate (2.6mM) in succinic acid (0.1M) pH 5.0;

5'-nucleotidase: nuclei ($\sim 1 \times 10^7$) were incubated (20 minutes, 37°C) with 5'-AMP (Sigma) (8.3mM) in Tris-HCl (0.3M) pH 7.8 containing $MgCl_2$ (12.5mM).

Enzyme reactions were terminated by the addition of ice-cold TCA to a final concentration of 5% (v/v). Precipitated protein was pelletted and the enzyme activities estimated from the released inorganic phosphate in the supernatants. The ammonium molybdate, 1-amino-2-hydroxynaphthalene-6-sulphonic acid reaction (Fiske and Subba-Row, 1925) was used for this determination.

Controls to estimate the quantity of inorganic phosphate extracted from nuclei under these conditions of incubation were also performed in which the specific substrates were omitted. Glassware for these assays was washed six times in tap-water, twice in distilled water and dried before use.

Succinate dehydrogenase activity was determined from the extent of reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium-chloride (INT) (Aldrich Chemical Co.) (Pennington, 1961) in 0.12M succinic acid pH 7.4 after incubation (30 minutes, 37°C) with nuclei ($\sim 1 \times 10^7$). Controls were performed in which 0.12M malonic acid pH 7.4 replaced the succinate. The reaction was stopped by the addition of TCA as above, followed by 4ml of ethyl acetate. The mixture was carefully vortexed to extract the reduced INT from the precipitated protein and the optical density of the organic layer at 590nm then measured to determine the enzyme activity.

II.2.n. Photography

Phase contrast microscopy was performed on a Leitz Orthoplan

microscope with an Orthomat camera attachment containing Ilford Pan F film.

For electron microscopy, samples were fixed with 2% glutaraldehyde in sodium cacodylate (0.1M) and post-fixed in 1% osmium tetroxide in sodium cacodylate (0.1M). After dehydration they were embedded in "Emix" (Agar Aids Ltd), sliced on an LKB Ultrotome 3, mounted on coated copper grids and stained with uranyl acetate and lead acetate. They were then examined and photographed using an AEI EM 6B electron microscope.

RESULTS

III.1 Development of Methods

The method was based on that of Blobel and Potter (1966) designed for hepatocyte nuclei. This required some modification when it was applied to cells cultured in vitro. Initially experiments were conducted to find the optimum clearance of homogeniser and extent of homogenisation. The optimum pH and ionic content of the suspension media were also investigated. Nuclei were examined by phase contrast microscopy before and after purification through the dense sucrose cushion and their yield, usually approximately 40%, was monitored by the DNA distribution. The appearance of the pellet of purified nuclei, with impure material above the dense sucrose, is shown in Fig. 2. Homogenisation in low ionic strength media with no divalent cations present (5mMTris-HCl, pH 7.4) was found to be particuarly suitable for these cells, which lyse almost immediately and require homogenisation only to remove cytoplasmic material from the nuclear surface. Divalent cations were omitted from the procedure until the final purification when the addition of Ca^{2+} was found to substantially improve the yield of nuclei, without jeopardising their purity as monitored by the enzyme profile (Tables 1 & 2).

The conditions of the homogenisation stage appeared to be critical to the subsequent quality of the nuclei. Accordingly, experiments were designed to discover whether reduction of either the ionic strength or the Ca^{2+} concentration had caused the improvement in the nuclei. The results of this investigation are shown in Table 3. The enzyme activities of the fractions are not greatly changed by replacing Ca^{2+} with Mg^{2+}, nor by removing divalent cations completely. The significant difference occurs when homogenisation takes place in a solution of low ionic strength.

Fig. 2.

Purification of Nuclei by Centrifugation through a concentrated sucrose solution

Nuclei prepared as described, and purified through a small volume (1ml) of 2M sucrose are seen in the pellet. Less pure nuclei and cytoplasmic debris are trapped at the interface above the 2M sucrose as indicated.

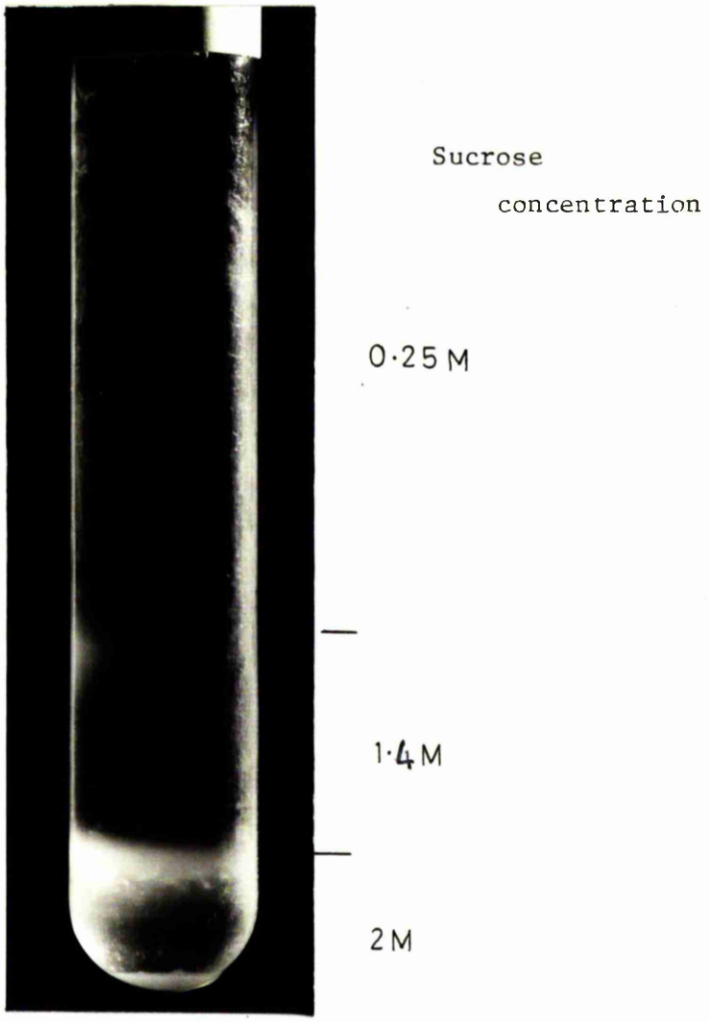

Sucrose
concentration
0·25 M
1·4 M
2 M

Table 1

Enzyme Activities in Nuclei

Cells were harvested as described (II.2a) and pooled in BSS. Nuclei were prepared as described in section II.2.b. The enzyme activities were determined at the various stages as outlined in section II.2.m and corrected for DNA recovery at each stage. In part b) figures in parentheses indicate percentage of homogenate activity.

a) Specific Activities of Various Enzymes

Preparation and Fraction	Glucose-6-phosphatase	Acid phosphatase	5'-nucleotidase	Succinate dehydrogenase
	(μmoles product/min.μg protein)			(△OD/min. μg protein)
Homogenate	3.81×10^{-5}	5.76×10^{-5}	1.70×10^{-5}	8.33×10^{-5}
Mitochondria	3.94×10^{-5}	7.45×10^{-5}	4.23×10^{-5}	1.95×10^{-4}
Nuclei	1.94×10^{-5}	3.29×10^{-5}	5.90×10^{-6}	8.71×10^{-6}

b) Total activities of various Enzymes

Preparation and Fraction	Glucose-6-phosphatase	Acid phosphatase	5'-nucleotidase	Succinate dehydrogenase
	(μmoles product/min.μg protein)			(△OD/min. μg total protein)
Homogenate	4.33	6.41	1.89	9.28
Mitochondria	0.68	0.13	0.73	0.34
Nuclei	0.74 (17%)	1.25 (19%)	0.23 (12%)	0.06 (0.7%)

Table 2

Enzyme Activities of Nuclei purified with and without Calcium

Crude nuclei prepared as described and suspended in 0.25M sucrose/5mM Tris-HCl, pH 7.4 were mixed with two volumes of 2M sucrose/5mM Tris-HCl, pH 7.4 (either with or without 5mM $CaCl_2$) and purified by centrifugation through a layer of the appropriate 2M sucrose solution. Enzyme activities were determined as described in Section II.2.m.

Specific Activities of Various Enzymes

Purification	Glucose-6 phosphatase	Acid phosphatase	5' nucleo-tidase	Succinate dehydrogenase
	(μmoles product/min. μg protein)			(ΔO.D/min. μg protein)
5mM Tris-HCl	2.15×10^{-5}	3.8×10^{-5}	6.61×10^{-6}	7.69×10^{-6}
5mM Tris-HCl + 5mM $CaCl_2$	1.95×10^{-5}	3.29×10^{-5}	5.99×10^{-6}	8.71×10^{-6}

Table 3

Succinate dehydrogenase activities of cell fractions after homogenisation in various conditions

Nuclei were prepared as described in Section II.2.b. but the homogenisation was carried out in various solutions as follows:

a) homogenisation in a solution of Tris-HCl, pH 6.9 (20mM), KCl (25mM) and $CaCl_2$ (5mM) (TKC);

b) homogenisation in a solution of Tris-HCl, pH 6.9 (20mM), KCl (25mM) and $MgCl_2$ (5mM) TKM);

c) homogenisation in a solution of Tris-HCl, pH 6.9 (20mM) and KCl (40 mM) i.e. having the same ionic strength as above but without divalent cations;

d homogenisation in a solution of Tris-HCl, pH 7.4 (5mM).

The procedure was not modified in any way apart from the use of the solutions described above. These solutions were present throughout their respective procedures.

Fraction and homogenisation	Specific Activity of Succinate Dehydrogenase (ΔOD/min/μg protein)
TKC pH 6.9	
Homogenate	7.02×10^{-5}
Mitochondria	6.9×10^{-5}
Nuclei	9.6×10^{-5}
TKM 6.9	
Homogenate	3.3×10^{-5}
Mitochondria	2.2×10^{-4}
Nuclei	1.07×10^{-4}
Tris KCl, pH 6.9	
Homogenate	4.02×10^{-5}
Mitochondria	2.4×10^{-5}
Nuclei	1.66×10^{-5}
Tris-HCl, pH 7.4	
Homogenate	6.33×10^{-5}
Mitochondria	2.20×10^{-4}
Nuclei	5.12×10^{-6}

b. Preparation of Nuclear Envelope

Nuclear envelope was prepared under various conditions and examined by density gradient analysis and polyacrylamide gel electrophoresis, as described in the Methods. The result of the density gradient analyses are shown in Figs. 3 to 5 which indicate that: a) the action of DNAase during the heparin treatment causes DNA fragments to band with nuclear envelope; b) divalent cations increase the density of isolated nuclear envelope and c) divalent cations do not have a similar effect on isolated endoplasmic reticulum. Densitometry scans of nuclear envelope electrophoresed on polyacrylamide gels are shown in Fig. 6a to c. These clearly show that large amounts of histone are found in the nuclear envelope after its isolation in the presence of divalent cations. Nuclear envelope isolated by the standard preparation contains much less histone. The standard nuclear envelope isolation procedure, described in Section II.2.c. was devised as a result of this series of experiments.

The use of PMSF to reduce the activity of serine proteases was investigated during the preparation of both nuclei and nuclear envelope. However, as it appeared to cause no change in the protein profiles of the resulting preparations its use was discontinued.

Fig.3.

Effect of DNA-ase upon DNA distribution in the purification of Nuclear Envelope

After labelling with ^{3}H-thymidine, nuclear envelopes were isolated by the heparin method described previously (DNA/heparin = 1.0), either with DNAases I & II (DNA/DNAase = 10.0) (-o-), or without DNAases, (-•-). Envelopes were washed and purified on sucrose density gradients, then fractionated and counted as described in section II.2.c. The percentages of counts applied to gradients which were recovered in the fractions totalled 104% without DNAases and 110% with DNAases.

Fig. 3.

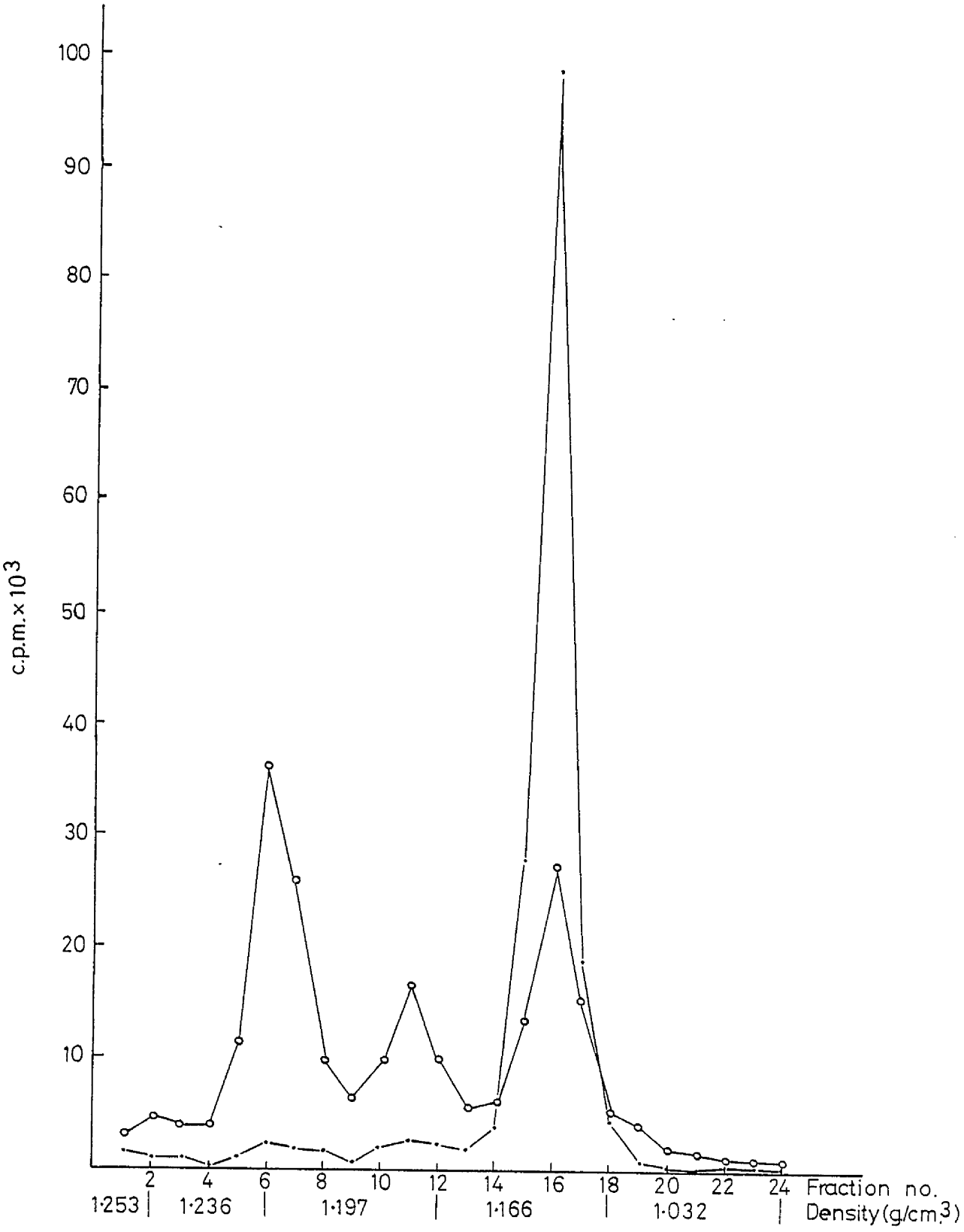

100
90
80
70
60
50
40
30
20
10
c.p.m. x 10³
2
4
6
8
10
12
14
16
18
20
22
24
Fraction no.
1·253
1·236
1·197
1·166
1·032
Density (g/cm³)

Fig.4.

Distribution of Membrane Components in Nuclear Envelope Purification after preparation with or without Divalent Cations

After labelling with [1-^{3}H]ethan-1-ol-2-amine, nuclear envelopes were isolated by the heparin method as described (DNA/heparin = 1.0), either with divalent cations (b) or without (a). No DNAase was used in any of these preparations. Percentages of applied counts recovered from the gradients were: 5mM Ca^{2+}, 60.4%; 5mM Mg^{2+}, 54.9%; standard preparation, 87.9%.

Fig. 4.a.

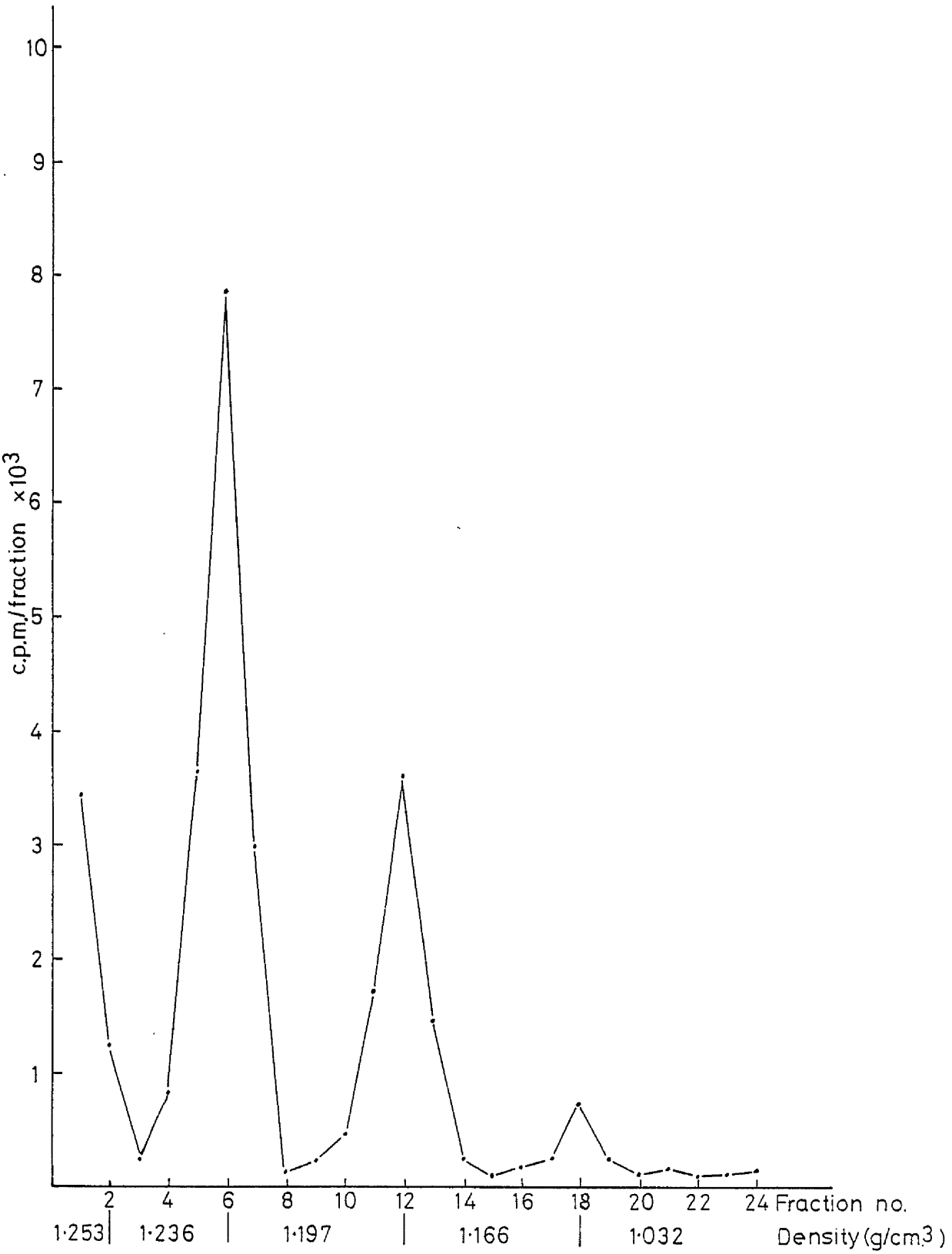

c.p.m./fraction ×10³
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
24
Fraction no.
1·253
1·236
1·197
1·166
1·032
Density (g/cm³)

Fig. 4.b. Ca^{2+} —o—
Mg^{2+} —•—

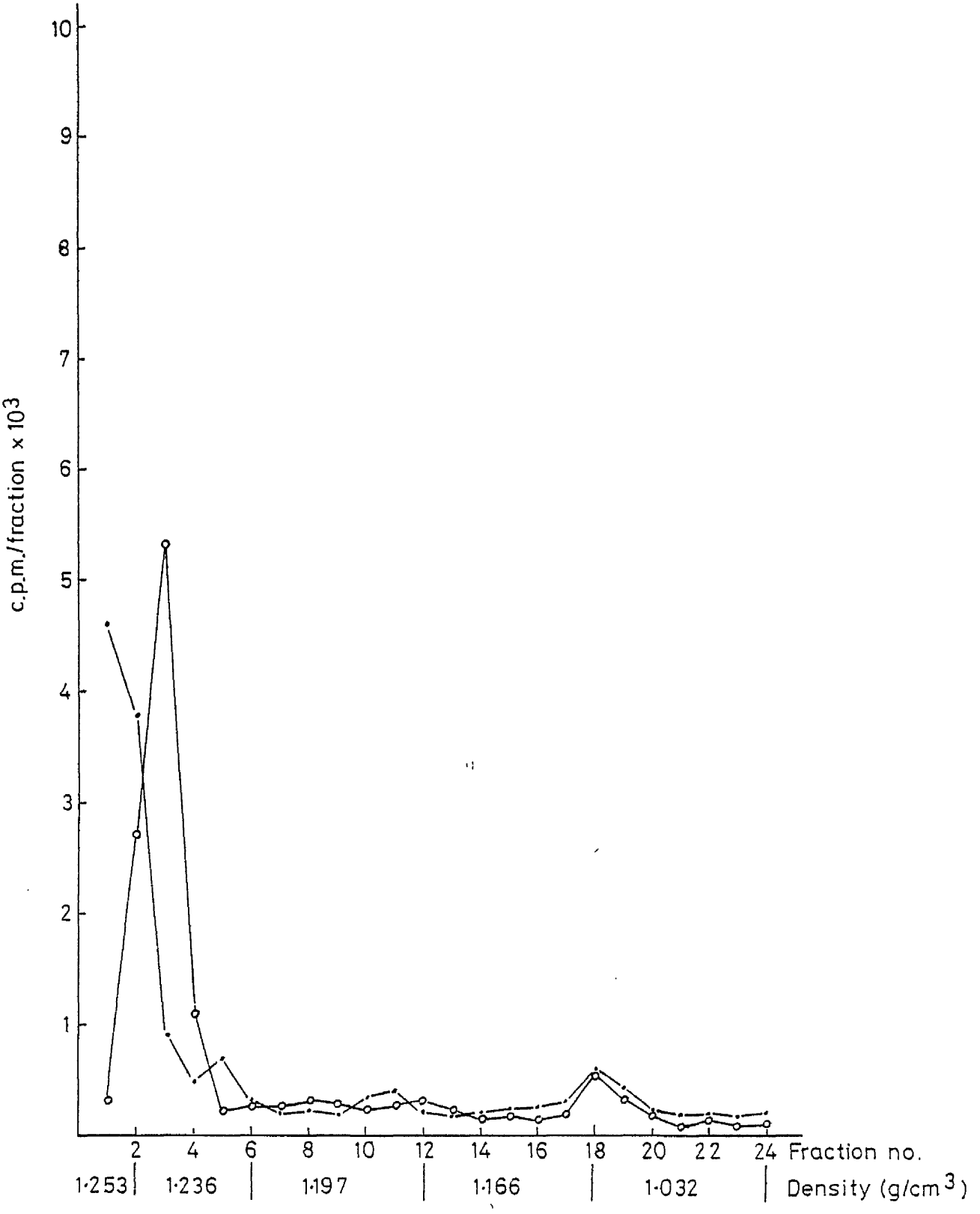


Fig.5.

Distribution of ^{3}H-Ethanolamine in Endoplasmic Reticulum fractionated on sucrose gradients with or without calcium.

Endoplasmic reticulum was isolated from the post mitochondrial supernatant of cells labelled with [1-^{3}H] ethan-1-ol-2-amine by centrifugation (180,000 g.av. 2 hr.). Membrane pellets were then either resuspended in 0.25M sucrose/50mM Tris-HCl pH 6.9, and fractionated on sucrose gradients without Ca^{2+}, - • -; or resuspended in 0.25M sucrose/50mM Tris-HCl, 5mM $CaCl_2$, pH 6.9, and fractionated with 5mM Ca^{2+}, - o -. Percentage recoveries of applied counts were: 5mM Ca^{2+}, 30.5%; no Ca^{2+}, 24.9%.

Fig. 5.

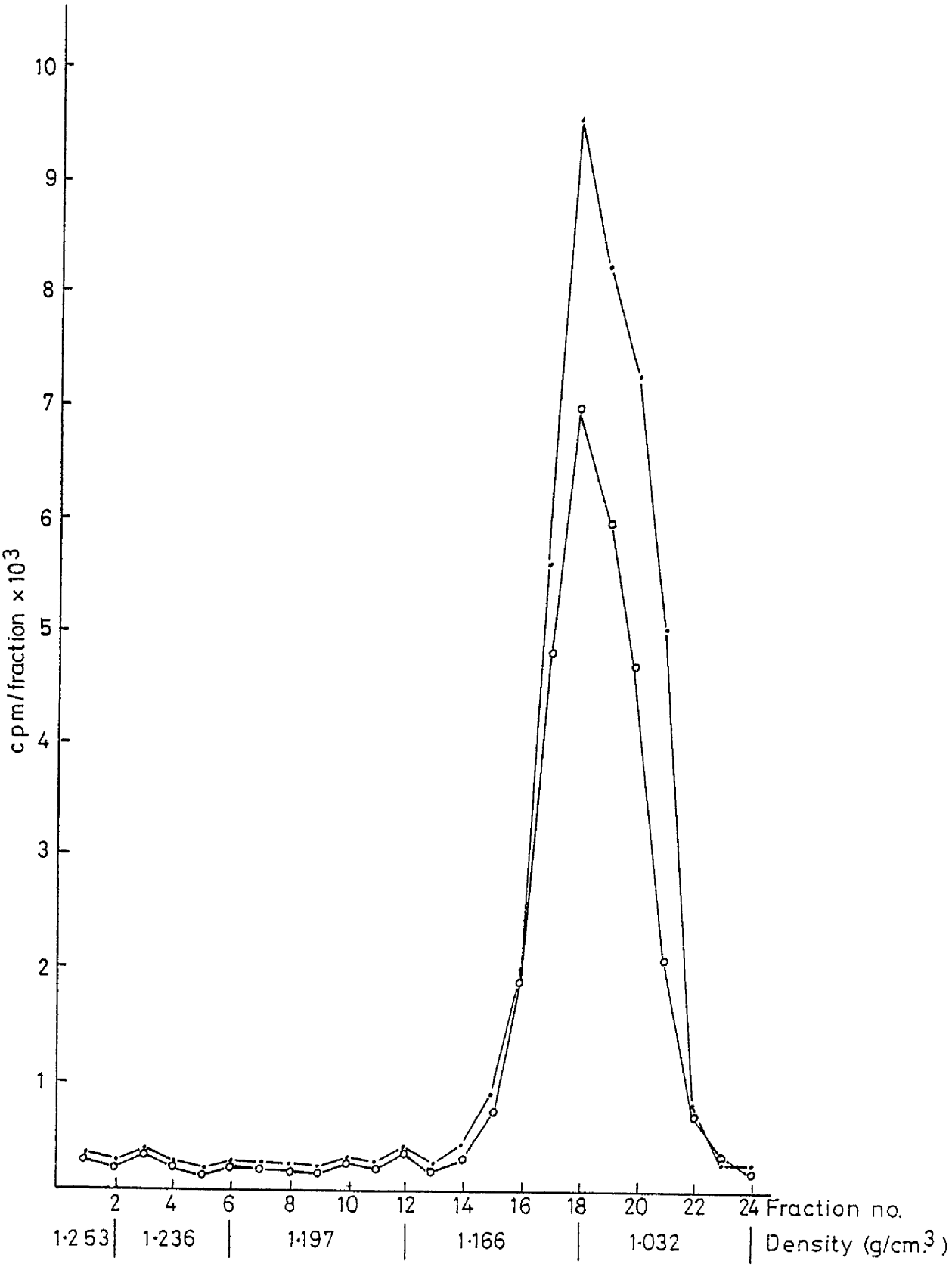

10
9
8
7
6
5
4
3
2
1
cpm/fraction x 10³
2
4
6
8
10
12
14
16
18
20
22
24
Fraction no.
1·253
1·236
1·197
1·166
1·032
Density (g/cm.³)

Fig.6.

Protein profiles of Nuclear envelope prepared by the standard procedure, or with Mg^{2+} or Ca^{2+}.

Nuclear envelopes were prepared either as described in Section II.2.c.(a) or with the addition of Mg^{2+} (b) or Ca^{2+} (c), (5mM) throughout that procedure. Polyacrylamide gel electrophoresis was performed on 5.8% acrylamide column gels containing 1% SDS. Samples were prepared for electrophoresis by the method of Fairbanks et al. (1971).

Fig. 6.a.

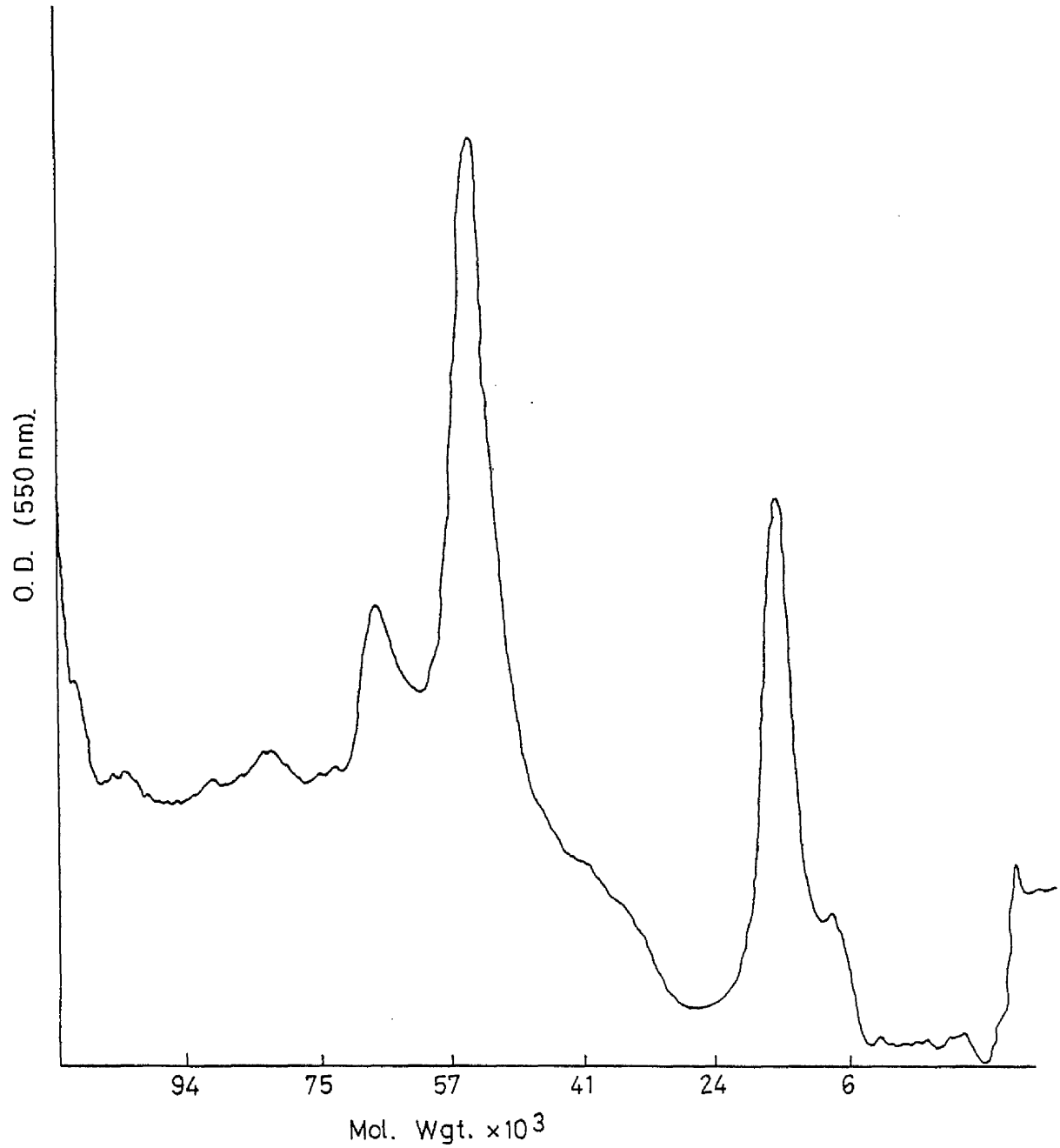


Fig. 6.b.

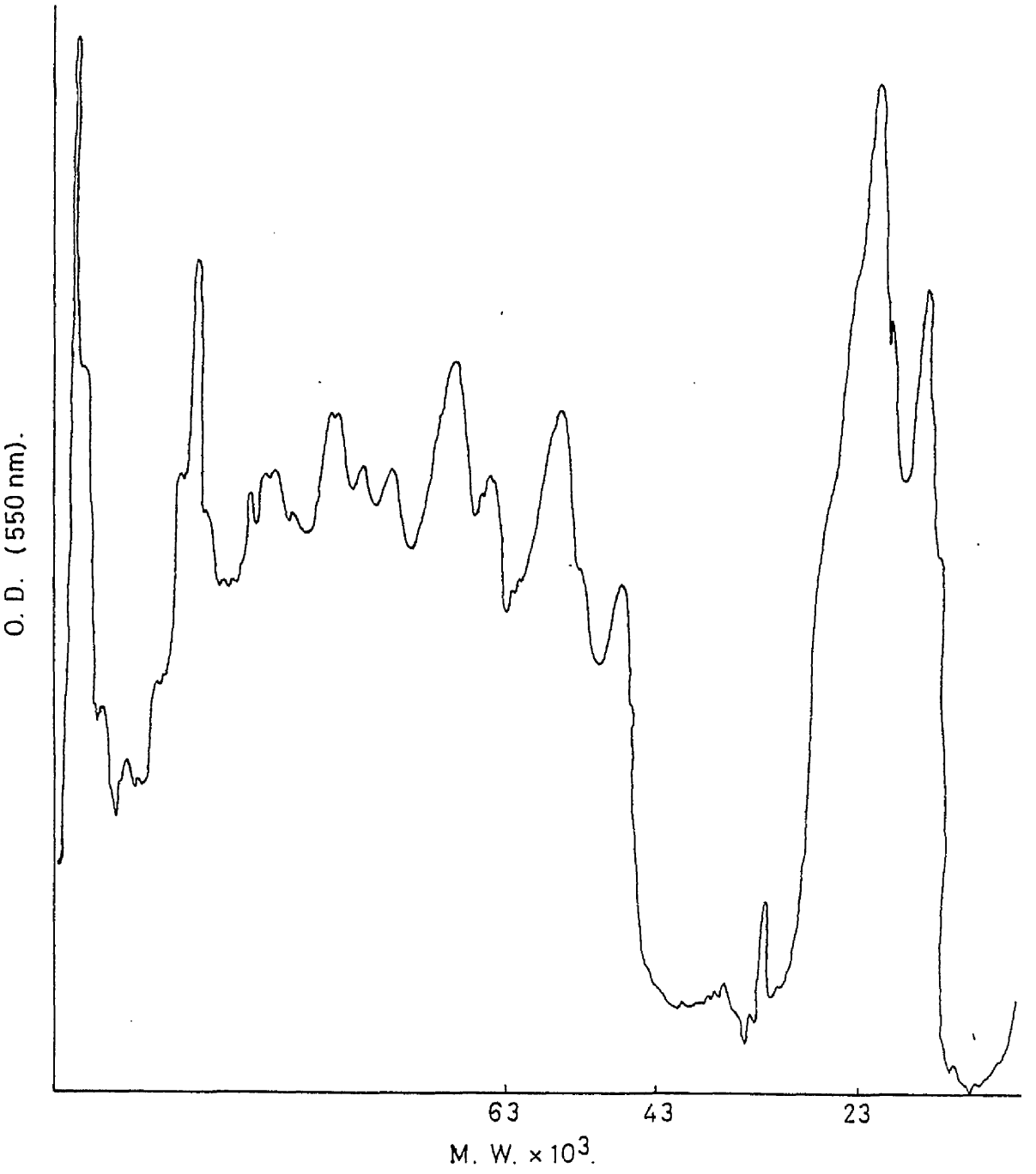
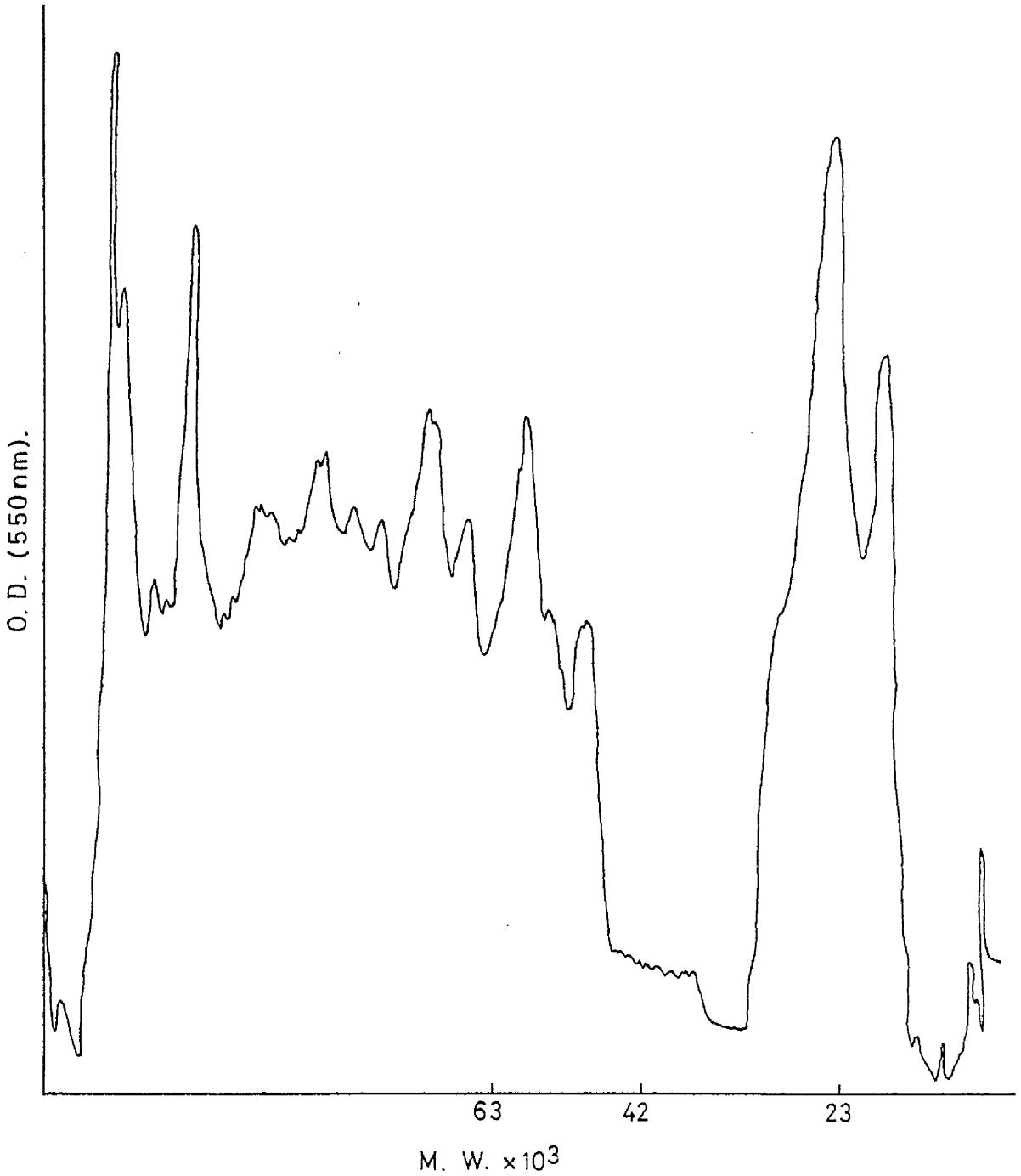


Fig. 6.c.



III.2. Characteristics of Nuclei

a) Morphology

The morphology of nuclei prepared as described in Section II.2.b and examined by phase contrast and electron microscopy is shown in Fig. 7. Nuclei had an average diameter of 8.5 ± 0.4 µm with a surface area of 228 μm^2. A small degree of expansion was noted in nuclei after resuspension in 0.25M sucrose/5mM Tris-HCl. Electron microscopy shows that nuclei have a double membrane with ribosomes on the cytoplasmic surface. The outer membrane extends away from the nucleus to form blebs of various sizes, and there is some attached extranuclear material (Fig. 7b).

The morphology of whole cells as shown by electron microscopy is presented in Fig. 8. This shows that -

a) the blebbing phenomenon is not an artifact of the nuclear isolation procedure;

b) the nucleus of this type of cell is relatively large, occupying approximately 13% of the cell volume;

c) the inner nuclear membrane appears straighter and thicker than the outer membrane and has an electron dense layer immediately inside it, which may be the nuclear lamina.

b) Chemical Characteristics

The DNA/protein ratios of nuclei were 0.25 ± 0.14. This was discussed in Section III.1.

c) Enzymology

The enzyme profiles of the various cell fractions were presented in Section III.1.

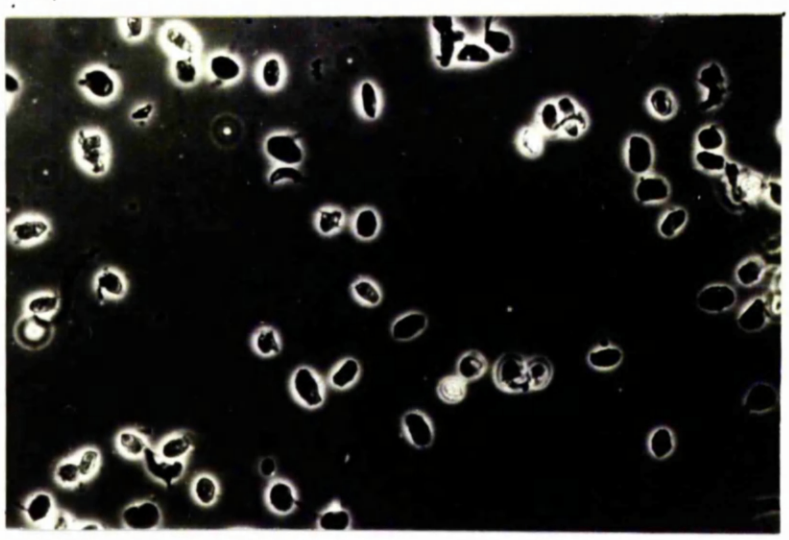
Fig. 7.

Morphology of purified nuclei

a) Nuclei, prepared as described in Section II.2.b. were examined by phase contrast microscopy and photographed as described in Section II.2.n.

b) Nuclei, prepared as described and examined by electron microscopy and photographed as described in Section II.2.n.

a)



×300

b)

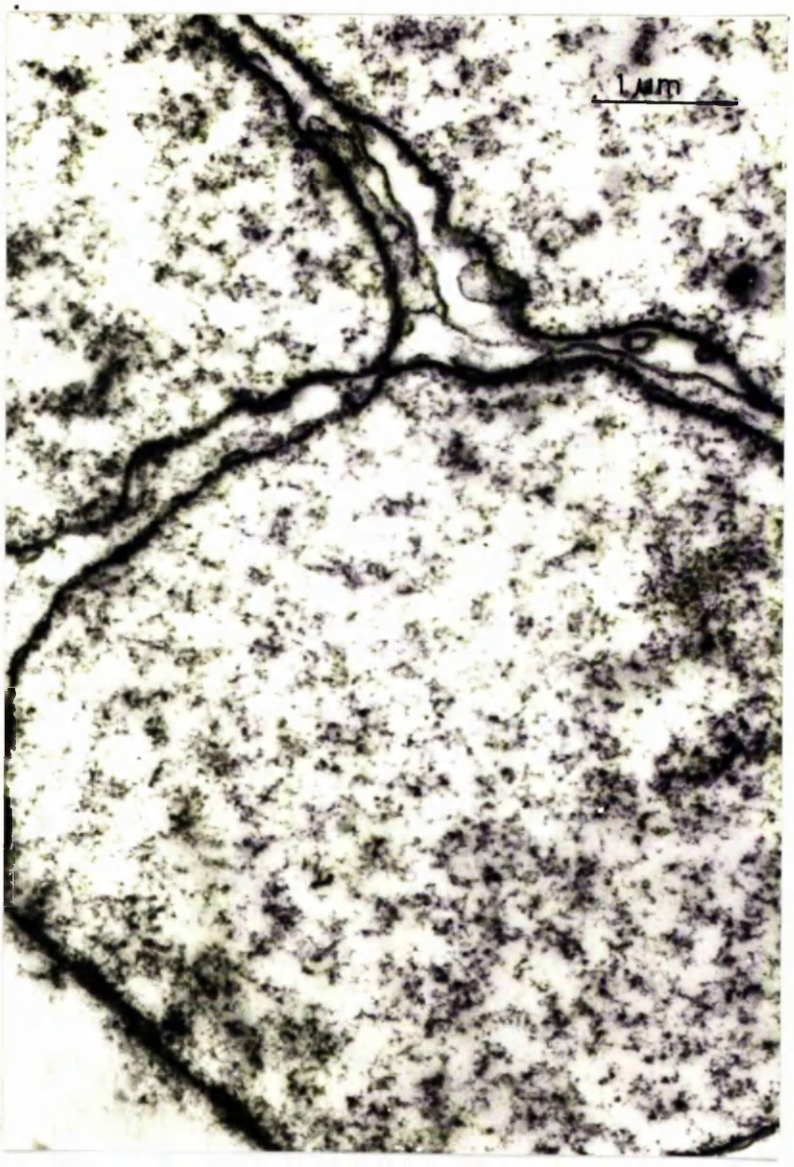
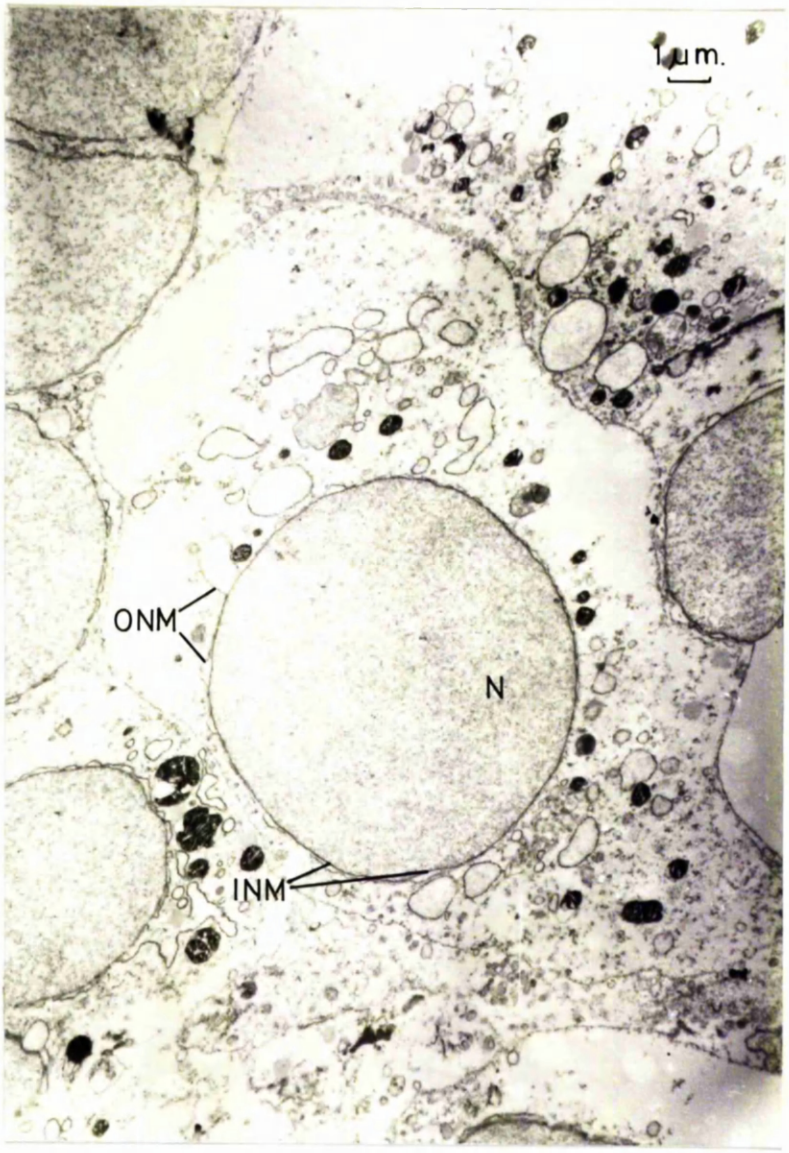


Fig. 8.

Morphology of BHK Cells

BHK cells (C13) harvested into BSS as described in Section II.2.a. were examined by electron microscopy and photographed as described in Section II.2.n.

N = nucleus, ONM = outer nuclear membrane, INM = inner nuclear membrane.


1 µm.
ONM
N
INM

III.3. Characterisation of Nuclear Envelope Fractions

a) Nuclear Envelope

Morphology

The morphology of nuclear envelope isolated from nuclei is shown in Fig. 9. There are sheets of membrane, small vesicles, pore complexes and amorphous material which possibly originated in the lamina. The pore complexes have an outer diameter of ~ 80 nm which is close to the values reported by Riley and Keller (1978) and by Gall (1967) for this structure.

Chemical Composition

The chemical composition of the nuclear envelopes were: DNA, 0.75%; RNA, 3.9%; protein, 80.56% and phospholipid 5.3% by weight.

The proteins of all the nuclear fractions were analysed by gel electrophoresis as described in Section II.2.g. and are shown together in Fig. 10. Each slot contained (as nearly as possible) 20 µg of protein. The fractions, as seen by this method, are usually very similar after transformation. The profiles of the various fractions, with densitometry scans and molecular weight analyses, are discussed individually in the appropriate sections.

The protein profiles of the whole nuclear envelope are shown in Fig. 11. This shows that there are many proteins present in the nuclear envelope with a wide range of molecular weights. The profile is dominated by two groups of proteins having apparent Mr of 72,000-60,000 and 53,000-47,000. There are definite differences in the profiles in the regions of apparent Mr 57,000 and 53,000 after transformation. The other parts of the profiles

Fig. 9.

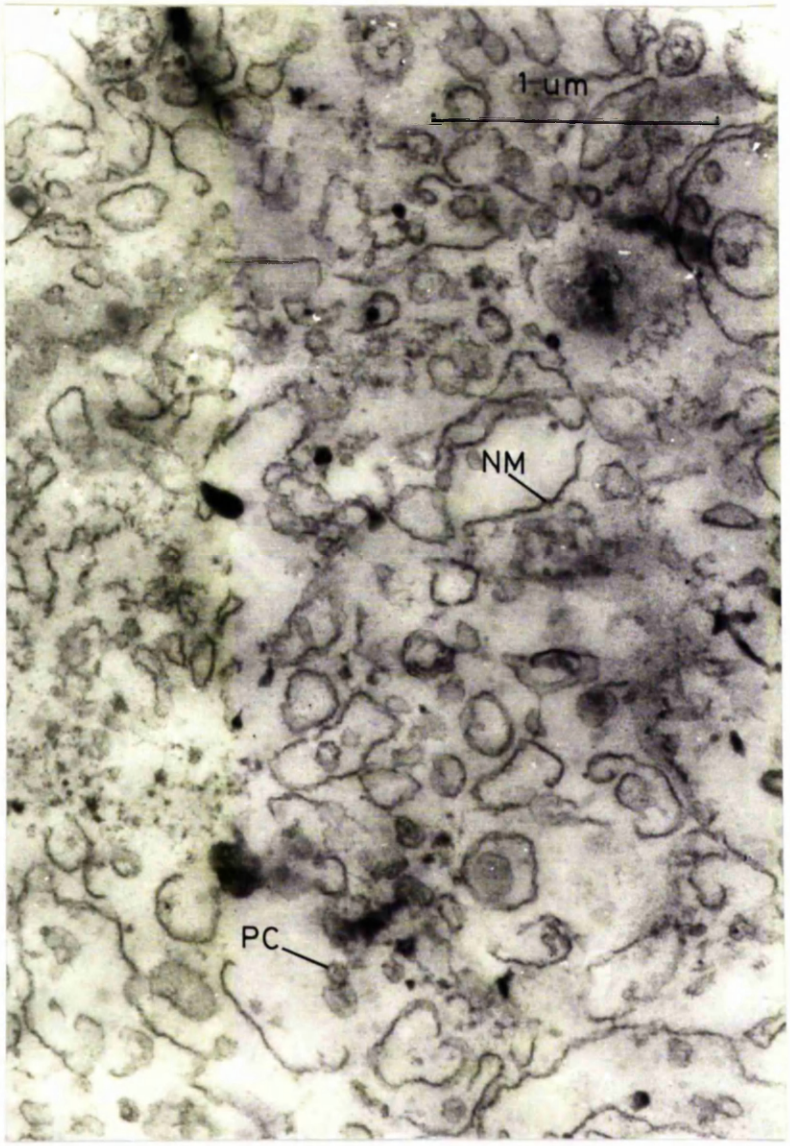
Morphology of Isolated Nuclear Envelope

Nuclei were isolated as described in Section II.2.b. Nuclear envelopes were isolated as described in Section II.2.c.

P.C. = pore complex

NM - nuclear membrane

Pore complexes were recognised as detached hollow rings with a globular appearance with an outer diameter of $\sim$ 80 μm.


1 um
NM
PC

are quite similar although there are occasional changes in the relative proportions of proteins, e.g. at apparent molecular weight 93,000.

Fig.10.

Protein profiles of Nuclear and Microsomal fractions before and after Transformation

A 20µg sample of each fraction was analysed on 8% polyacrylamide slab gels as described in Section II.2.g.

A & B = cell homogenates (C_{13} & PyY respectively)

C & D = whole nuclei " " "

E & F = nuclear envelope " " "

G & H = pore-complex-lamina " " "

I & J = membrane proteins " " "

K & L = rough E.R. (C_{13} & PyY respectively)

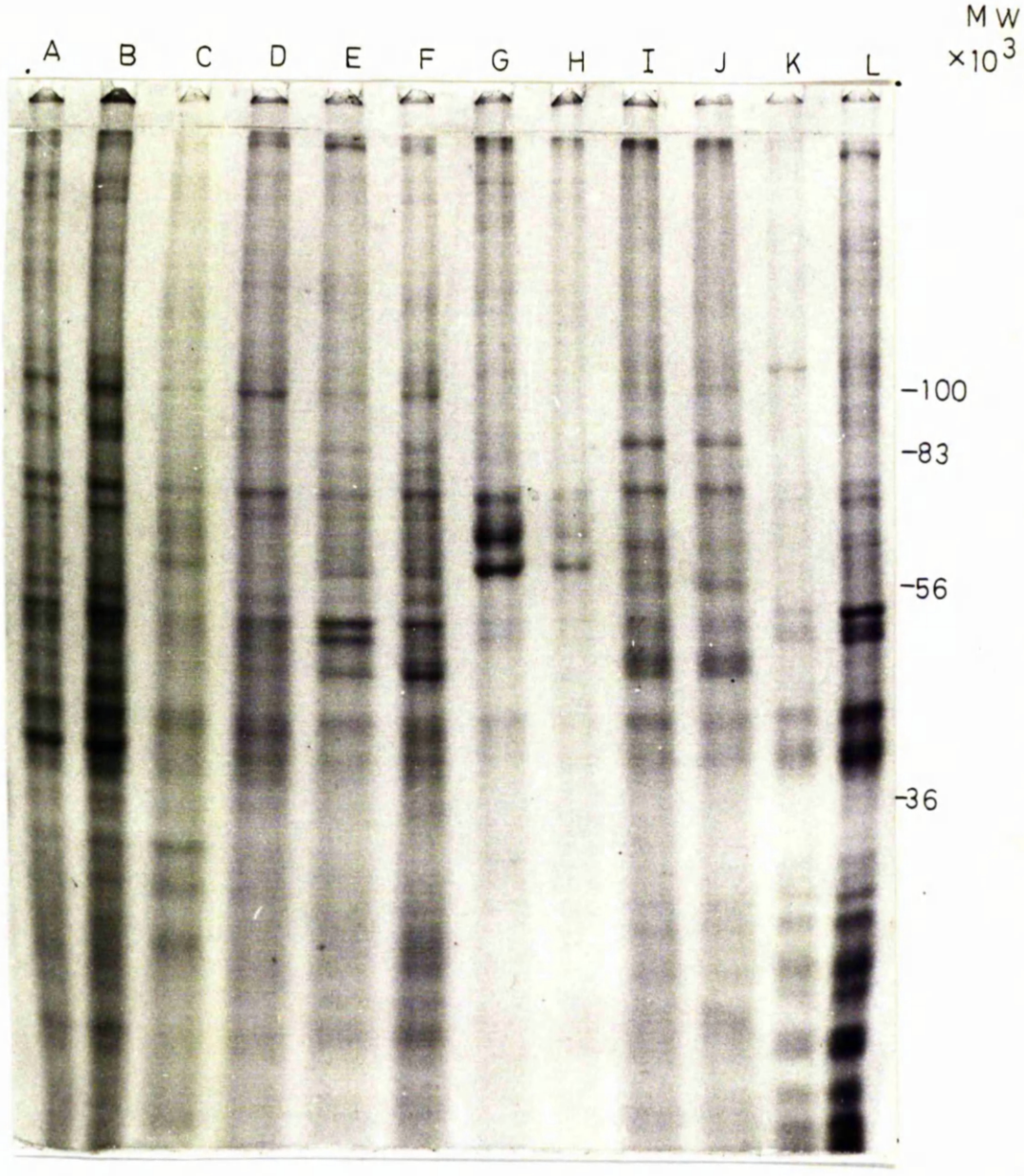

A
B
C
D
E
F
G
H
I
J
K
L
MW
×10³
100
83
56
36

Fig.11.

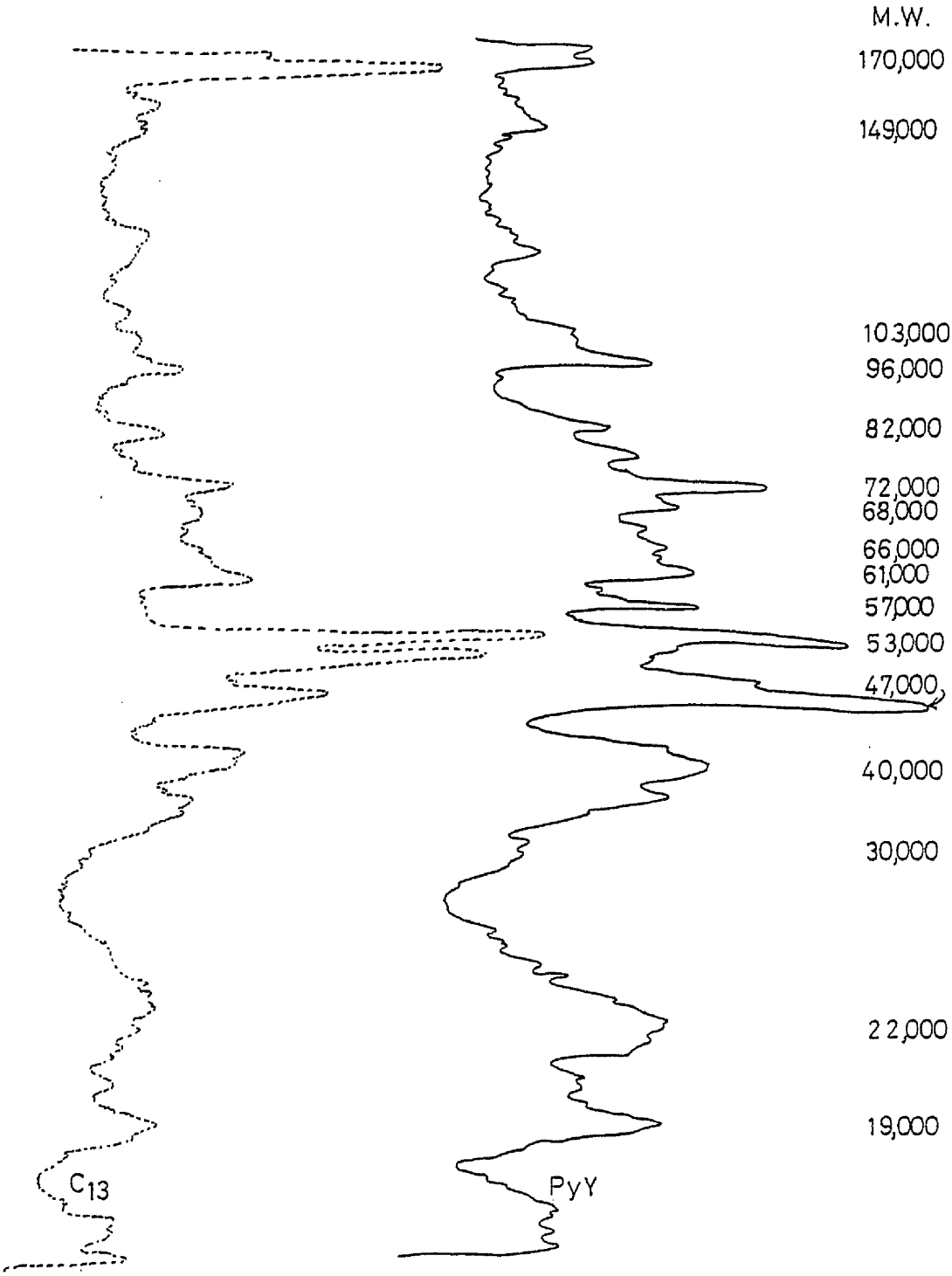
Protein Profile of the Nuclear Envelope before and after Transformation

Nuclear envelope was prepared from nuclei (C_{13} or PyY) isolated as described in sections II.2.b & c. Portions (20µg) were analysed on 8% polyacrylamide slab gels and their optical densities scanned as described in Section II.2.g.

------- = C_{13} nuclear envelope

——— = PyY nuclear envelope.

Fig. 11


M.W.
170,000
149,000
103,000
96,000
82,000
72,000
68,000
66,000
61,000
57,000
53,000
47,000
40,000
30,000
22,000
19,000
C13
PyY

III.3.b. Pore-Complex-lamina fraction

The morphology of this fraction is shown by electron microscopy in Fig. 12. This reveals the fibrous structure of the protein lamina with occasionally pore complexes (arrows).

The protein profiles of this fraction are shown in Fig. 13. The profile is dominated by the triplet of proteins of apparent Mr 72,000-60,000 which belong to the lamina and have been described by many authors. (Sections I.1 and I.3). There are many other minor proteins, the functions of which are not known.

Fig. 12.

Morphology of Pore Complex Lamina

The pore complex-lamina fraction was isolated from C_{13} nuclei as described in Section II.2.d. It was examined and photographed by electron microscopy as described in Section II.2.n.

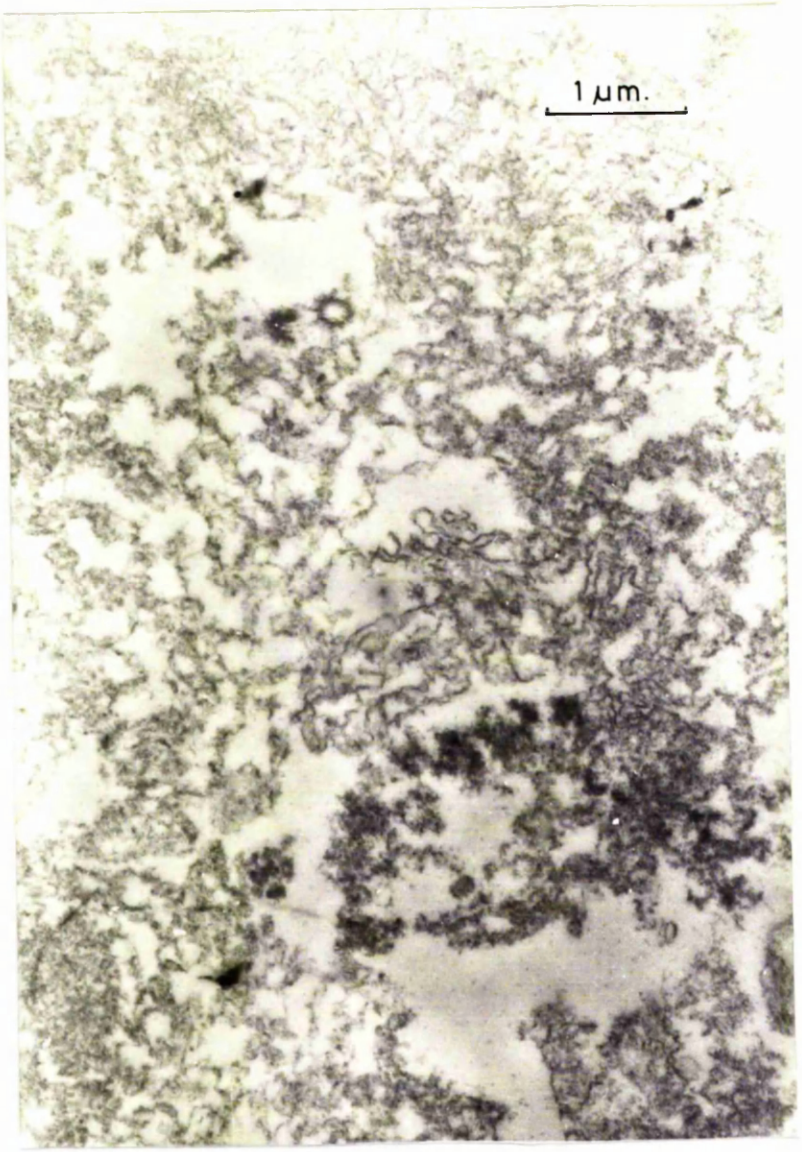
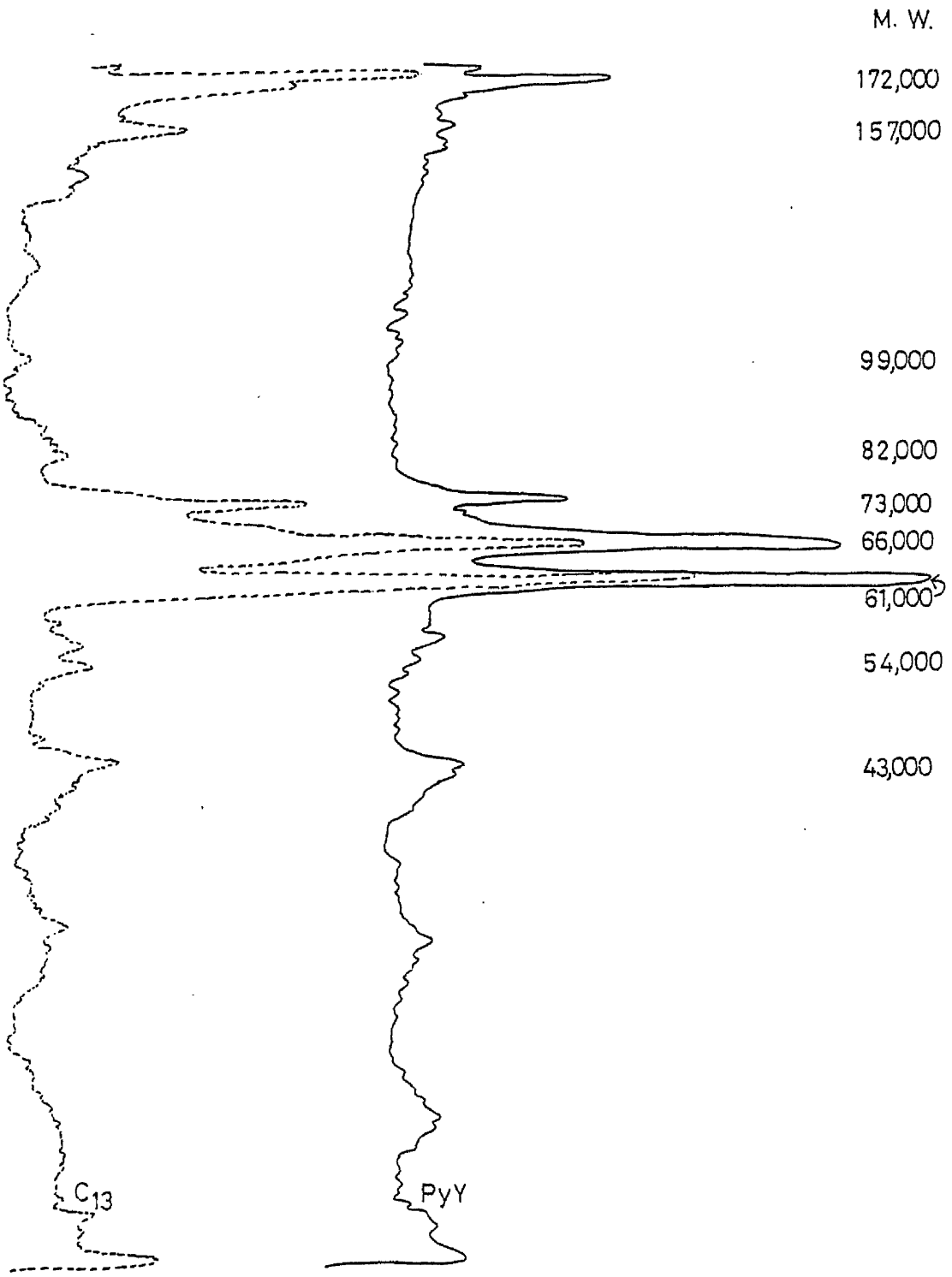

1 µm.

Fig. 13.

Protein Profiles of the Pore-complex-lamina Fraction before and after Transformation

The pore complex lamina fraction was isolated from nuclei (C_{13} or PyY) as described in Section II.2.d. Portions($\sim$ 20µg) were analysed by polyacrylamide gel electrophoresis and densitometry as described in Section II.2.g.

Fig. 13


M. W.
172,000
157,000
99,000
82,000
73,000
66,000
61,000
54,000
43,000
C13
PyY

III.3.c. Membrane Proteins

In this thesis the term "nuclear membrane proteins" refers to those extracted by Triton-X100 (2%) from nuclei which have been digested with DNAase. The protein profiles of this fraction, before and after transformation, are shown in Fig. 14 and appear very similar. The nuclear membrane proteins of normal cells are compared with those of endoplasmic reticulum in Fig. 15. There are substantial differences between these profiles in spite of the supposed physical continuity between the membranes, especially in the region of apparent Mr 85,000 to 60,000. There are similarities however. The proteins of apparent Mr 100,000, 53,000, 41,000, 25,000, 22,000 and 20,000 apparently occur in both fractions, though in different proportions.

A comparison between the profiles of the nuclear membrane and the nuclear envelope shows that proteins of apparent Mr 82,000, 74,000, 53,000, 48,000 and 42,000 are found in both fractions. There is also some similarity between the nuclear membranes and the pore complex lamina fraction in that proteins of apparent Mr 42,000, 53,000, 61,000, 65,000 and 74,000 appear in both profiles, although in very different relative proportions.

Investigation of Affinity between membrane Proteins and the Pore-complex Lamina

The experiments of Wunderlich et al.(1978, 1979, 1980) suggest that there may be some form of communication between the lamina and the nuclear membrane. Accordingly, the possibility of an affinity between isolated membrane proteins and the lamina was investigated. Membrane proteins isolated from cells labelled with L- 4,5-^{3}H leucine were dialysed as described until the concentration of

Triton-X100 was less than 0.1%. This suspension was incubated with unlabelled pore complex lamina for periods up to four hours. The lamina was then pelletted, washed repeatedly and counted. No significant affinity between isolated membrane proteins and pore complex lamina was found although various conditions were investigated.

Fig. 14.

Protein Profiles of the Nuclear Membrane before and after Transformation

The nuclear membrane proteins were isolated from nuclei (C_{13} or PyY) as described in Section II.2.e. Portions ($\sim$ 20µg) were analysed by polyacrylamide gel electrophoresis and densitometry as described in Section II.2.g.

Fig. 14

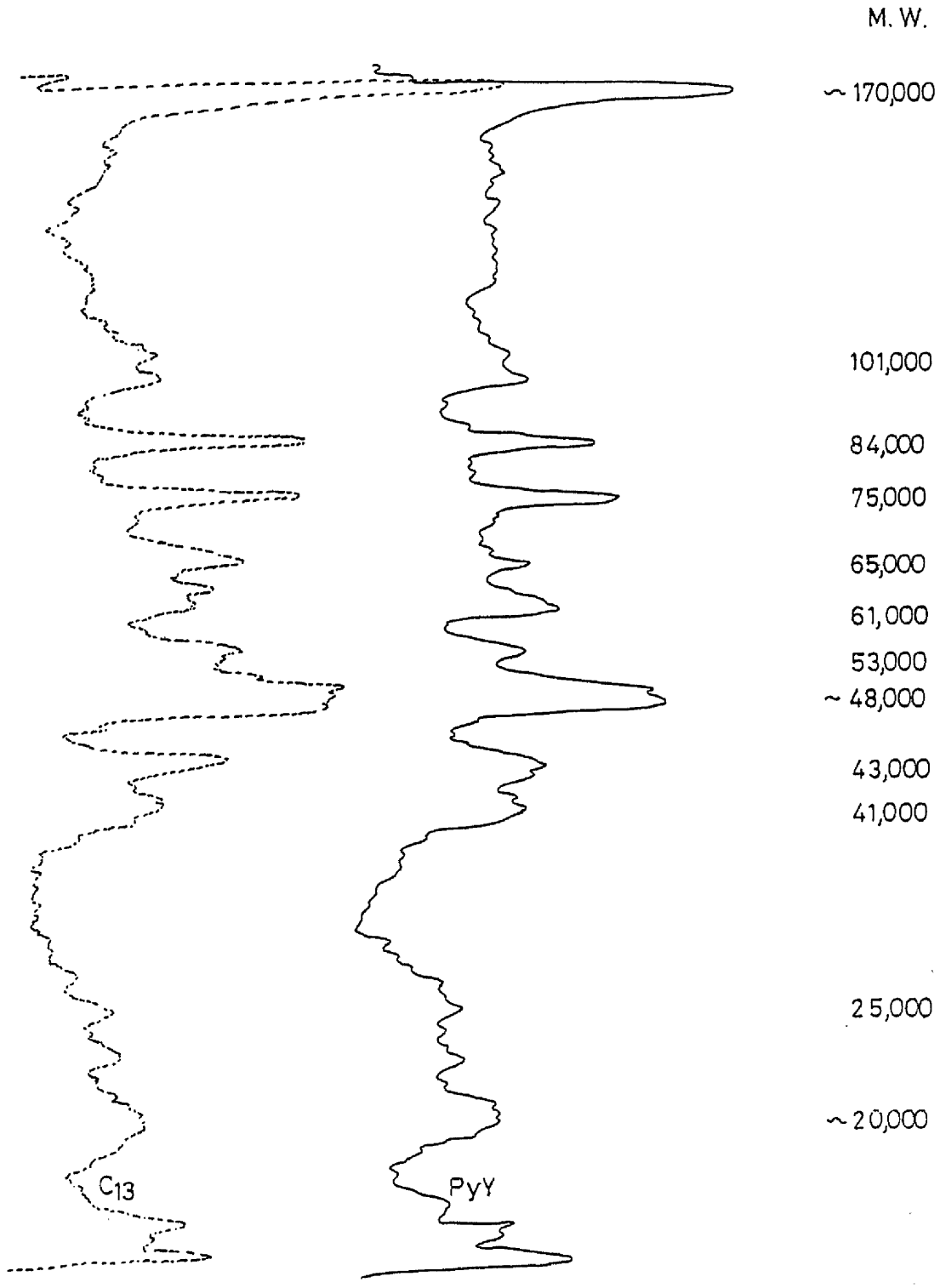
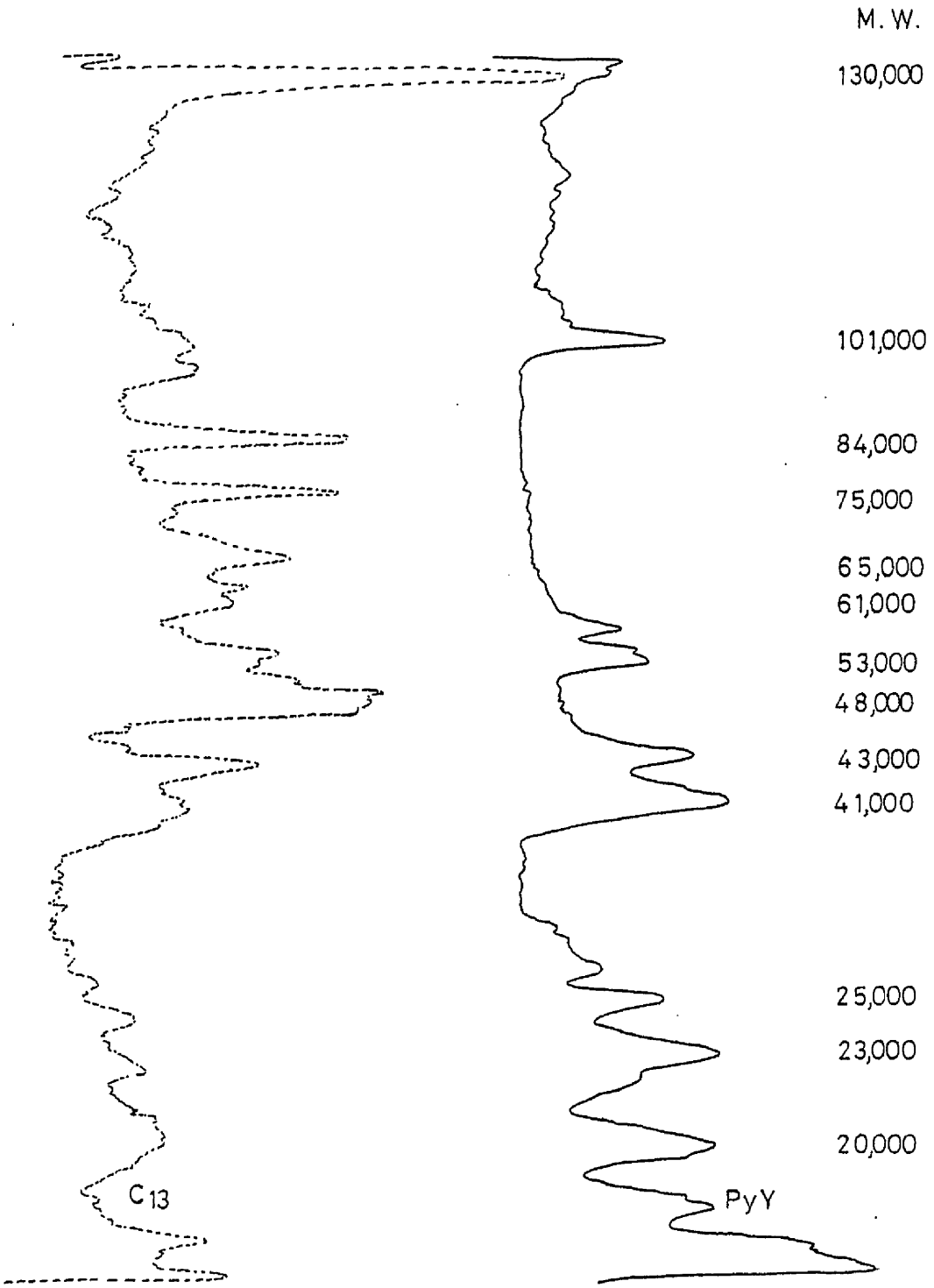

M. W.
~ 170,000
101,000
84,000
75,000
65,000
61,000
53,000
~ 48,000
43,000
41,000
25,000
~ 20,000
C13
PyY

Fig. 15.

Protein Profiles of Nuclear Membrane and Rough Endoplasmic Reticulum

Nuclear membrane and rough endoplasmic reticulum were isolated from C_{13} cells as described in Sections II.2.e. and II.2.f. respectively. Portions ($\sim$ 20μg) of the proteins were analysed by polyacrylamide gel electrophoresis and densitometry as described in Section II.2.g.

Fig. 15


M. W.
130,000
101,000
84,000
75,000
65,000
61,000
53,000
48,000
43,000
41,000
25,000
23,000
20,000
C13
PyY

P.104 Addendum to III.4.a.1 Activities of iodinated lectins.

The results presented in Table 4 indicate that the lectin activities were reduced by iodination. However, it was concluded that enough activity remained for the purposes of the experiment.

III.4. Carbohydrates of the Nuclear Envelope

a) Characterisation of the lectin overlay method

1) Activity of iodinated lectins

The changes in carbohydrate binding activities of the various lectins caused by iodination was investigated by the agglutination of red cells as described in Section II.2.j. The results of this investigation are presented in Table 4.

In order to ascertain that the lectins were active in the conditions of the lectin overlay, a 10µg sample of red cell membrane proteins was electrophoresed on the usual 8% acrylamide slab gel and overlaid with the various lectins as described in Section II.2.i. Densitometric scans of the resulting autoradiographs are presented in Fig.16 a-c. RCA was not included in this experiment.

2) Fixation procedure for gels prior to lectin overlay

Several techniques of gel fixation were investigated before the lectin overlay method was regularly employed. Variations in the Tanner and Anstie (1976) procedure were investigated using fixation and staining in 25% isopropanol/10% acetic acid containing 0.05% Coomassie blue as the control. These variations included increasing the methanol concentration to 75%, increasing the glutaraldehyde concentration to 0.10% and replacing the methanol with 25% isopropanol. None of these improved upon the result obtained by the Tanner & Anstie procedure which produced a profile identical to the control. Increasing the concentration of glutaraldehyde reduced the affinity of Coomassie blue for the proteins, probably by blocking the groups to which the stain binds.

Table 4

Activity of Lectins Before and After Iodination

Assays were performed in round-bottomed wells containing lectins dissolved in PBS (1-12.5µg/ml in the first well with serial dilution thereafter). Trypsinised, washed, human red cells were added to a final concentration of 0.75% and the trays were shaken gently and left to settle at room temperature for 16 hours before reading.

*Activity expressed as the lowest concentration (µg/ml) capable of agglutinating erythrocytes.

Lectin	Activity*		
	Before iodination	After iodination	% remaining
Con A	3.12	12.5	25
Lentil	0.39	3.12	13
WGA	1.0	3.12	32
RCA	0.015	0.5	3

Fig. 16.

Activity of Lectins in the conditions of the Lectin Overlay

Cell membrane (10µg) from human erythrocytes was electrophoresed in the standard 8% acrylamide slab gels as described in Section II.2.g. Gels were overlaid with ConA (0.48µg/ml), WGA (1.62µg/ml) or lentil lectin (0.80µg/ml) in PBS as described in Section II 2.i. and stained with Coomassie blue. Densitometry scans of the protein profiles and autoradiographs of the overlaid gels are presented.

a) ConA overlay

b) WGA overlay

c) Lentil lectin overlay.

Fig. 16a.

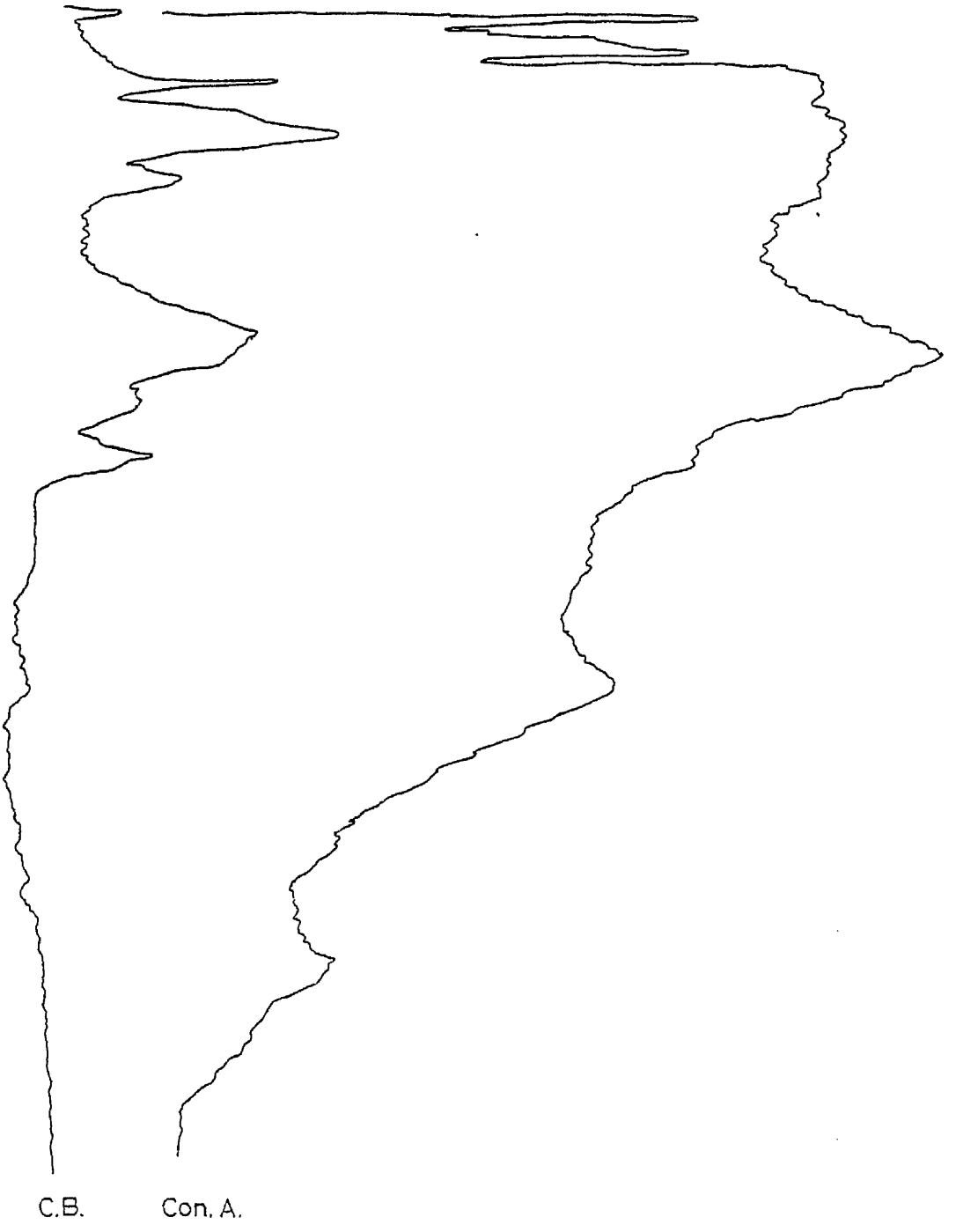


Fig. 16b.

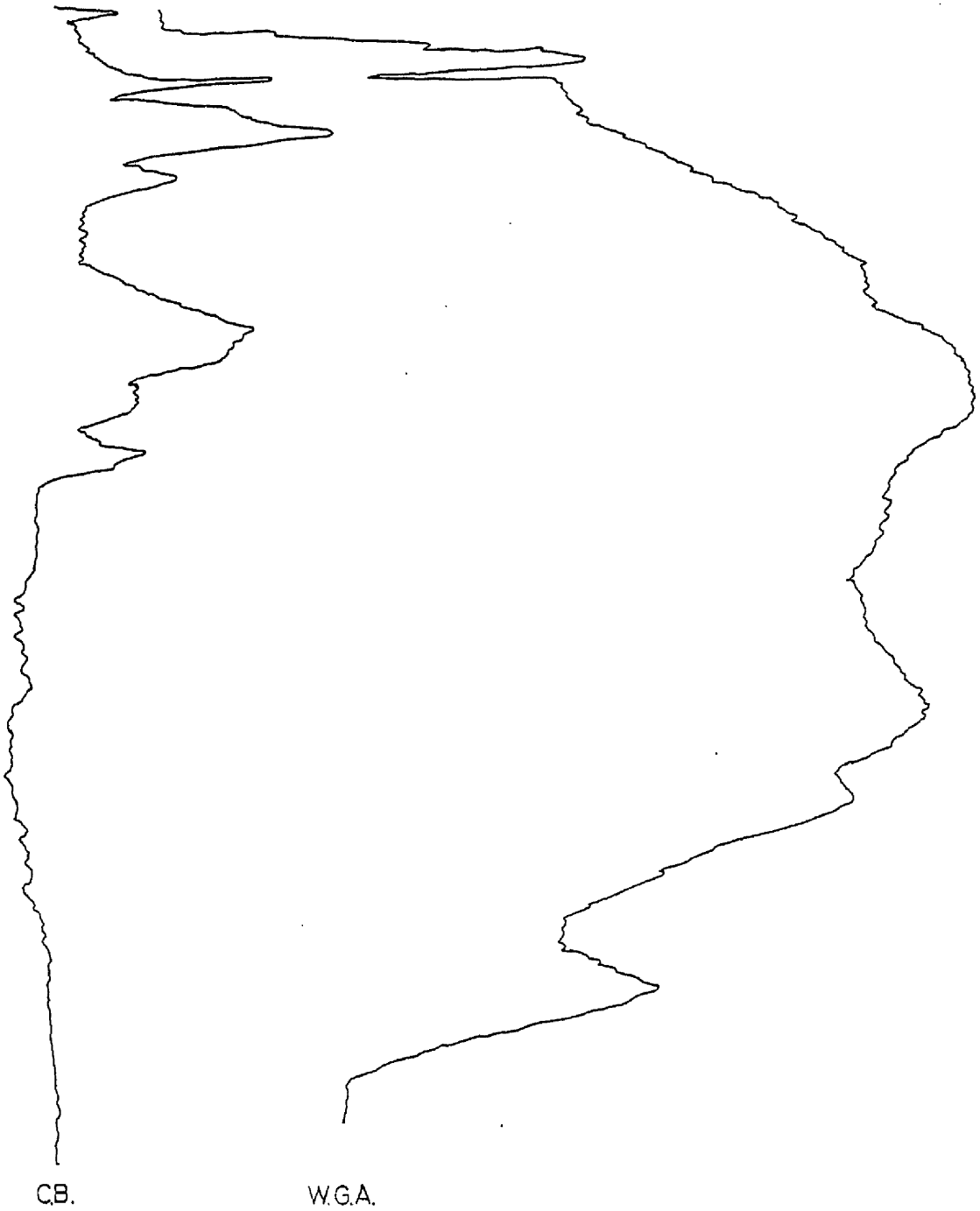
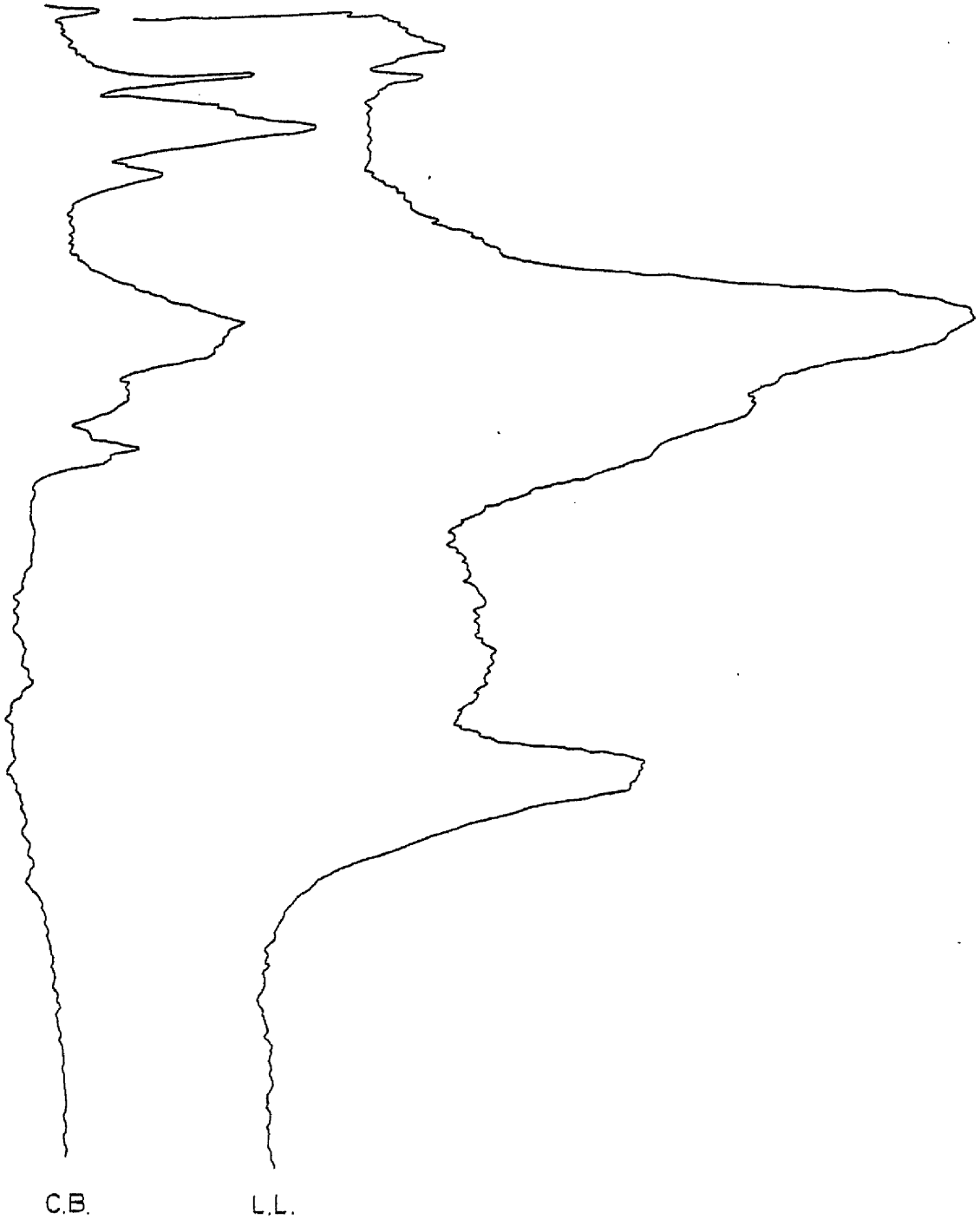


Fig. 16c.



3) Specificity of the Lectin Overlays

To ensure that the observed affinities of the lectins were caused by specific interactions, gels containing cell homogenate were overlaid with the various lectins in the presence of their specific monosaccharides (0.1M). In some experiments controls contained the same fraction as the overlay. Results of these controls are presented with the densitometric scans of the various autoradiographs.

III.4.b. Carbohydrates of Nuclear Fractions before and after Transformation

After transformation, changes in the carbohydrates of the various cell membranes have been reported (e.g. Buck et al., 1970). Such changes have also been described in nuclei, as discussed previously. In order to localise these changes within the nuclear envelope, various fractions, isolated from normal and transformed cells, were electrophoresed on gels identical to that shown in Fig. 10. These were fixed by the method of Tanner and Anstie (1976), overlaid with $[^{125}I]$-lectins as described in Section II.2.i. and autoradiographed. Densitometric scans of the autoradiographs of nuclei, nuclear fractions and rER, are shown in Figs. 17 to 21.

Although these overlays were only performed once in the case of lentil lectin and RCA, ConA and WGA overlays have been used routinely during the project. For the various fractions profiles visualised by both the lectin overlay (with ConA and WGA) and by Coomassie blue staining were reproducible. It is reasonable to assume therefore that the other lectins have produced profiles which are typical for the fraction concerned.

No conclusions can be drawn as to the absolute quantities of carbohydrates that are present in the various fractions because, although a quantitative calibration of these lectins was attempted, it was not successful. However, these results do provide information concerning the relative quantities of each carbohydrate in the various fractions. This is possible because all the tracks contained, as nearly as possible, 20µg of protein and, for each lectin used, all the tracks were overlaid and autoradiographed in exactly the same conditions.

As previously mentioned, PMSF was investigated as an inhibitor of serine proteases during the preparation of nuclei and nuclear envelopes, but was not found to make an appreciable difference to the protein profiles visualised by Coomassie blue. The PMSF was routinely present during the isolation of nuclear membrane proteins and pore complex lamina, and there is no evidence of proteolysis in the results obtained for these fractions or for nuclei. There may be some evidence for proteolysis in the carbohydrate profiles of the nuclear envelope fraction, for example in the large low molecular weight peak of the ConA overlay. This is possibly because the overlay technique is more sensitive than Coomassie blue in the detection of proteins.

As can be seen in Figs. 17 to 21, ConA, WGA and lentil lectin all show some affinity for all the various fractions. RCA showed affinity only for normal nuclei and nuclear envelope, in which the profile was suggestive of proteolysis. The carbohydrate profiles of the various fractions with the other lectins all appear generally similar before and after transformation, the major differences being quantitative rather than qualitative. For example, 20µg of denatured C_{13} nuclear protein binds less ConA than does the same amount of PyY nuclear protein. Conversely PyY pore-complex-lamina binds much less ConA than its untransformed counterpart. Also PyY nuclear membrane binds less WGA than does C_{13} nuclear membrane although their ConA profiles appear very similar. Nuclear envelope of both normal and transformed nuclei appear to bind less WGA than the other fractions, the proteins of apparent Mr 90,000 to 70,000 having lost their affinity for the lectin. This may indicate proteolytic

activity during the procedure, although low molecular weight cleavage products do not appear in the profile.

Comparison of the carbohydrate profiles between the various fractions indicates that the pore-complex-lamina retains quite significant amounts of carbohydrate even after extraction with 2% Triton-X100 and 2M NaCl followed by two washes in 0.25M sucrose. There is substantial binding of ConA and WGA and some binding of lentil lectin to this fraction. Previous ConA overlays performed on this fraction also show this result.

The glycoprotein profiles of nuclear membrane, pore-complex-lamina and rough endoplasmic reticulum are distinctly similar in their affinity for WGA and lentil lectin. These fractions show a marked difference in their affinity for ConA however, especially in the region of apparent molecular weight 70,000 to 50,000. Nuclear membrane proteins bind more ConA/μg and have a wider range of glycoproteins with ConA-affinity than does rough E.R.

In Figs. 17 to 21 the protein profiles shown by Coomassie blue are presented for comparison with the glycoproteins. These profiles indicate that lectin overlays detect a different population of glycoproteins, present in smaller amounts than can be fully resolved by Coomassie blue. The heterogeneity of the glycoprotein population is apparent from the broadness of its peaks compared to those stained by Coomassie blue. This heterogeneity is due to the variation in the polysaccharide moiety, in that some chains may be less complete than others. None of the peaks detected by Coomassie blue can be unequivocably ascribed to glycoprotein peaks because they are diffuse and so unlike the peaks stained by Coomassie blue. It is possible that the proteins of apparent molecular weight 53,000 and 47,000 in the nuclear envelope and those of apparent molecular weight 75,000 and 84,000 in the nuclear membrane may be glycoproteins.

These putative glycoproteins all have an apparent affinity for ConA and, in the nuclear membrane, possibly also for WGA. In whole nuclei there is only one peak (molecular weight 55,000) which may correspond to a glycoprotein. Although many of the other peaks coincide with those of glycoproteins their shapes are very different. This indicates that the nuclear membrane glycoproteins are a very small fraction of the total nucleus. It can be concluded that the prominent peaks of the pore complex lamina, and the rough E.R. are not glycoproteins.

Fig. 17.

Glycoproteins of Whole Nuclei Before and After Transformation

Nuclei (C_{13} or PyY) were isolated as described in Section II.2.b. Portions ($\sim$ 20µg) were analysed by polyacrylamide gel electrophoresis as described in Section II.2.g. and overlaid with various $\left[{}^{125}\text{I}-\right]$-lectins as described in Section II.2.i.

Control gels contained 20µg of cell homogenate and were overlaid in the presence of the specific monosaccharides (0.1M).

a) Protein profile stained with Coomassie blue (CB)

b) ConA overlay

c) WGA overlay

d) Lentil lectin overlay

e) RCA overlay.

Fig. 17a. Coomassie blue.

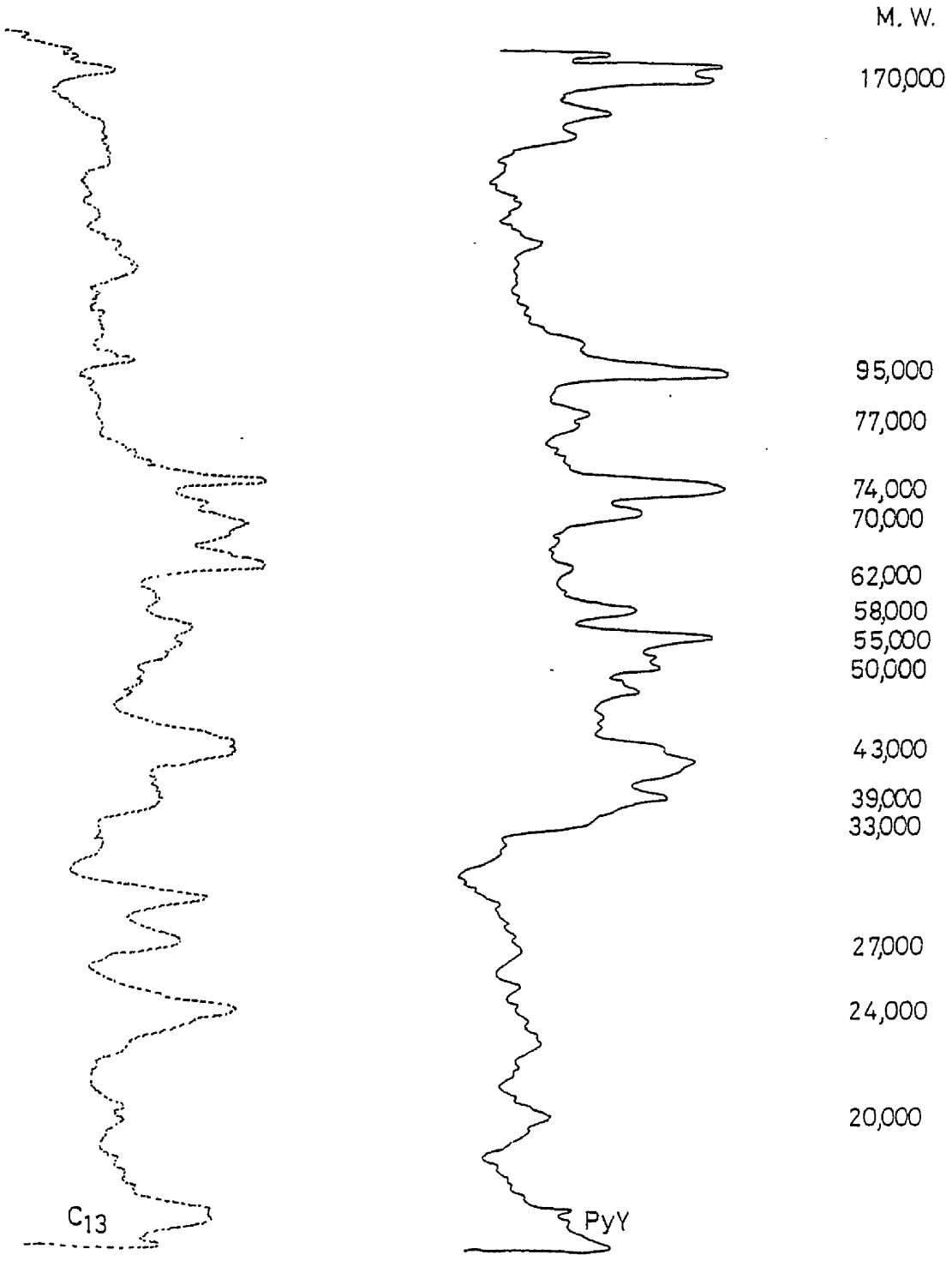


Fig. 17b. Con A.

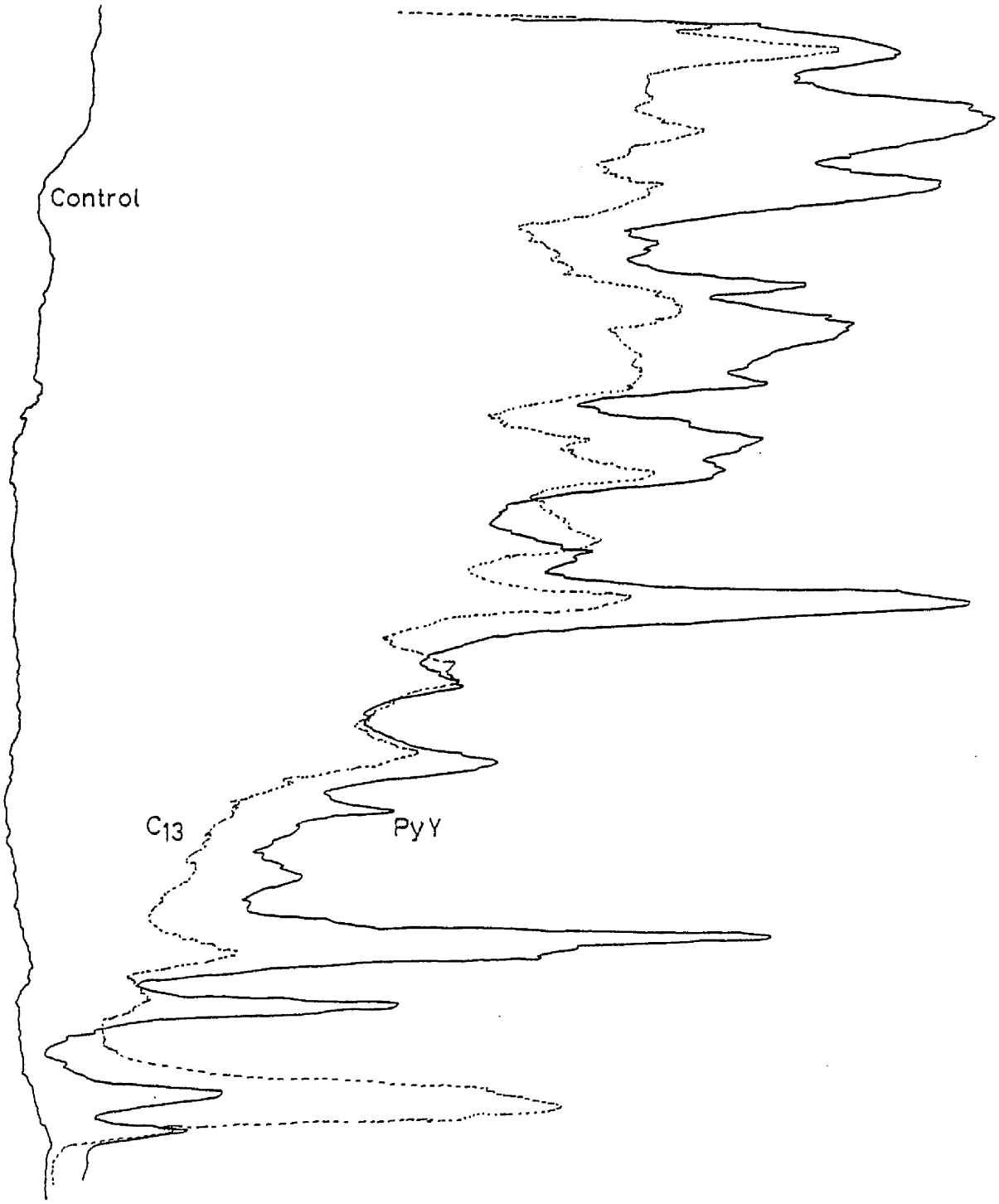


Fig. 17c. W.G.A.

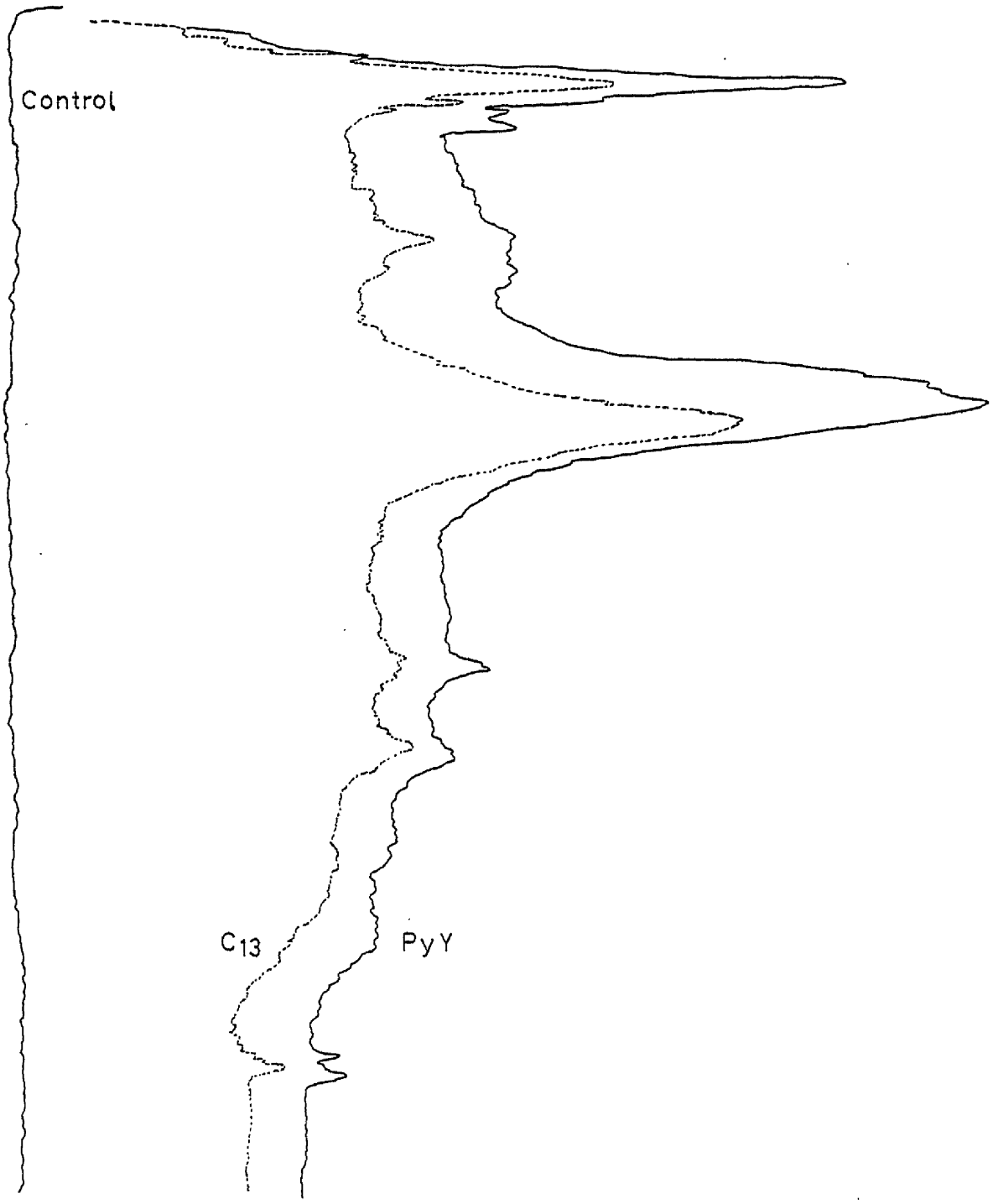


Fig. 17d.. L.L.

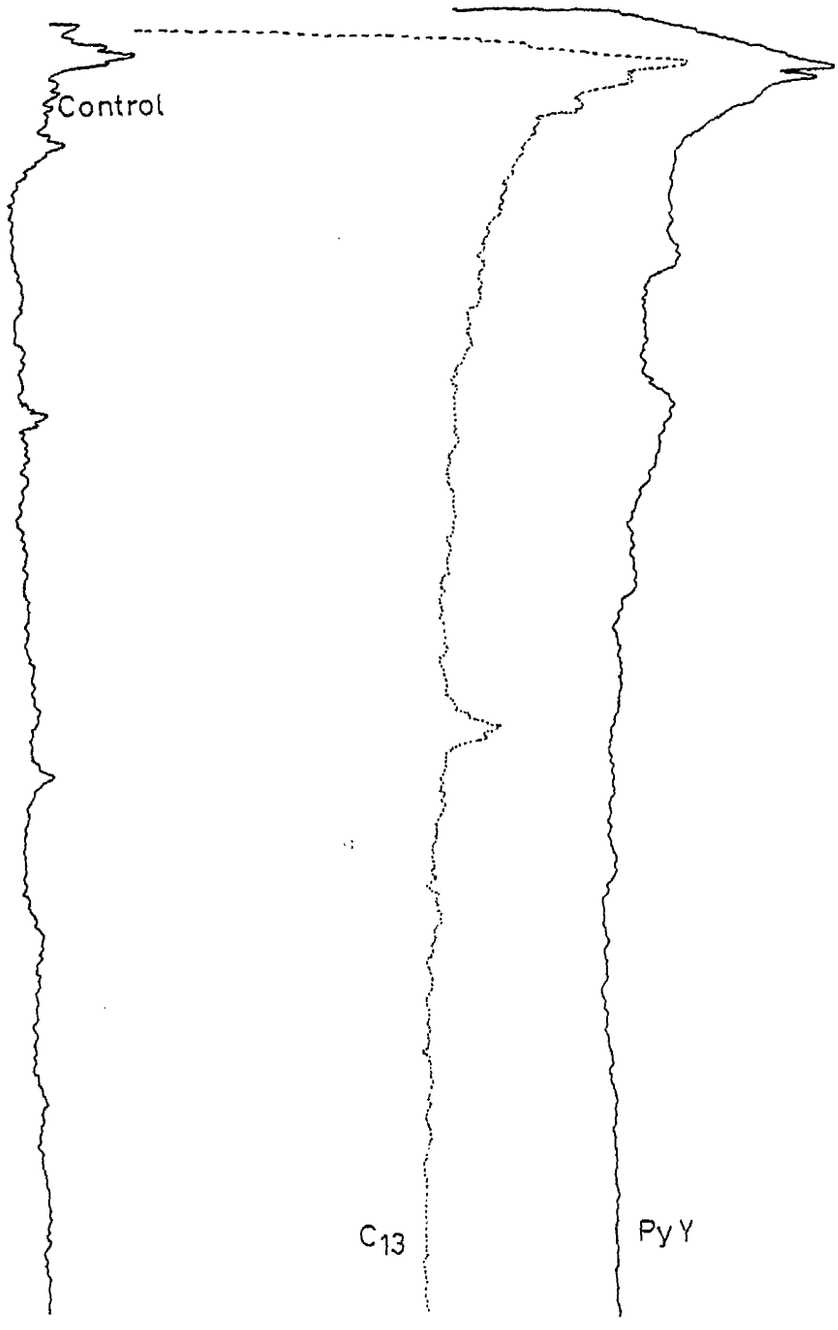


Fig. 17e. R.C.A.

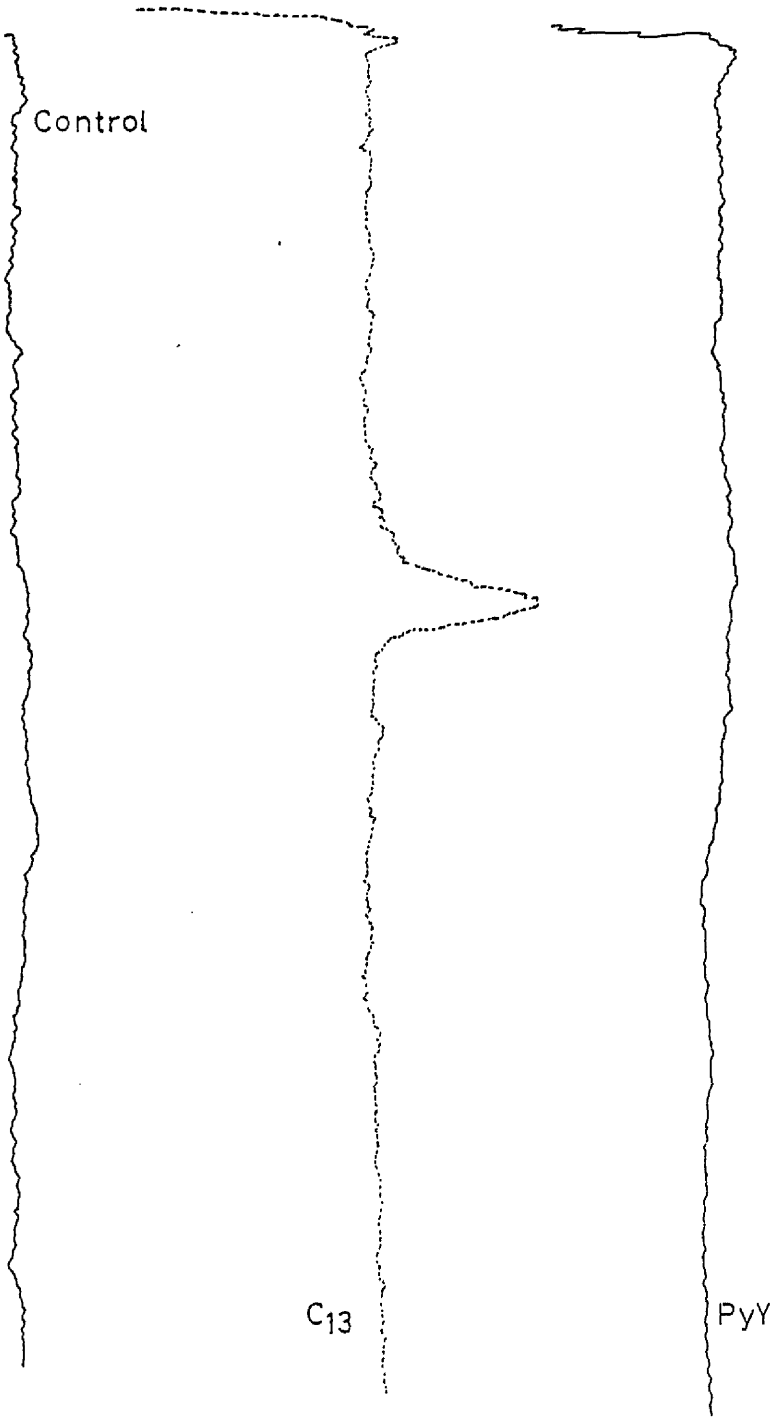


Fig.18.

Glycoproteins of Nuclear Envelope Before and After Transformation

Nuclear envelopes were isolated as described in Section II.2.c. from nuclei (C_{13} or PyY) prepared as described. Portions (20µg) were electrophoresed as described in Section II.2.g. and overlaid with various $\left[^{125}I-\right]$ lectins as described in Section II.2.i. Control gels contained 20µg of cell homogenate protein and were overlaid in the presence of the specific monosaccharides (0.1M).

a) Protein profile stained with Coomassie blue

b) ConA overlay

c) WGA overlay

d) Lentil overlay

e) RCA overlay.

Fig. 18a. Coomassie blue.

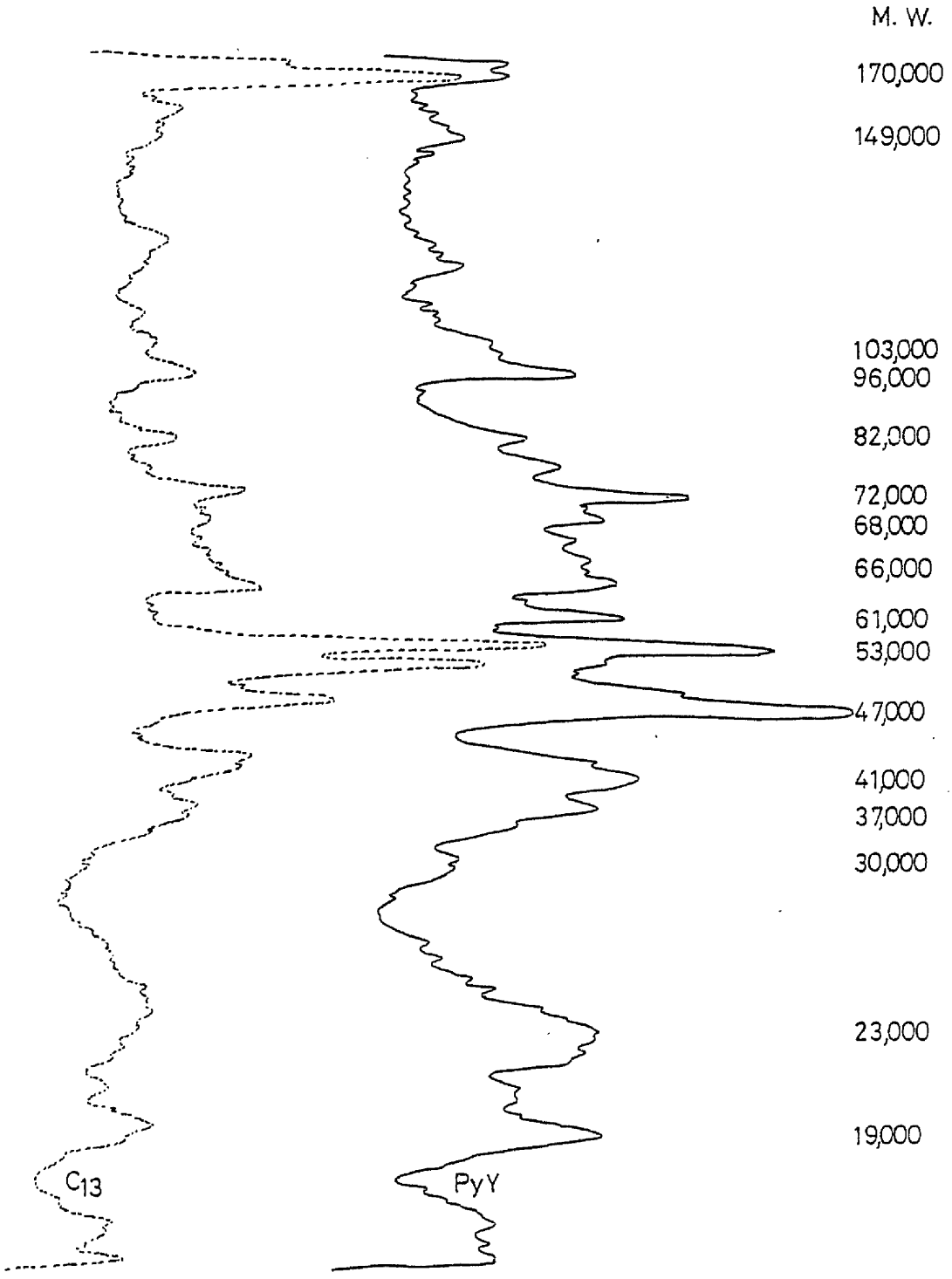


Fig. 18b. Con A.

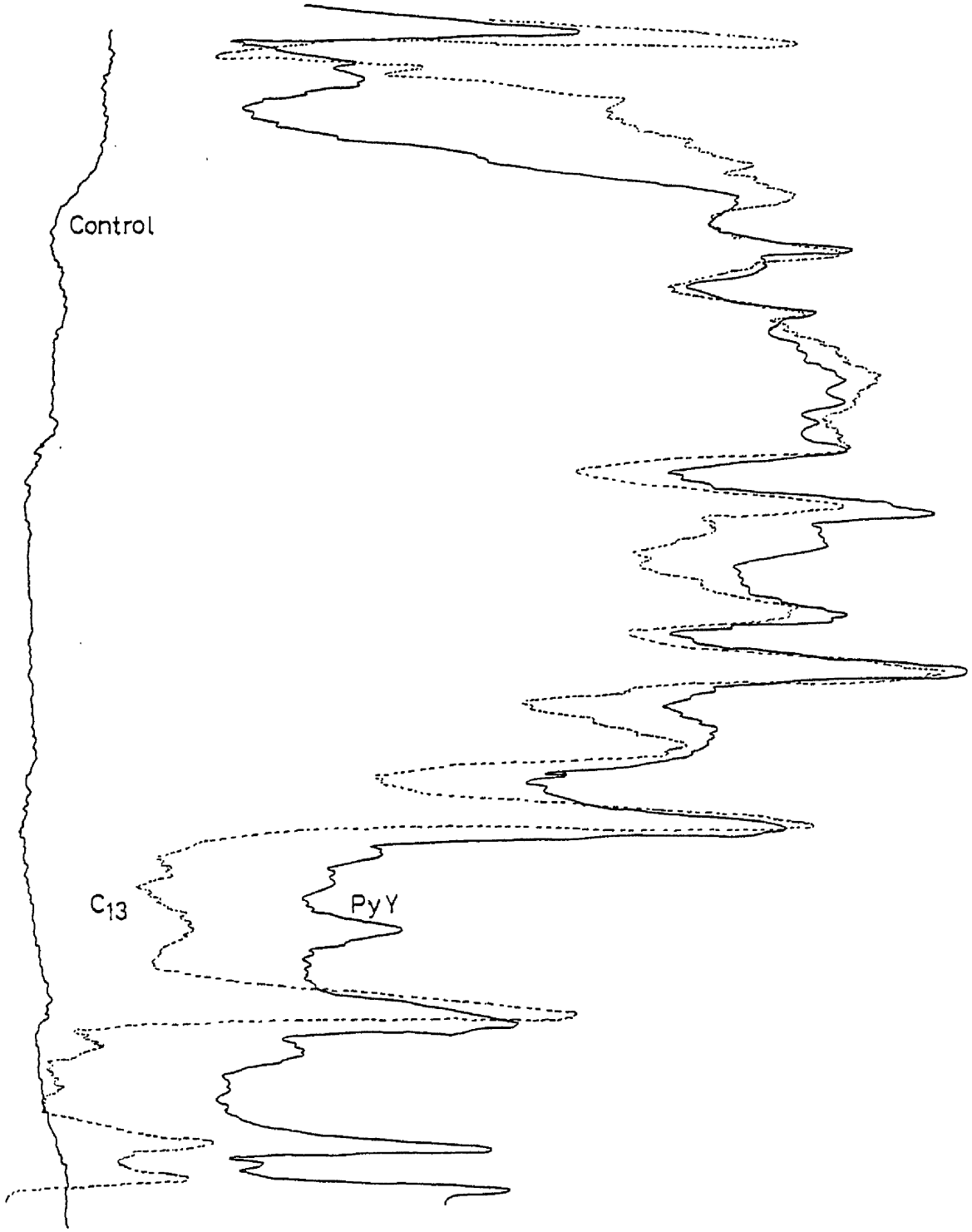


Fig. 18c. W.G.A.

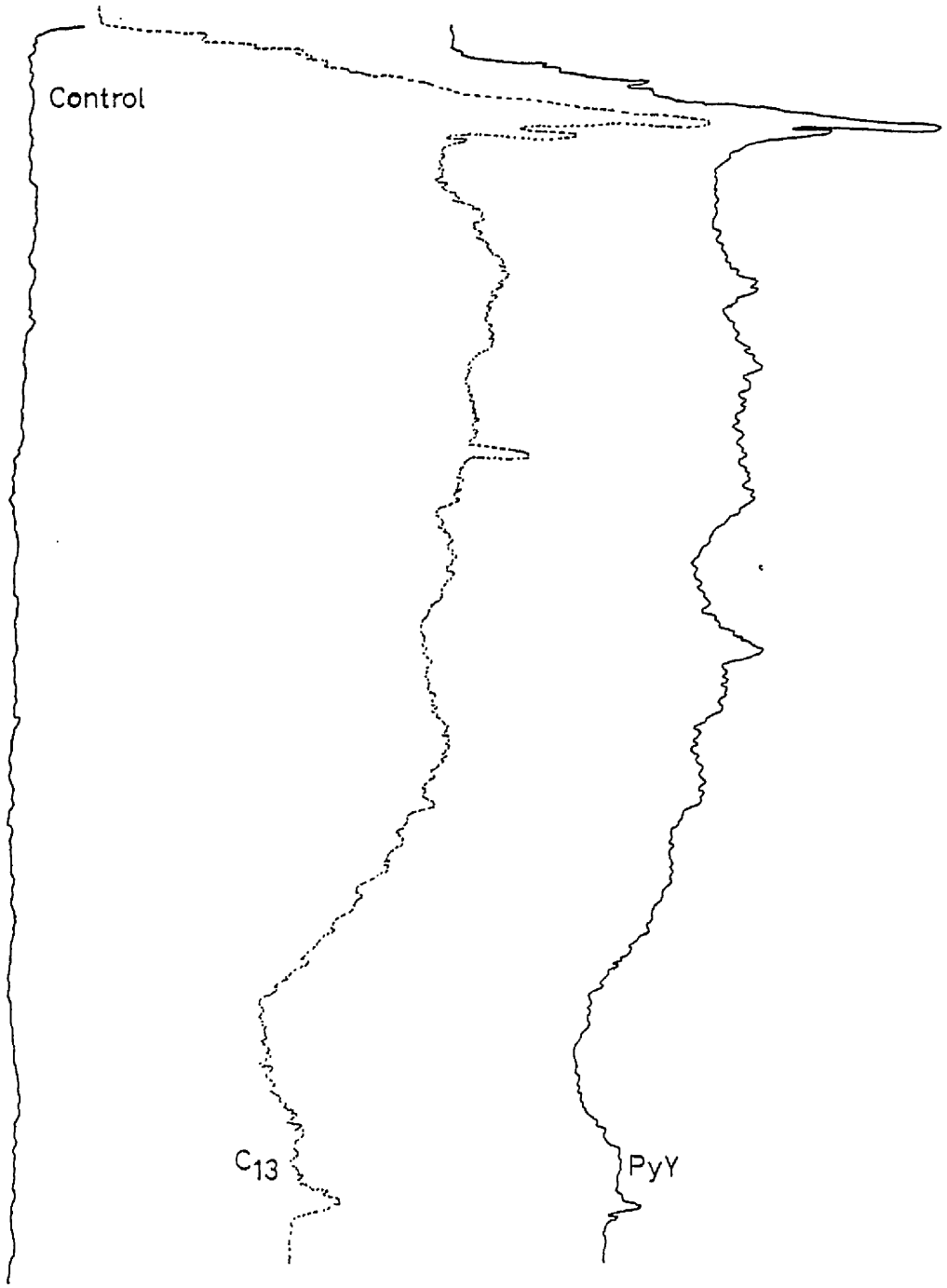


Fig. 18d. L.L.

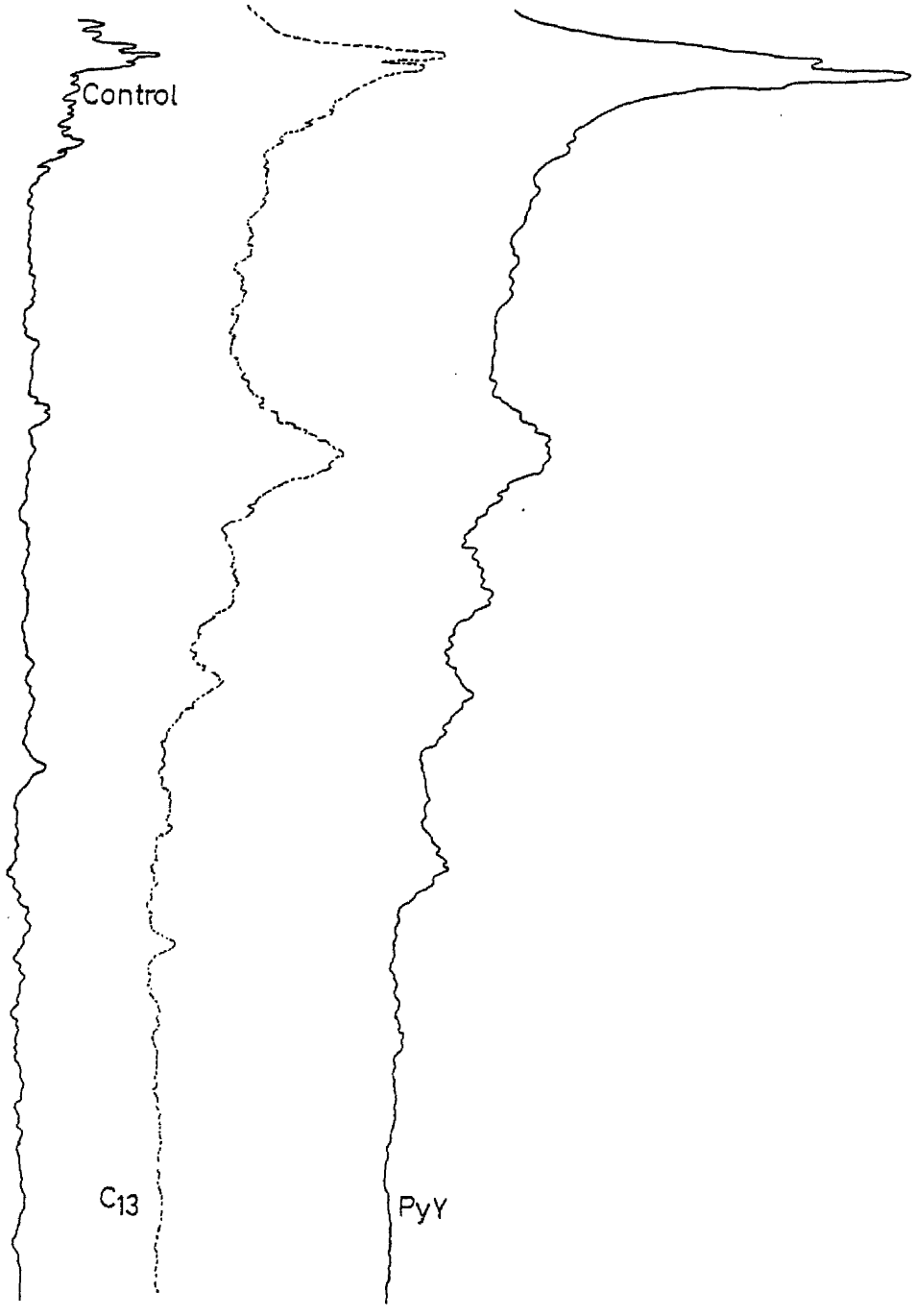


Fig. 18e. R.C.A.

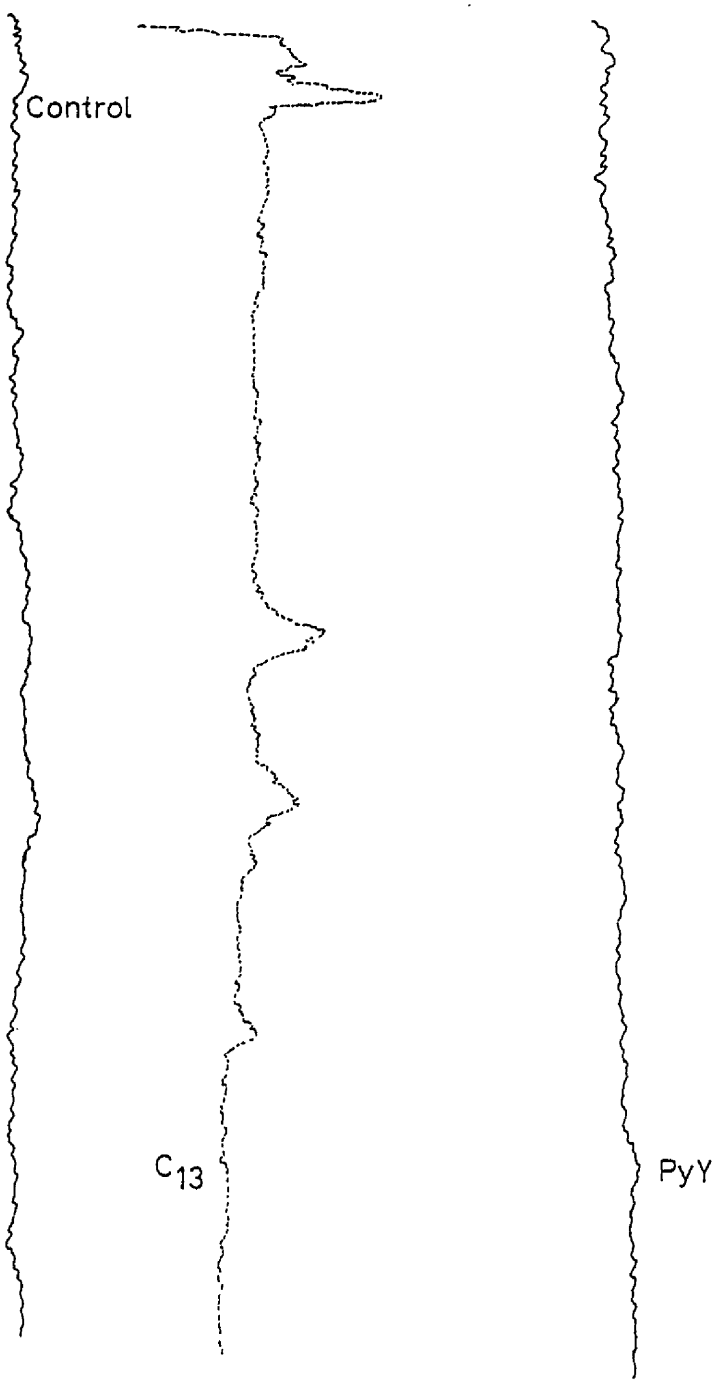


Fig. 19.

Glycoproteins of the Pore-Complex-lamina Before and After Transformation

Pore complex lamina was isolated as described in Section II.2.d. from nuclei (C_{13} or PyY) prepared as described. Portions (20µg) were electrophoresed and overlaid with various [^{125}I-] lectins as described previously. Controls contained cell homogenate protein (20µg) and were overlaid in the presence of the specific monosaccharides (0.1M).

a) protein profile stained with Coomassie blue

b) ConA overlay

c) WGA overlay

d) Lentil lectin overlay.

Fig. 19a. Coomassie blue.

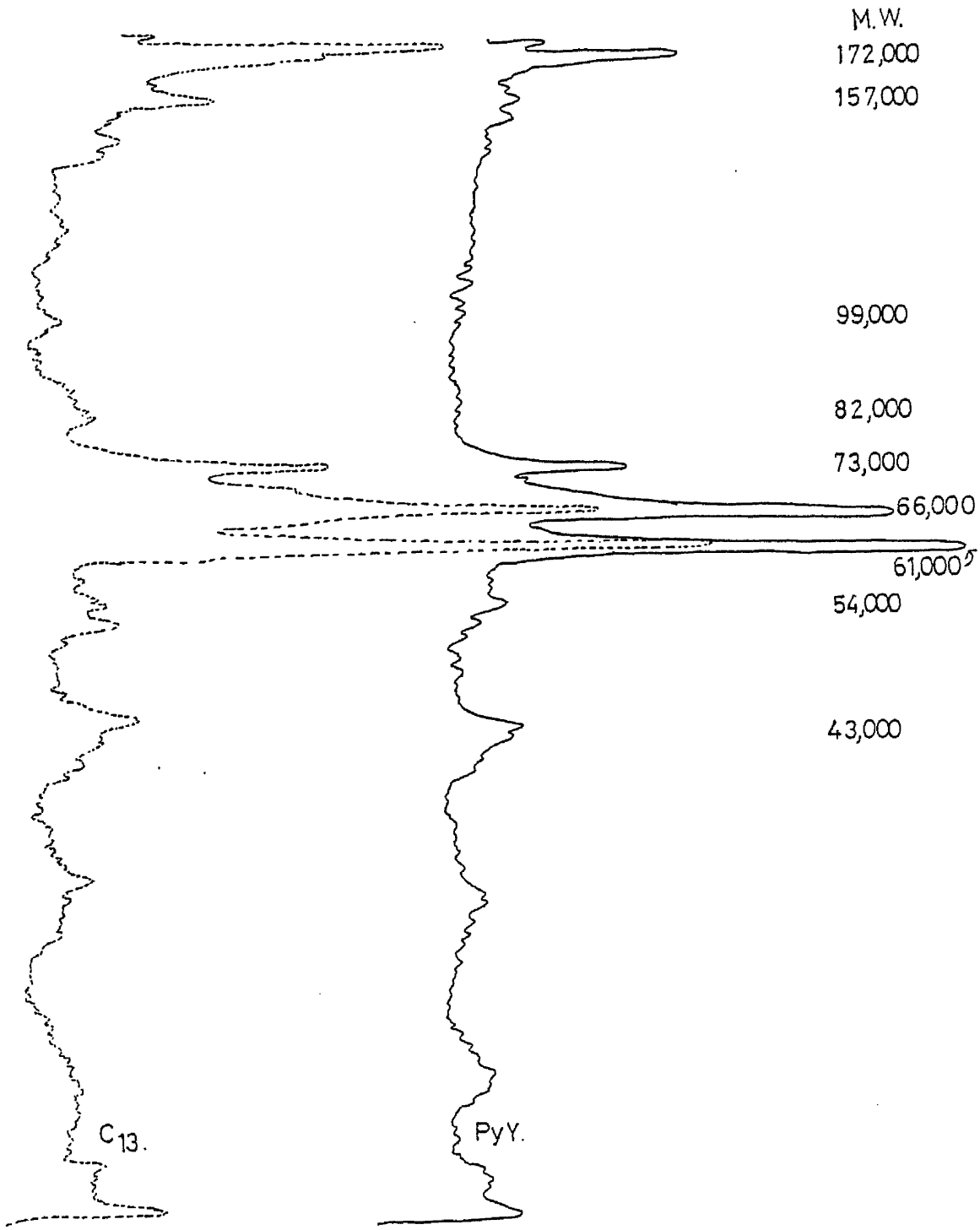


Fig. 19b. Con. A.

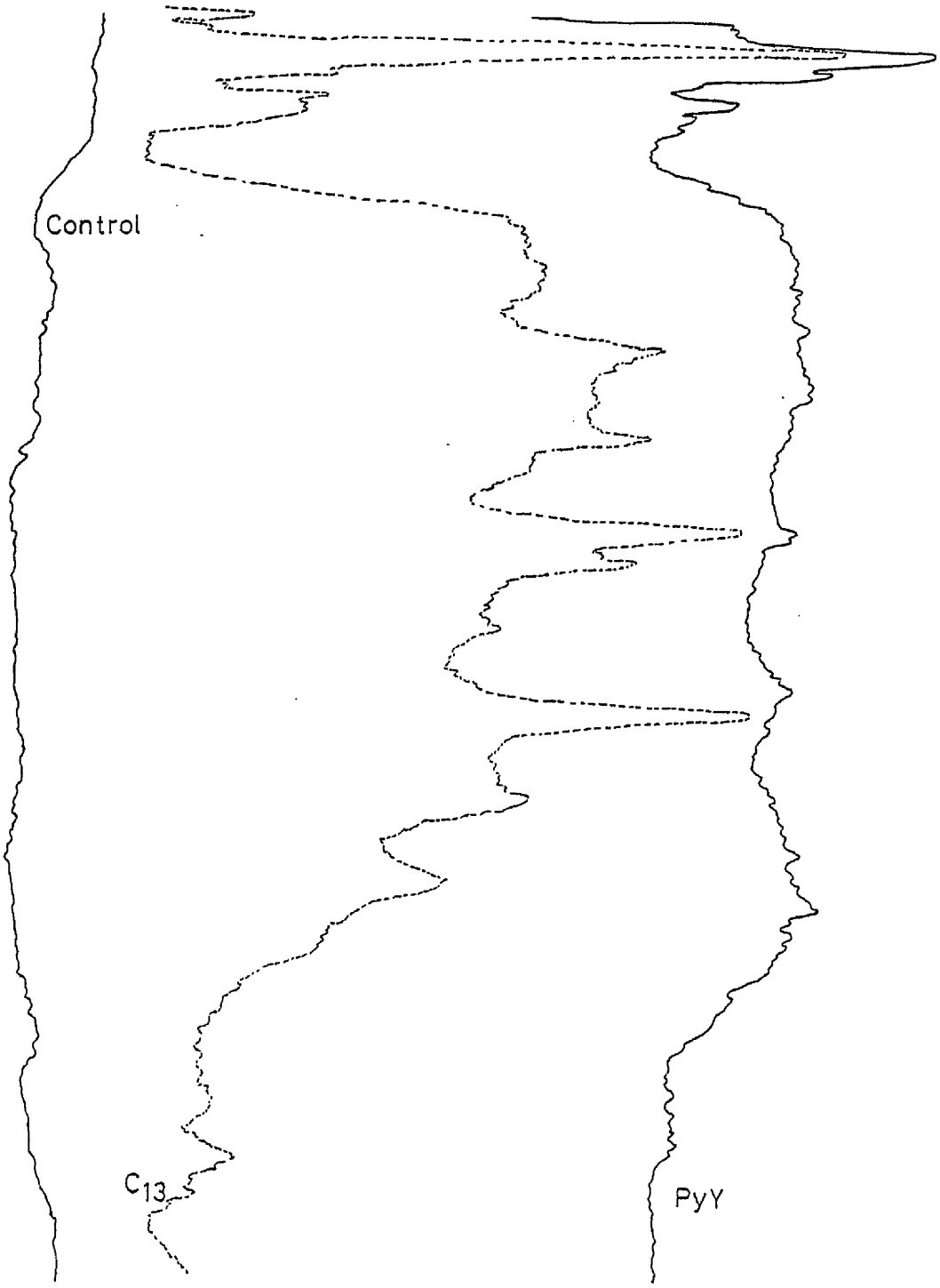


Fig. 19c. W.G.A.

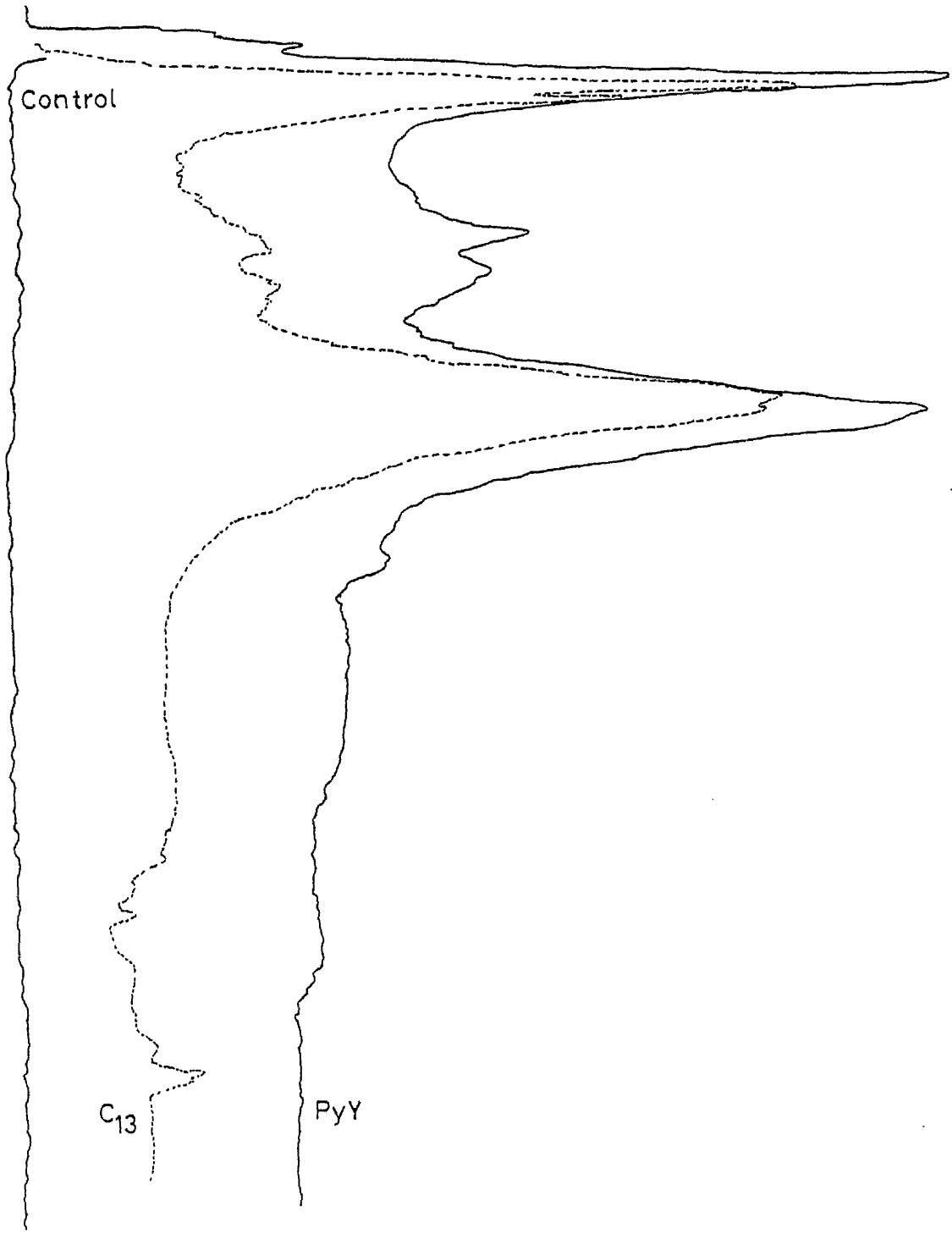


Fig. 19d. L.L.

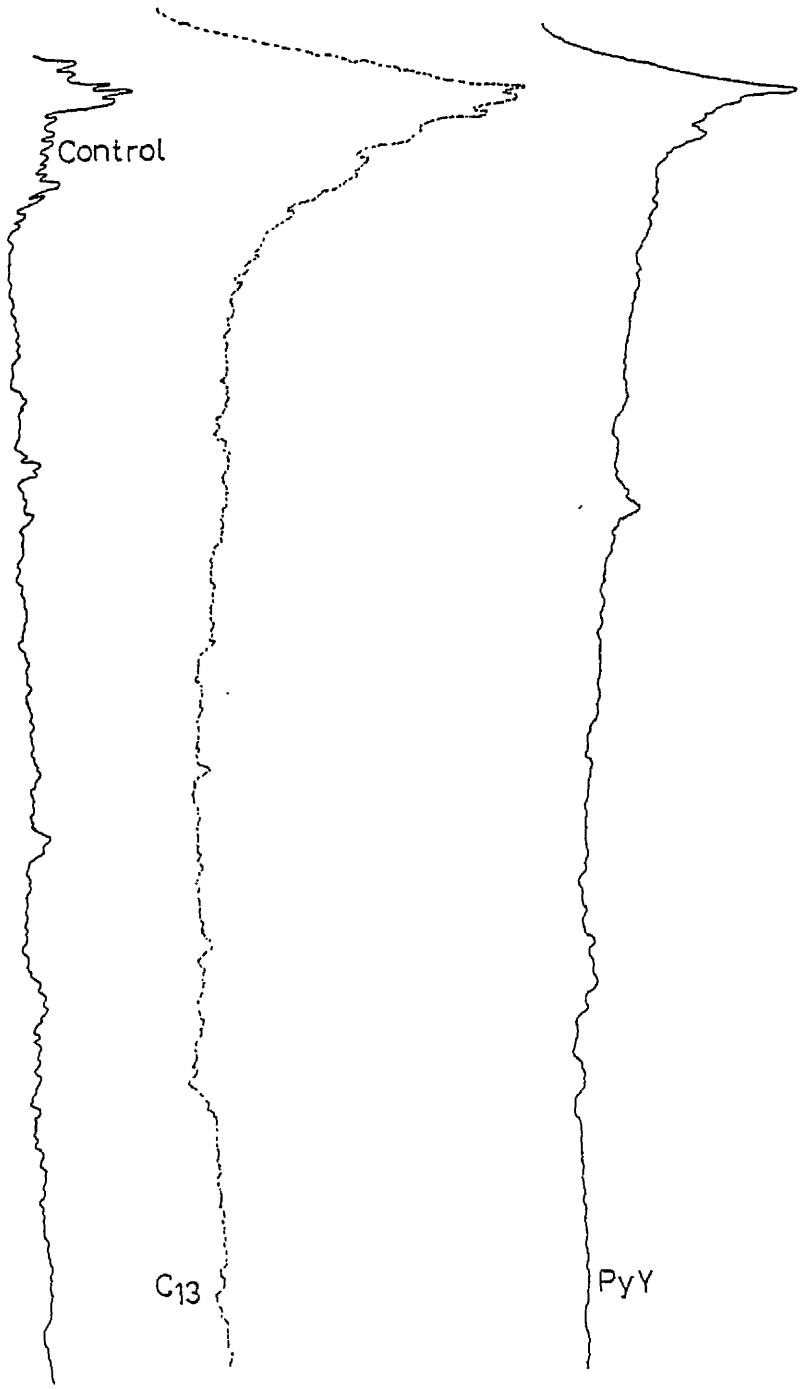


Fig. 20.

Glycoproteins of Nuclear Membrane Before and After Transformation

Nuclear membrane proteins were extracted from DNA-ase-digested nuclei (C_{13} or PyY) as described in Section II.2.e. Portions ($\sim$ 20µg) were electrophoresed and overlaid with varous $\left[{}^{125}\text{I}-\right]$-lectins as described in Sections II.2.g. and II.2.i. Controls contained 20µg of cell homogenate proteins and were overlaid with the specific monosaccharides (0.1M).

a) protein profile stained with Coomassie blue

b) ConA overlay

c) WGA overlay

d) Lentil lectin overlay

Fig. 20.a. Coomassie blue.

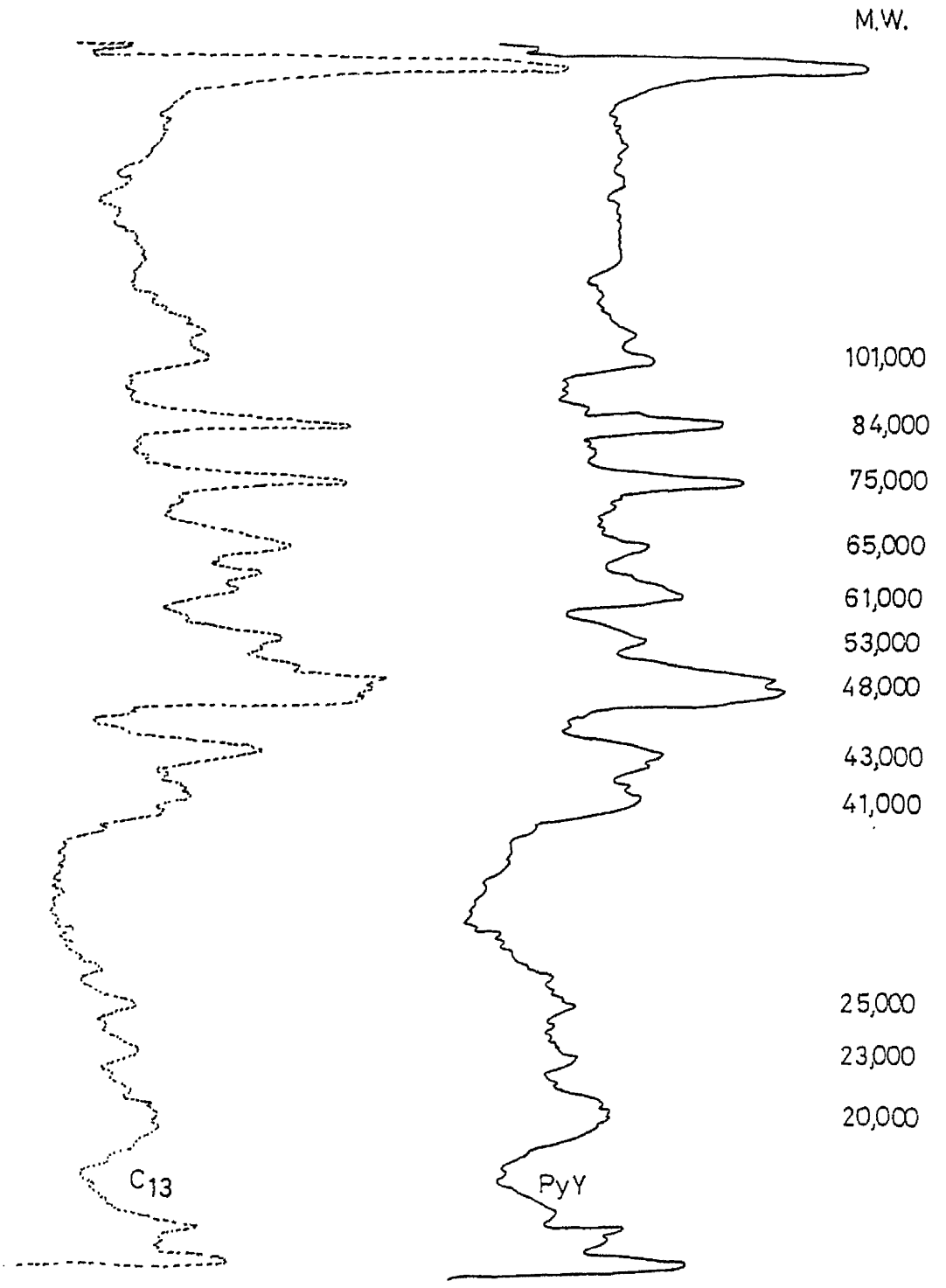


Fig. 20.b. Con A.

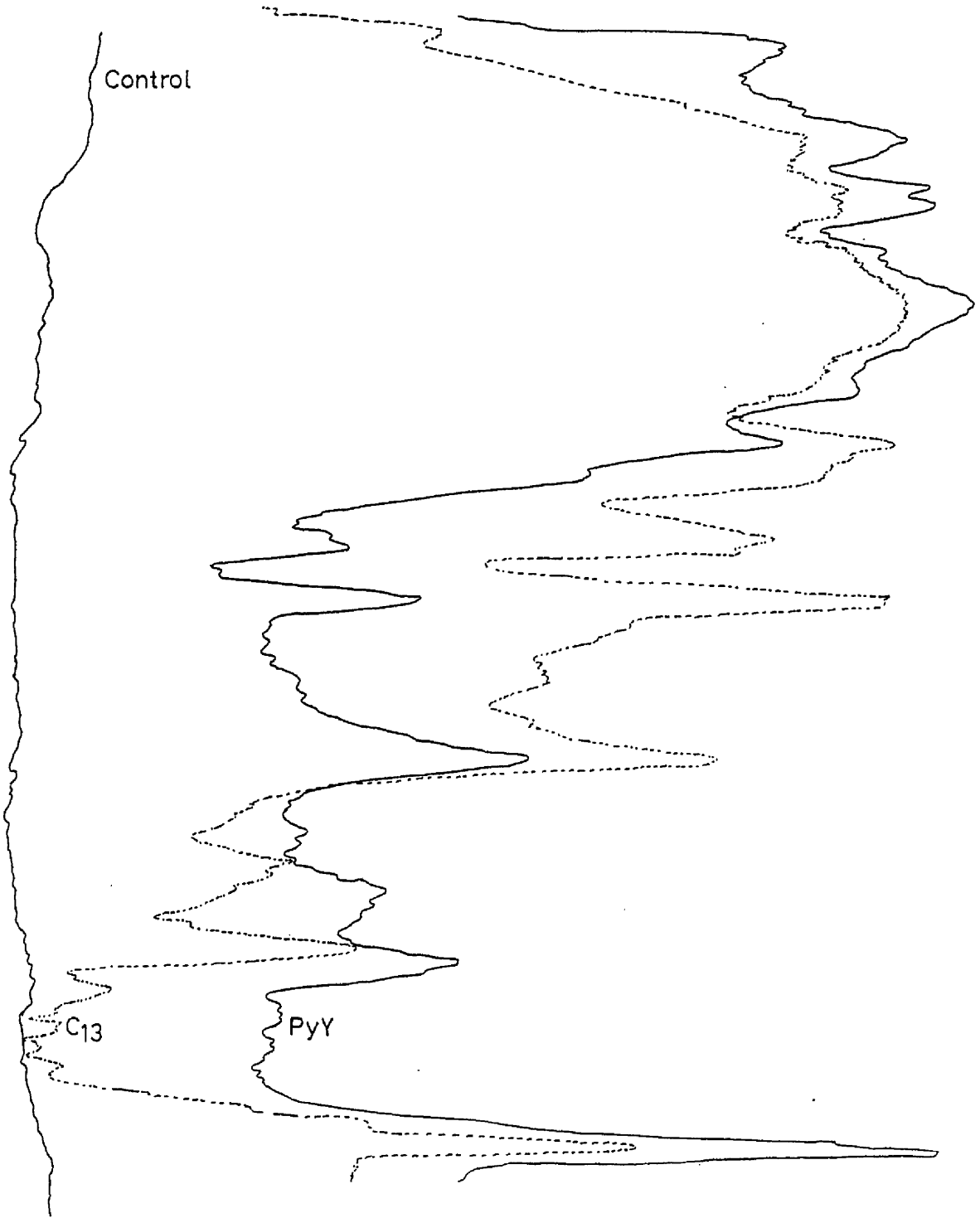


Fig. 20.c. WGA.

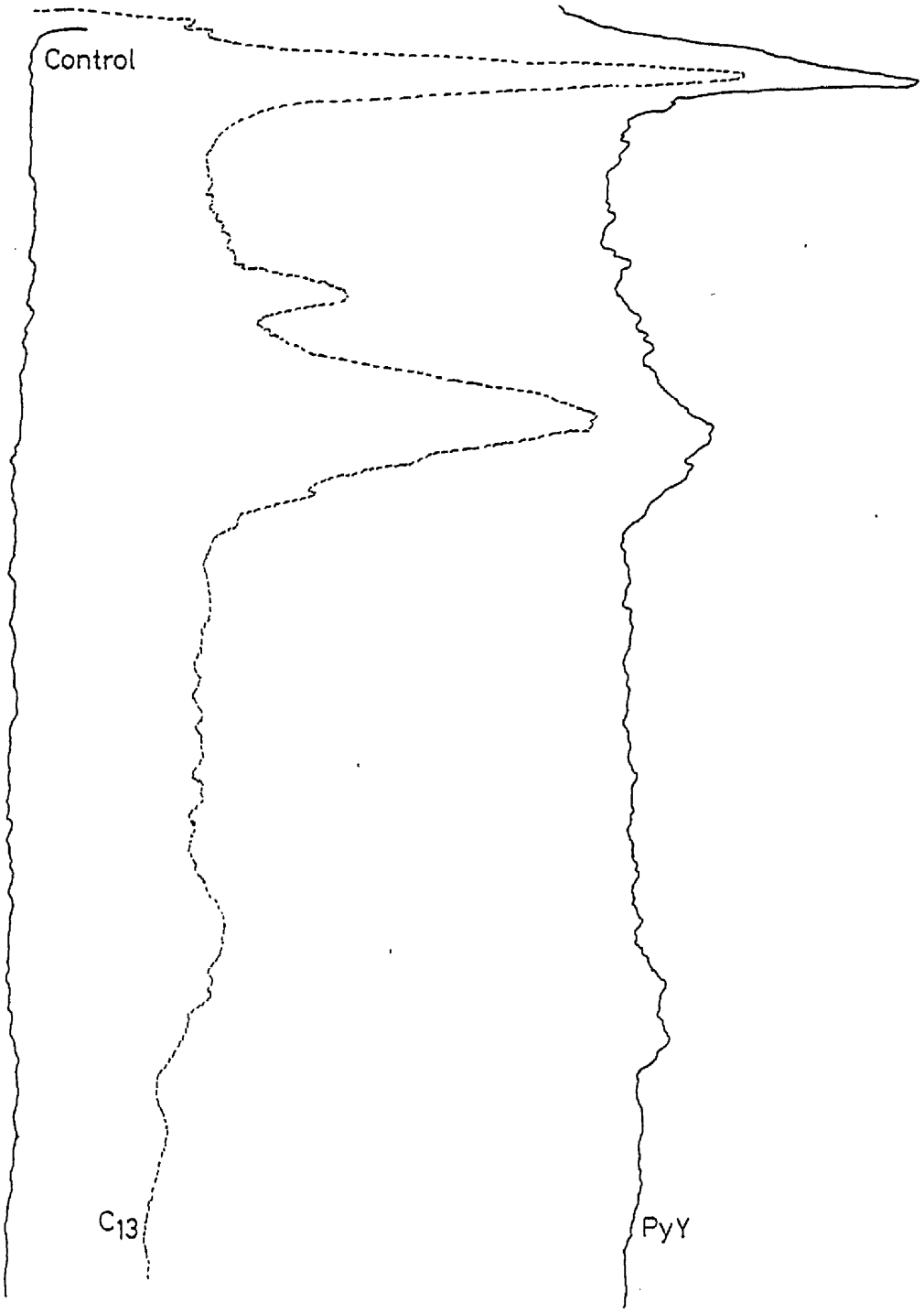


Fig. 20.d. L.L.

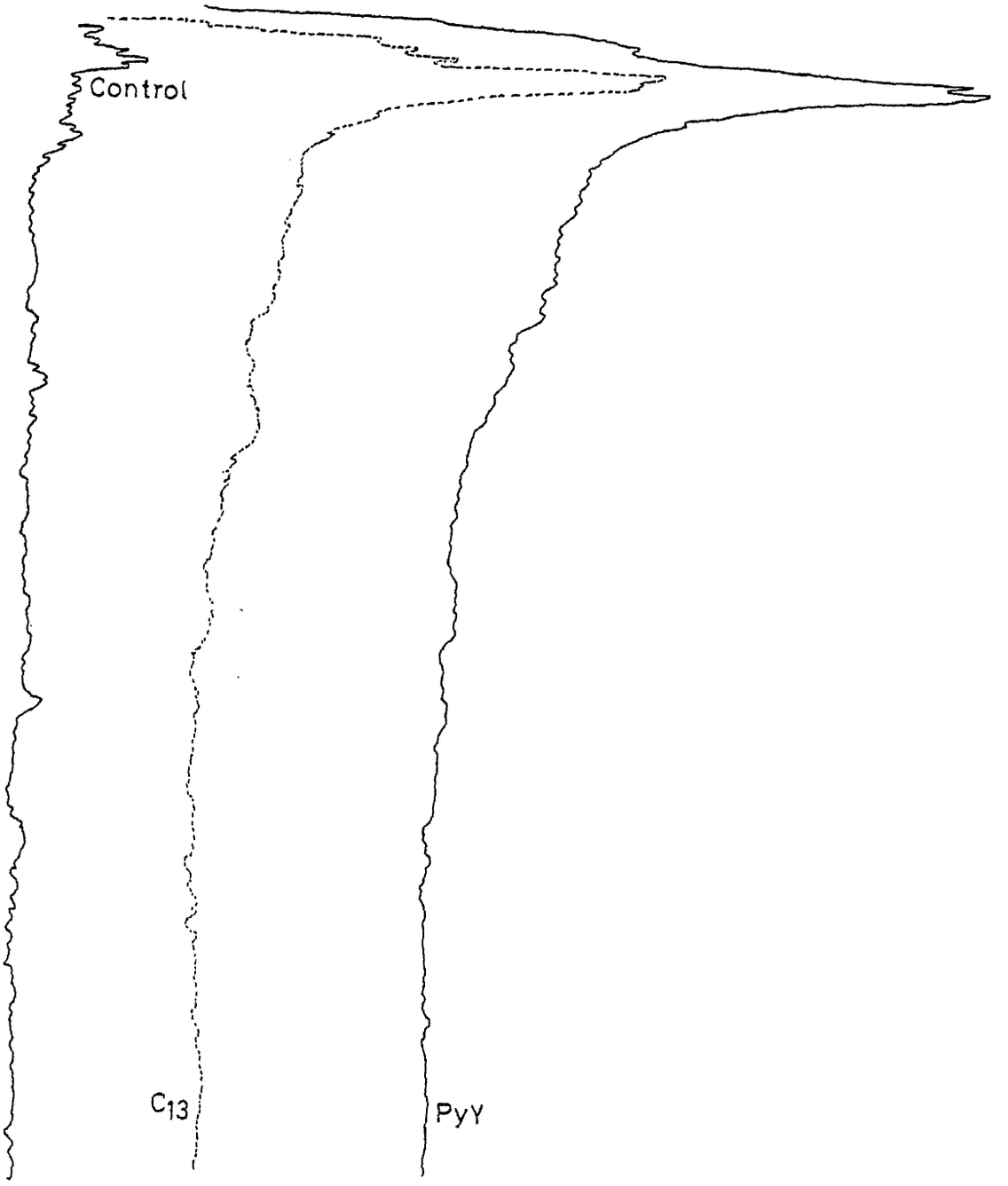


Fig. 21.

Glycoproteins of Rough Endoplasmic Reticulum Before and After Transformation

Rough endoplasmic reticulum was isolated from C_{13} or PyY cells as described in Section II.2.f. Portions ($\sim$ 20µg) were electrophoresed and overlaid with various [^{125}I-]lectins as described in Sections II.2.g. and II.2.i. Controls contained 20µg cell homogenate protein and were overlaid in the presence of specific monosaccharides (0.1M).

a) protein profile stained with Coomassie blue

b) ConA overlay

c) WGA overlay

d) Lentil lectin overlay

Fig. 21.a. Coomassie blue.

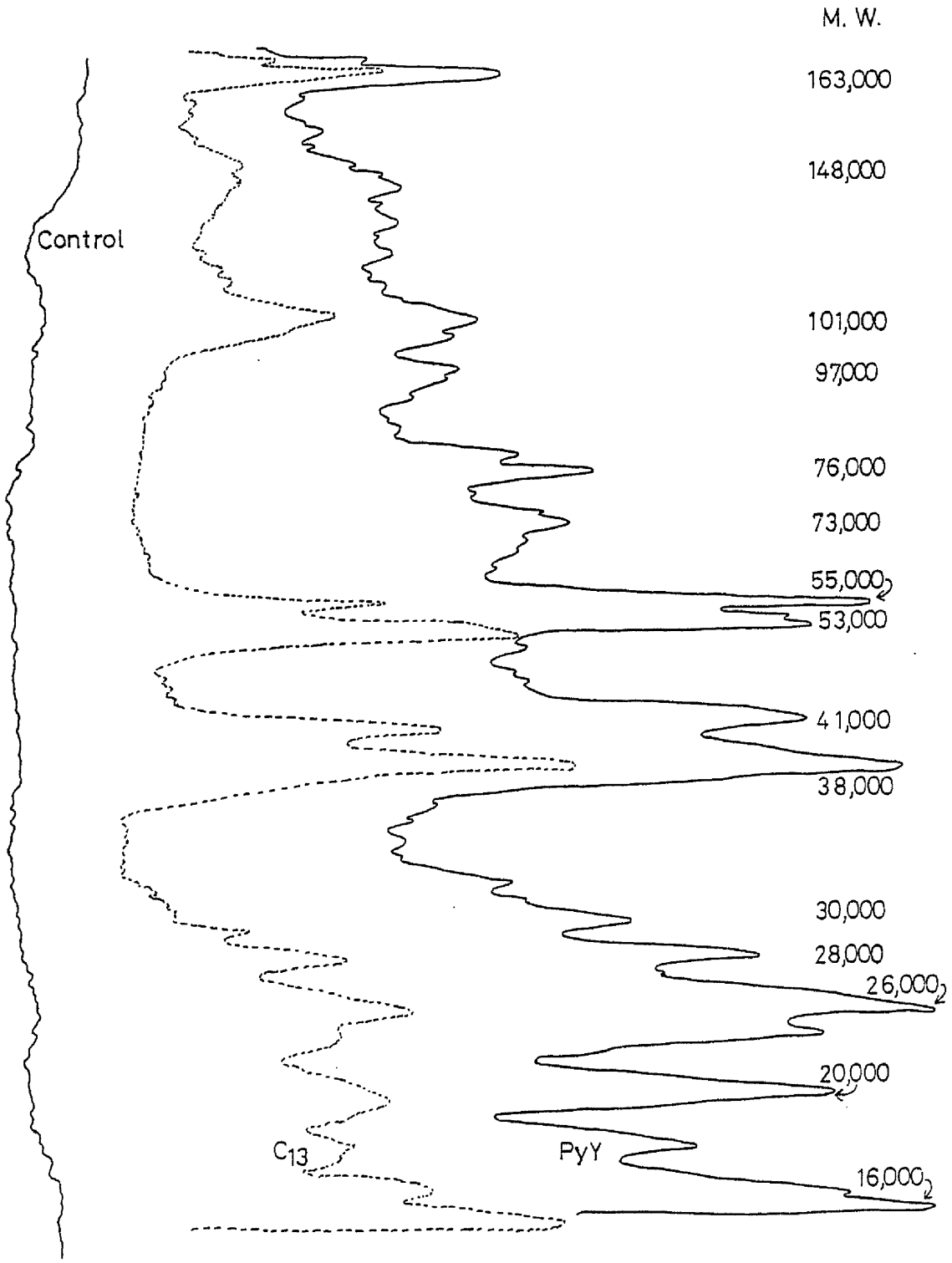


Fig. 21.b. Con A.

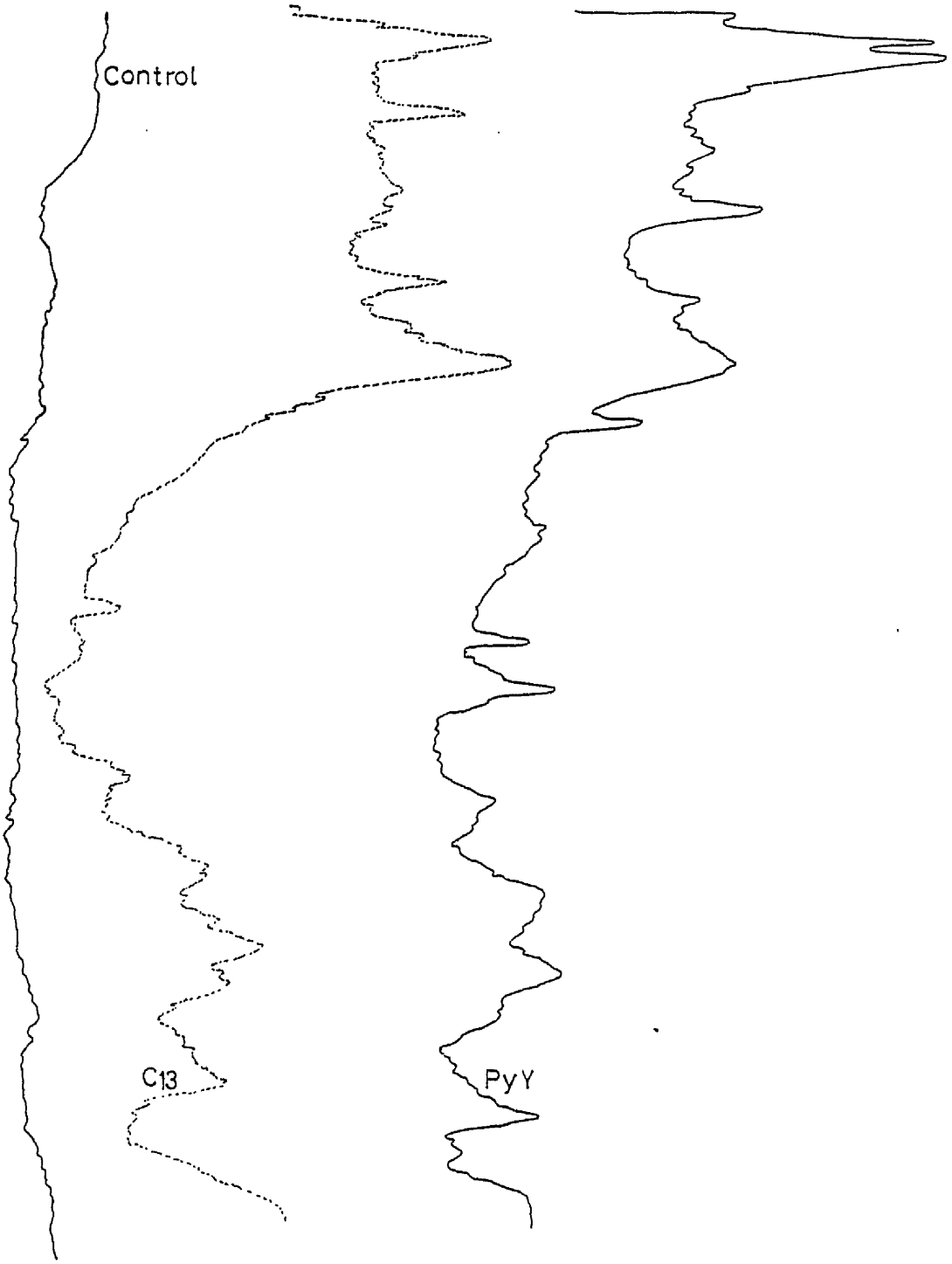


Fig. 21.c. W.G.A.

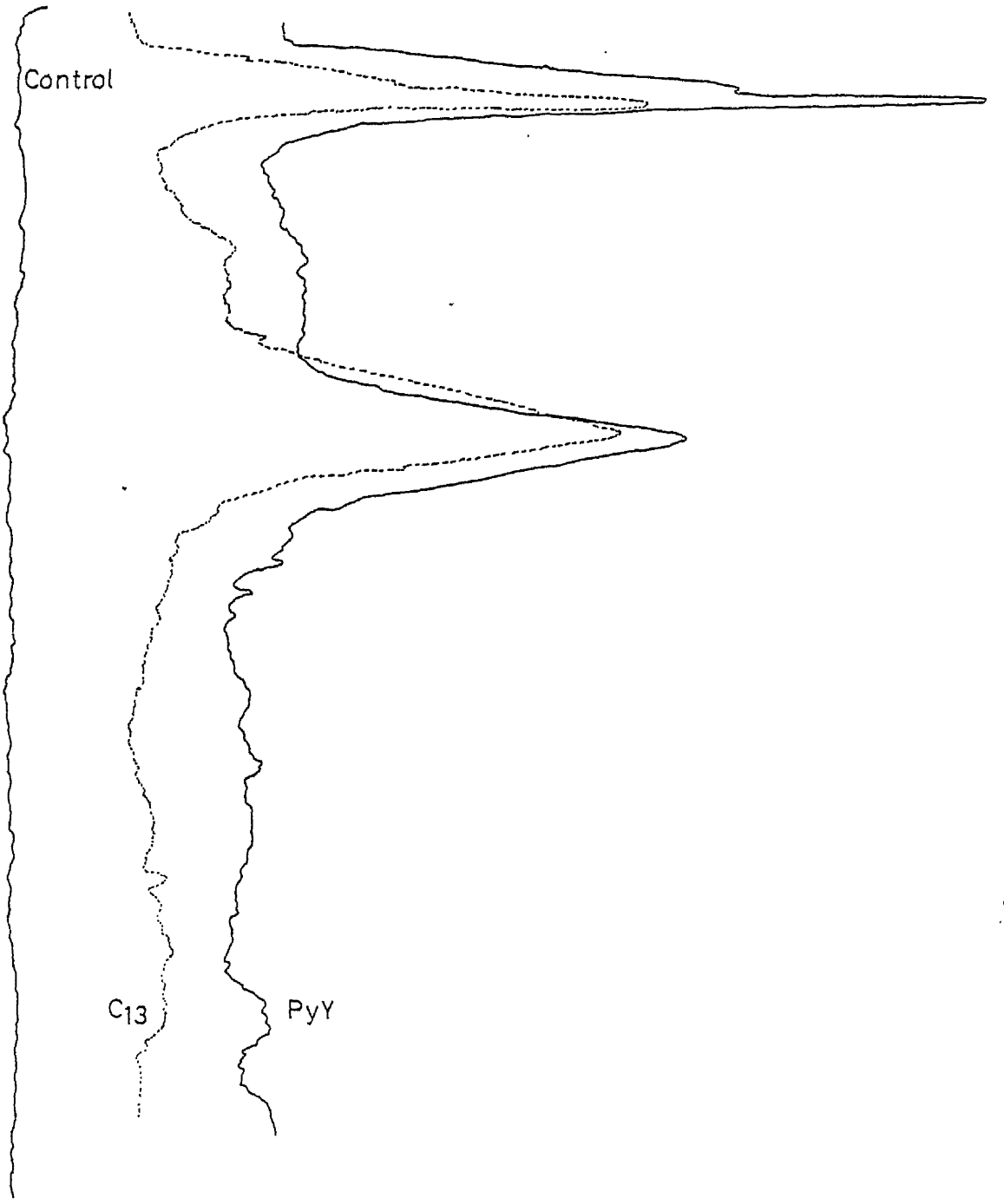
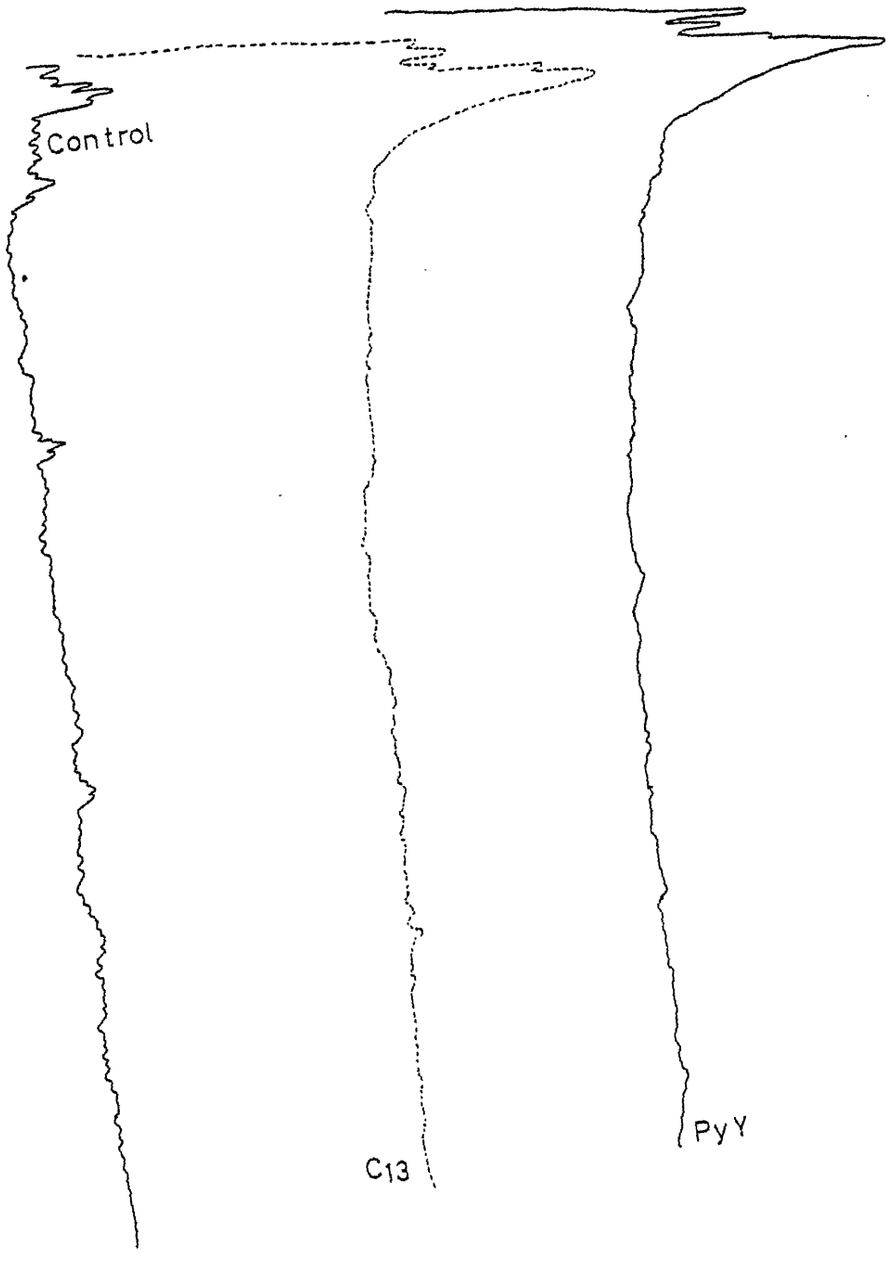


Fig. 21.d. L.L.



III.5. Topography of Nuclear Envelope Proteins

a) Extraction of Nuclei with Various Solvents

In addition to the fractionation of nuclei to produce nuclear envelope, lamina and membrane, whole nuclei were extracted with various solvents to investigate the type of interaction between the extracted proteins and the nuclear structure. The solvents used were Triton-X100 (2%, 1%, 0.1% w/v) to extract membrane proteins, deoxycholate ("DOC", sodium salt, 0.05%, w/v) as a comparison for the proteins extracted by Triton, and NaCl (1M) and EDTA (1mM), these last two being used to extract proteins attached to the structure by ionic interactions. The proteins extracted by Triton-X100 were precipitated with ethanol and electrophoresed in slab gels with the residual nuclear structures. Standard proteins were electrophoresed in the presence of small amounts of Triton-X100 to investigate its effect on their electrophoretic mobility in the event of incomplete removal of the detergent from the proteins. There was no difference in the mobility of the standard proteins. The quantity of protein removed from nuclei appeared to increase with the Triton concentration up to 1% (w/v) after which a further increase to 2% (w/v) did not appear to remove more protein. The profiles of the proteins extracted from C_{13} nuclei by the various concentrations of Triton-X100 were very similar, and consisted of proteins of 100,000, 95,000, 84,000, 75,000, 65,000, 61,000, 59,000, 54-53,000, 49-47,000, 43,000, 41,000, 27,000, 23,000, 21,000 and 19,000. Extraction with EDTA removed so little protein that the profile could not be resolved.

Nuclei were extracted with 2% (w/v) Triton-X100 in different concentrations of Ca^{2+} in conditions of the same ionic strength.

These profiles are very similar, indicating that calcium concentration does not influence the selection of proteins removed by 2% Triton-X100.

The effect of transformation on the extraction of proteins in various conditions was also examined. In the presence of 5mM and 1mM Ca^{2+} the profiles of protein extracted by 2% Triton-X100 from the two types of nuclei were very similar. However, in the presence of 1mM EDTA there was a marked difference in the proteins removed from the normal and transformed nuclei by this detergent. (See Fig. 23) A protein of apparent Mr 63,000 removed from C_{13} nuclei was not found in the extract from PyY nuclei. Instead a doublet Mr, 65,000 and 62,000, was removed.

The extraction procedure in which 2% Triton-X100 is used closely resembles the third stage of the isolation procedure for pore-complex-lamina (Section II.2.d) during which the nuclear membrane is removed. The profiles of protein removed from whole nuclei are compared with the membrane proteins in Fig. 24. Similarly, the 1MNaCl extraction resembles the last stage of the procedure for isolating lamina. No real comparison is possible between the proteins removed from whole and DNAase-treated nuclei in this case, because the proteins removed by 1MNaCl from whole nuclei were always difficult to visualise after electrophoresis e.g. Fig. 22, track K. Fig. 24 shows that there are differences between the proteins extracted from whole and DNAase treated nuclei by Triton-X100. The most marked difference occurs in the proteins having apparent Mr of 65,000 to 43,000.

Differences occur both in the relative proportions of the extracted proteins and to a lesser extent in the actual proteins

extracted. For example, there is a protein which has an Mr of 50,000 which is removed from DNAase treated nuclei, but which is not present in the extract from whole nuclei.

Fig. 22.

Profiles of Proteins extracted from C_{13} nuclei by Various Solvents

Nuclei (C_{13}) were suspended in 0.25M sucrose/5mM Tris-HCl, pH 7.4 and PMSF added to a final concentration 0.2mM. Separate aliquots of the nuclear suspension (each containing 4×10^7 nuclei) were incubated (10 min, 4°C) in the following solutions: 2%, 1% and 0.1% w/v Triton-X100, 0.05% (w/v) deoxycholate and 1M NaCl, all in a final volume of 1ml. Nuclei were then pelletted (2000 g.av. 10 min) and extracted proteins precipitated from the supernatant. Portions of the residual nuclei and all of the extracted proteins were electrophoresed as described in Section II.2.g.

A = whole nuclei; B = nuclei extracted with 2% Triton; C = 2% Triton extract; D = nuclei extracted with 1% Triton; E = 1% Triton extract; F = nuclei extracted with 0.1% Triton; G = 0.1% Triton extract; H = nuclei extracted with 0.05% DOC; I = 0.05% DOC extract; J = nuclei extracted with 1M NaCl; K = 1MNaCl extract.

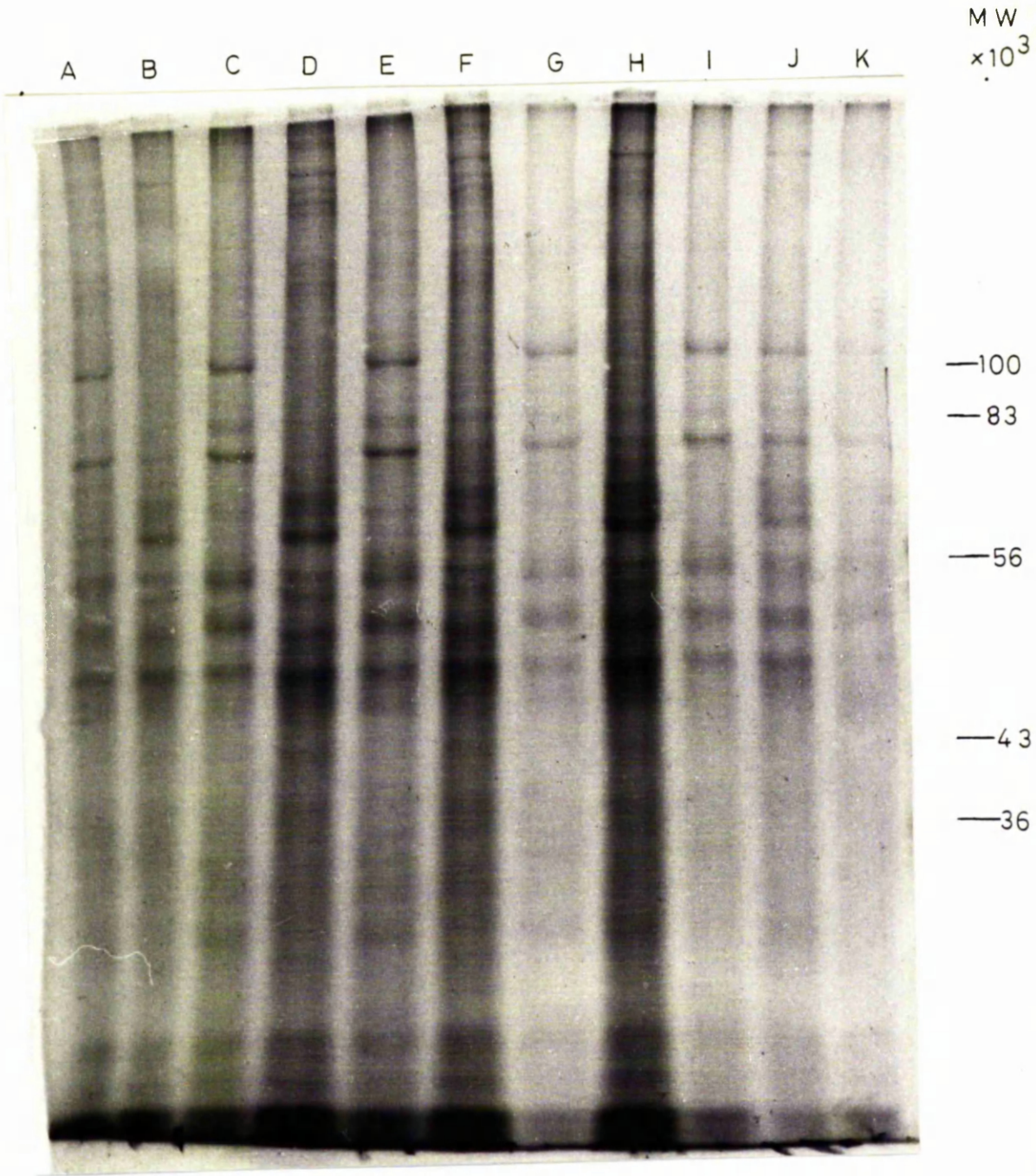

A
B
C
D
E
F
G
H
I
J
K
MW
×10³
100
83
56
43
36

Fig. 23.

Profiles of Proteins extracted by Triton-X100 from Nuclei of Normal and Transformed Cells

Purified C_{13} and PyY nuclei were suspended in 0.25M sucrose/5mM Tris-HCl, pH 7.4 containing 0.2mM PMSF, and either 1mM Ca^{2+} or 1mM EDTA. Triton-X100 was added to a final concentration of 2% (w/v) and the suspensions were incubated (10 min, 4°C). Nuclei were then pelletted and extracted protein precipitated from the supernatant. Extracted proteins and nuclei were electrophoresed as described in Section II.2.g.

A = C_{13} nuclei extracted by 2% Triton in 1mM Ca^{2+}

B = PyY nuclei extracted by 2% Triton in 1mM Ca^{2+}

C = C_{13} nuclei extracted by 2% Triton in 1mM EDTA

D = PyY nuclei extracted by 2% Triton in 1mM EDTA.

Fig. 23.

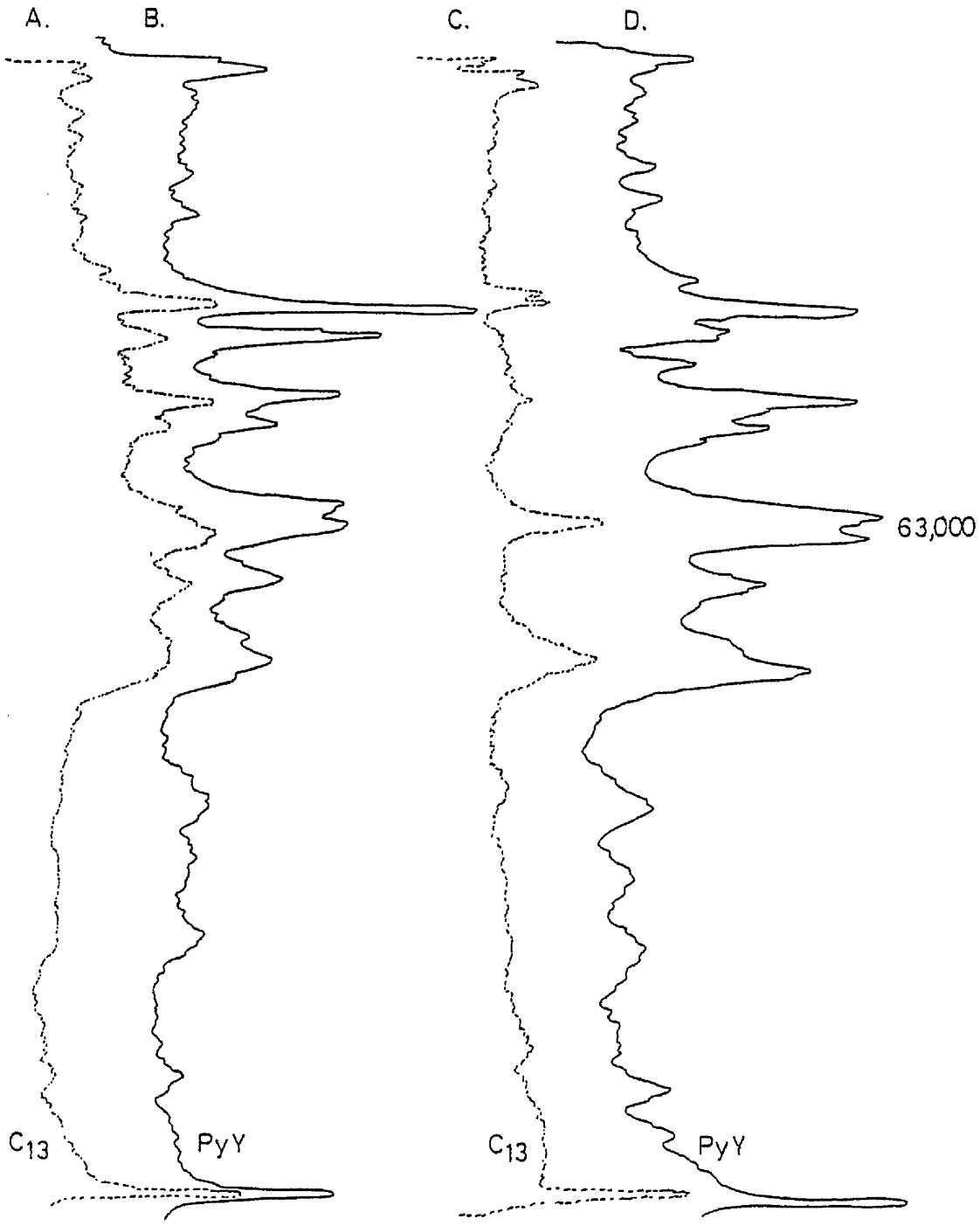


Fig. 24.

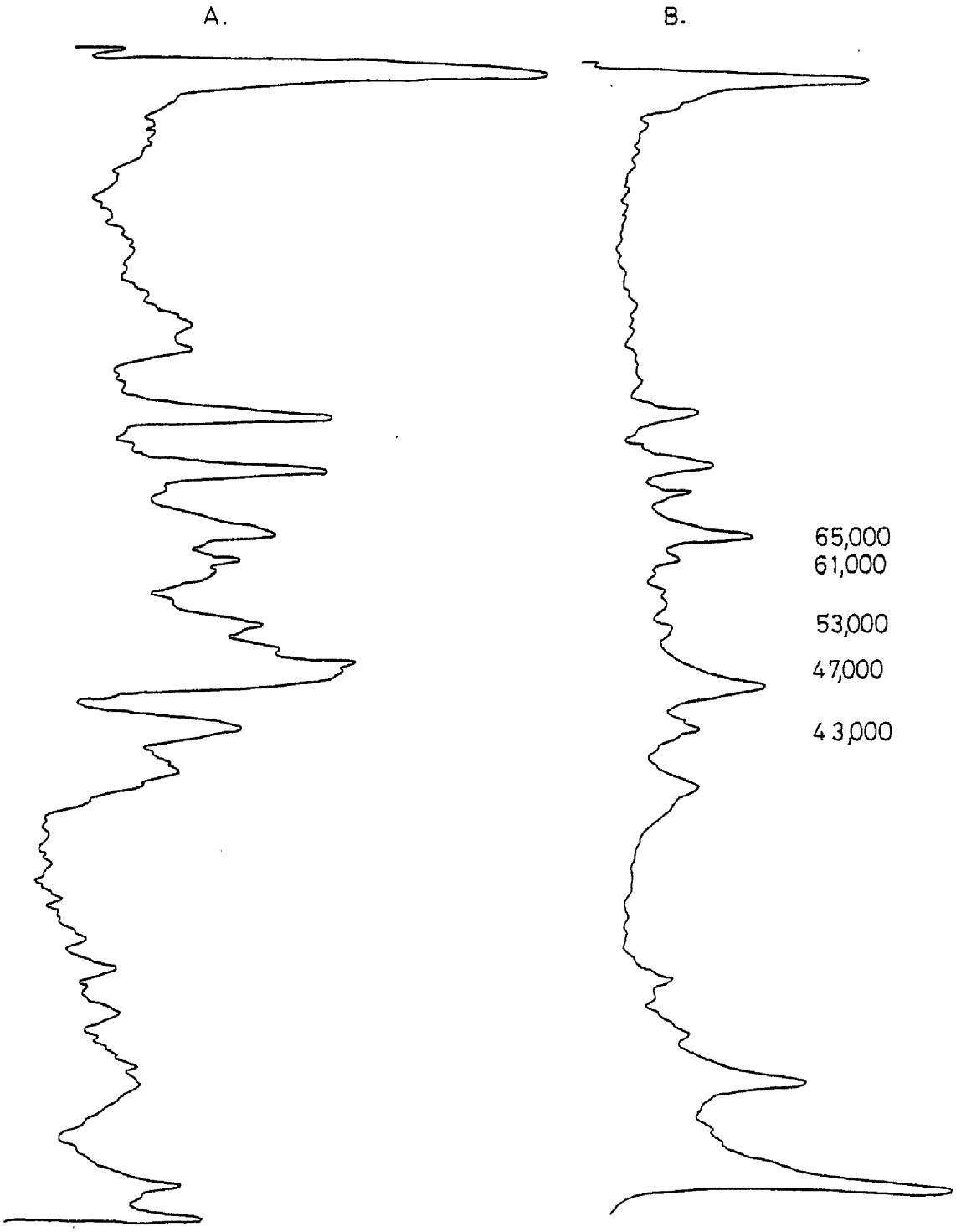
Proteins removed from whole and DNAase-treated nuclei by Triton-X100

Purified C_{13} nuclei were extracted twice with DNAase I exactly as described in Section II.2.d. They were then resuspended in 10mM triethanolamine-HCl, pH 7.5 containing 0.1mM $MgCl_2$, 10% (w/w) sucrose and 2% (w/v) Triton-X100, and incubated (10 min, 4°C). The nuclei were then pelletted and extracted proteins were pelletted from the supernatant, electrophoresed with proteins similarly extracted from whole nuclei.

A = proteins extracted from DNAase treated C_{13} nuclei

B = proteins extracted from whole nuclei.

Fig. 24.



III.5.b. Exposure of Proteins on the Nuclear Surface

The topography of the nuclear envelope was investigated by subjecting nuclei to iodination. Two types of iodinating system were employed in order to compare the range of proteins accessible to particles of different sizes. Soluble lactoperoxidase was used with either hydrogen peroxide or soluble glucose oxidase and glucose to initiate the reaction. Alternatively, lactoperoxidase and glucose oxidase immobilised on polyacrylamide beads ("Enzymobeads") were used. Lactoperoxidase is a large protein which cannot penetrate membranes but will probably penetrate the pore complex to reach the interior of the nucleus. This is to be expected because immunoglobulins can penetrate the nucleus as shown by Ely et al.(1978). Feldherr(1965) demonstrated that only particles with a diameter of less than 85Å could pass through the pore complex. Therefore it is probably safe to assume that "Enzymobeads", having a diameter of several μm , cannot penetrate the pore structure although they may enter its outer regions to some extent.

The proteins of the lamina were also investigated in this way by comparative iodinations of nuclei after extraction with Triton-X100. The dimensions of the lamina network are not known, so the extent of penetration by "Enzymobeads" is uncertain in this case. The iodination profiles produced by the two systems were compared in order to clarify this.

In the preliminary experiments "Enzymobeads" produced a higher percentage incorporation than did soluble lactoperoxidase and hydrogen peroxide, i.e. 3-5% against 1-2% with the soluble enzyme. As it was thought that the use of H_2O_2 to initiate the lactoperoxidase reaction was responsible for the low effeciency of the soluble enzyme, glucose oxidase and glucose were used instead. In this

case the percentage incorporation of ^{125}I produced by the soluble system rose to 29%, whilst that of the "Enzymobeads" increased to 17%, probably because of more efficient agitation. A control iodination in which the quantity of Enzymobeads was doubled produced a percentage incorporation of 35%. The iodination profile was not changed, however. In the absence of any enzymes, the percentage ^{125}I incorporation was only 0.009%.

In Fig. 25 the iodination profiles of whole nuclei produced by beads and lactoperoxidase are compared. Soluble lactoperoxidase iodinates proteins of apparent Mr 160,000, 150,000, 138,000, 80,000, 75,000, 72,000, 67,000, 63,000, 61,000, 57-56,000 and 38,000 together with many proteins of low molecular weight. "Enzymobeads" iodinated proteins of apparent Mr 63,000, 52,000, 26,000 and 24,000 and therefore have limited access to the nuclear structure. The protein of Mr 52,000 is very heavily iodinated. It may therefore be a prominent protein of the pore complex, or a prolific protein of the nuclear membrane.

After extraction with Triton-X100 lactoperoxidase iodinates proteins of apparent Mr 150,00, 138,000, 98.000, 90,000, 73,000, 69,000, 65,000, 63,000, 50,000, 48,000, 38,000, 35,000 and 23,000. The extra proteins iodinated by soluble lactoperoxidase after Triton extraction were those of Mr 98,000, 90,000, 65,000, 50,000, 48,000, 35,000 and 23,000. These proteins may lie towards the cytoplasmic surface of the lamina. Possibly, during the iodination of whole nuclei, they were shielded by the nuclear membranes on one side and the thickness of the lamina on the other.

After Triton extraction "Enzymobeads" also iodinated a wider range of proteins which had Mr of 74,000, 69,000, 63.000, 61,000, 52,000, 48,000, 36,000, 33,000, 29,000, 26,000,

25,000 and 22,000. The protein of Mr 63,000 is very heavily iodinated in this profile, whereas the protein of Mr 52,000 is not so heavily iodinated as before. This may indicate that the protein of Mr 63,000 is very prominent or prolific on the surface of the lamina. This protein corresponds in molecular weight to the smallest of the lamina triplet. The protein of Mr 52,000 may be a nuclear membrane protein extracted, at least partially, by Triton. The resistance of the pore complex to Triton extraction (Aaronsen and Blobel, 1975) argues against this protein existing in the structure.

This experiment has been repeated several times and has consistently given similar profiles. However, as the resolution of the earlier results was rather poor, these data must be regarded as preliminary. Any conclusions from this experiment must be drawn with reservation until such time as the work is repeated.

Fig. 25.

Profiles of Proteins iodinated in Whole and Triton-extracted Nuclei by Soluble and Immobilised Lactoperoxidase

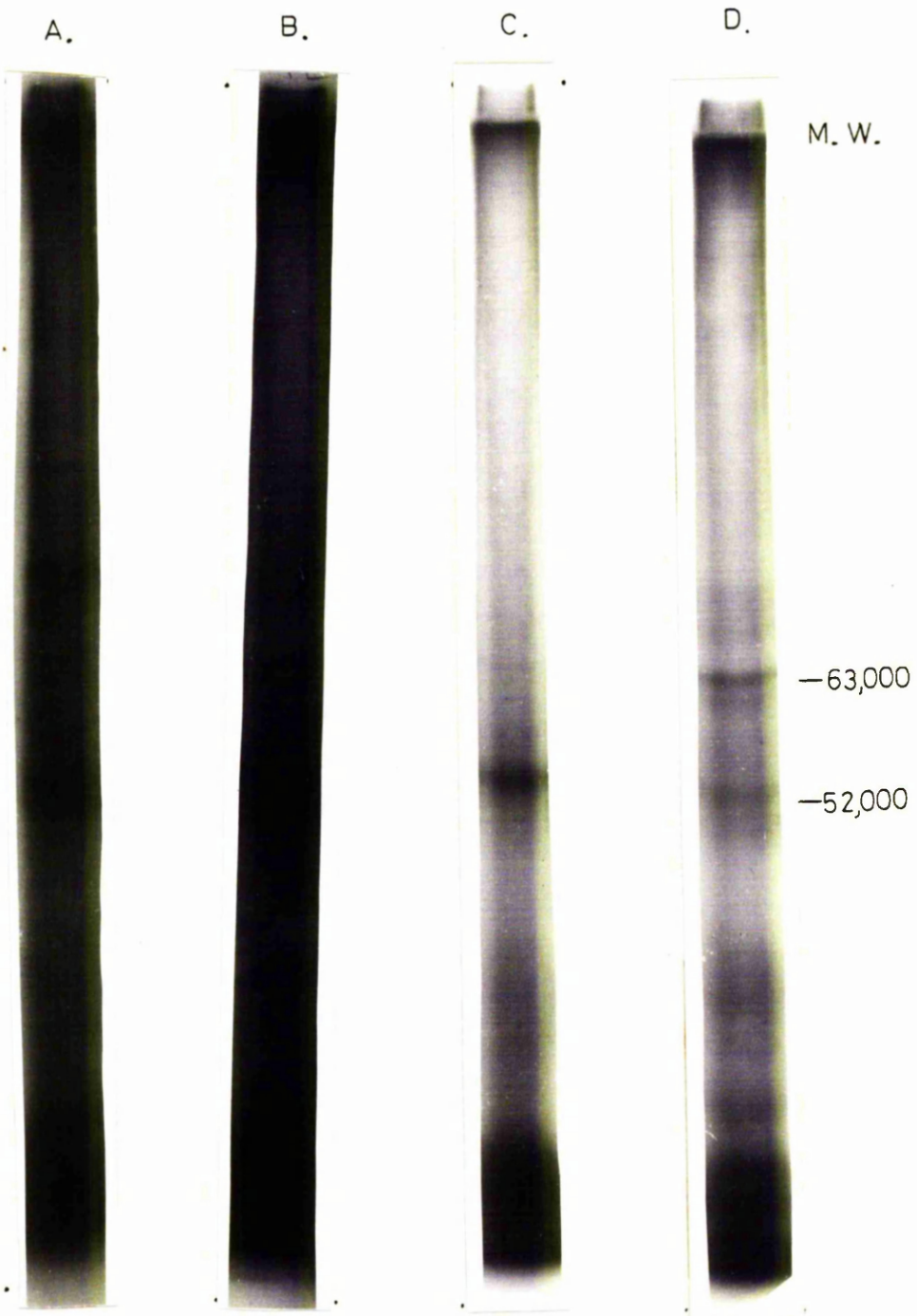
Freshly purified C_{13} nuclei were suspended in 0.25M sucrose/5mM Tris-HCl pH 7.4 containing 0.2mM PMSF ($\sim 3 \times 10^7$ nuc/ml). Triton-X100 was added to a final concentration of 2% (w/v) and the nuclei were incubated (10 min, 4°C) and pelletted. These nuclei, and others purified in the same batch but not extracted with Triton, were then iodinated with soluble or immobilised lactoperoxidase as described in Section II.2.k. They were then electrophoresed as described in Section II.2.g. and the iodination profile resolved by autoradiography.

A = whole nuclei iodinated with soluble lactoperoxidase

B = Triton extracted nuclei iodinated as in A

C = Whole nuclei iodinated with immobilised lactoperoxidase

D = Triton extracted nuclei iodinated as in C.


A.
B.
C.
D.
M. W.
—63,000
—52,000

III.6. Effect of Calcium Concentration upon Nuclei and Nuclear Envelope

Calcium, and to a lesser extent, magnesium, change the volume of nuclei, as originally noted by Anderson and Wilber (1951b) in rat hepatocyte nuclei. Such changes are found in BHK-21 cell nuclei as shown in Fig. 26 and Table 5. Nuclei were incubated with either 5mM $CaCl_2$ or 1mM EDTA at $4^{o}C$. The treatment was continued for 30 minutes, although the gross volume changes were complete within five minues. The ionic strengths of the solutions were equalised with KCl. This experiment showed that divalent cations would significantly reduce the nuclear volume. Conversely, the absence of divalent cations caused nuclei to expand, and this was readily reversible as seen in Fig. 26, C and D. Calcium had a greater effect than magnesium in these experiments. Similar changes in nuclear size were observed after extraction with 2% Triton-X100, showing that they are not dependent upon the presence of a membrane.

The presence of calcium also caused a marked increase in the density of nuclear envelope fragments as seen in Fig. 27. Conversely, EDTA caused a reduction in the densities of nuclear envelope (Fig. 27).

The effect of calcium concentration upon the topography of the nuclear lamina was investigated. Nuclei were extracted with 2% Triton-X100 and exposed to iodination by soluble lactoperoxidase and by "Enzymobeads" in both the expanded and contracted states, which were induced by adjustment of the calcium concentration. The results, shown in Fig. 28 indicate that in contracted nuclei proteins of Mr 95,000, 87,000, 83,000, 74,000, 65,000, 58,000, 52,000, 48,000, 44,000, 39,000, 36,000, 34,000, 30,000, 28,000 and 24,000 were iodinated by both agents. Two proteins of Mr 71,000

and 35,000 were iodinated in addition by soluble lactoperoxidase.

In expanded nuclei the range of proteins iodinated by the two agents is also very similar. The iodinated profile closely resembles that of contracted nuclei but includes in addition proteins of apparent Mr 194,000, 180,000, 79,000, 69,000, 50,000 and 32,000. Other proteins of Mr 70,000 and 45,000 are only iodinated by lactoperoxidase in expanded nuclei. The protein of Mr 39,000 is much more heavily iodinated by the soluble enzyme than by its immobilised counterpart. Conversely, the protein of Mr 29,000 is much less heavily iodinated by the soluble enzyme.

In the previous section the iodination of whole and Triton extracted nuclei by soluble and immobilised lactoperoxidase was discussed. The nuclei in those experiments were in a state intermediate between the expanded and contracted state described here. In these experiments all the nuclei had been extracted with Triton, in order to examine the topography of the lamina. The iodination profile of expanded nuclei resembles that of the Triton-extracted nuclei described in the previous section more than does that of contracted nuclei. It contains a larger range of proteins however, which may indicate a structural dislocation of the fully expanded state.

Fig. 26.

Changes in Nuclear Volume in Response to the Concentration of Divalent Cations

Freshly purified nuclei (C_{13}) were incubated (30 min, 4°C) either in 0.25M sucrose/5mM Tris-HCl, 5mM $CaCl_2$, pH 7.4 or in 0.25M sucrose/5mM Tris-HCl, 1mM EDTA, 11mM KCl, pH 7.4. After photography, nuclei previously incubated in EDTA were transferred to 0.25M sucrose/5mM Tris-HCl, pH 7.4 containing either 5mM $CaCl_2$ or 5mM $MgCl_2$ and photographed as described in Section II.2.n.

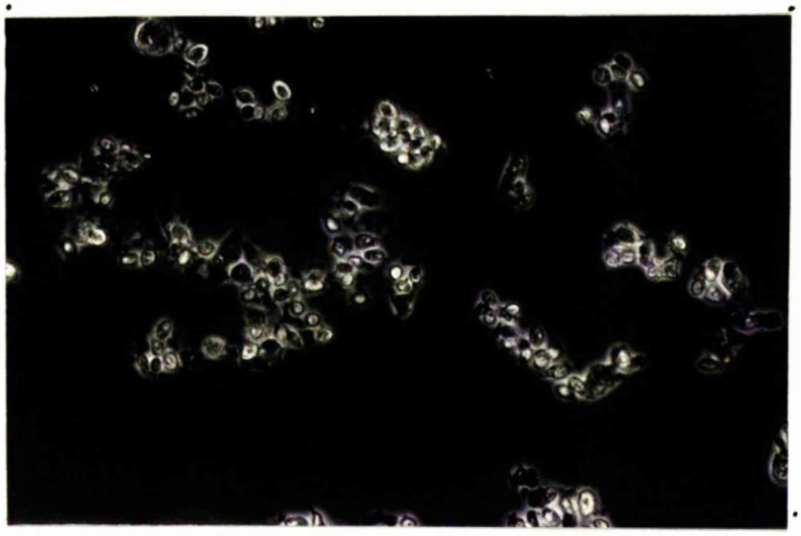
A = nuclei incubated in 5mM Ca^{2+}

B = nuclei incubated in 1mM EDTA

C = nuclei transferred from 1mM EDTA to 5mM Ca^{2+}

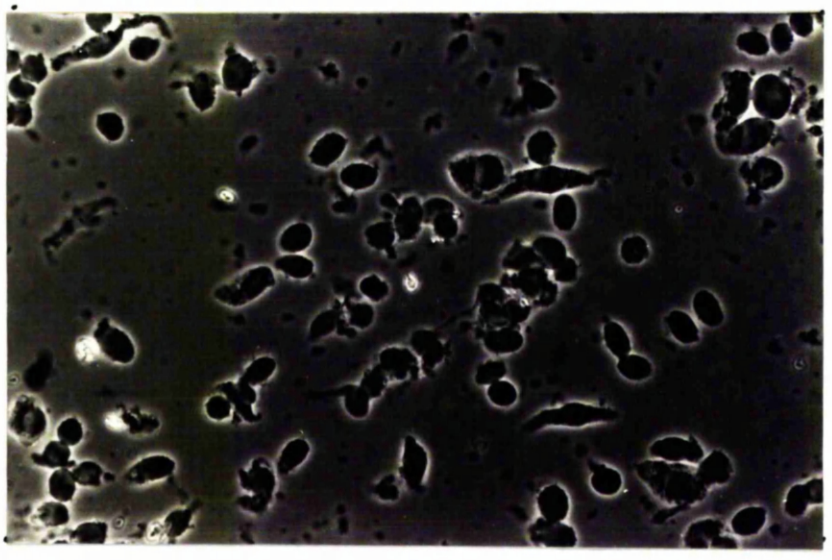
D = nuclei transferred from 1mM EDTA to 5mM Mg^{2+}.

A



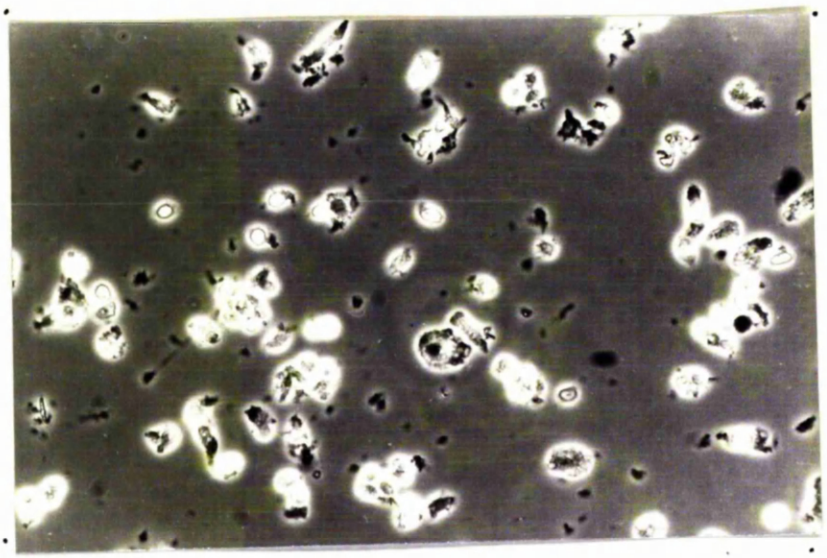
×305

B



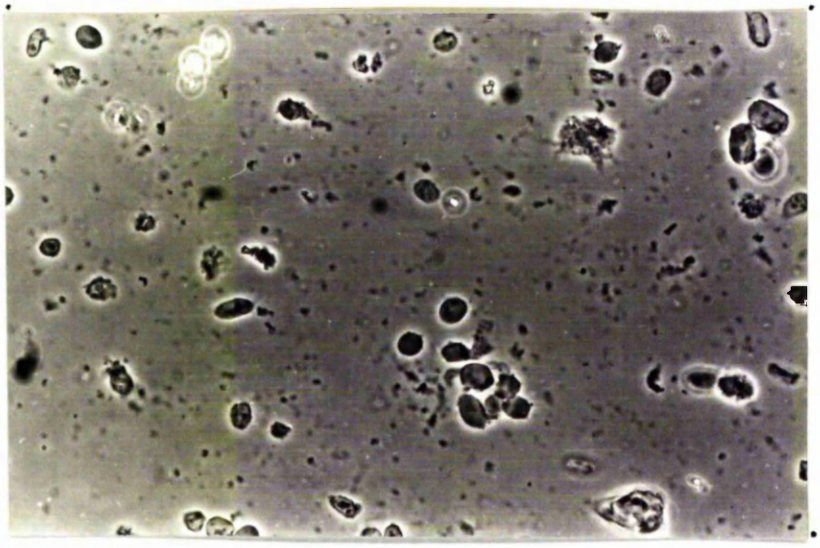
×305

C



×305

D



×305

Table 5

Changes in Nuclear Dimensions with Different Concentrations of Divalent Cations

Nuclei prepared and incubated as described in Fig. 32 were photographed as described in Section II.2.n. Dimensions were measured from prints, taking the average of three diameters of 50 nuclei in each case. Calculations were based on the assumption that nuclei are spherical. Standard deviations are shown in parentheses.

Treatment	Diameter (μm)	Volume (μm^3)	Surface Area (μm^2)
5mM Ca^{2+}	8.6 ($\pm$0.4)	338.8	235.0
1mM EDTA	13.7 ($\pm$0.6)	1349.3	590.0
1mM EDTA to 5mM Ca^{2+}	5.9 ($\pm$0.2)	105.4	107.9
1mM EDTA to 5mM Mg^{2+}	10.7 ($\pm$0.6)	636.0	358.2

Fig. 27.

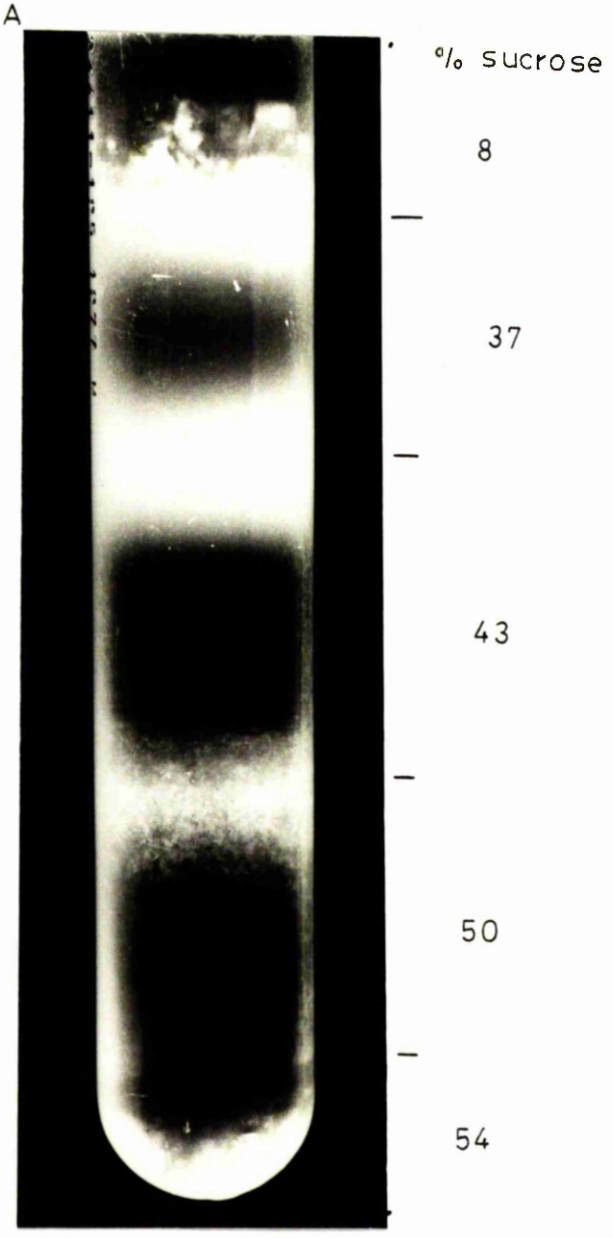
Effect of Calcium Concentration on the Density of Purified Nuclear Envelope

Nuclear envelope was prepared from C_{13} nuclei as described in Section II.2.c. in the presence of various concentrations of Ca^{2+}. Envelope fragments were then purified on sucrose gradients as described in Section II.2.c.

A = envelope prepared in the presence of 1mM EDTA

B = envelope prepared in the presence of 5mM Ca^{2+}.

A



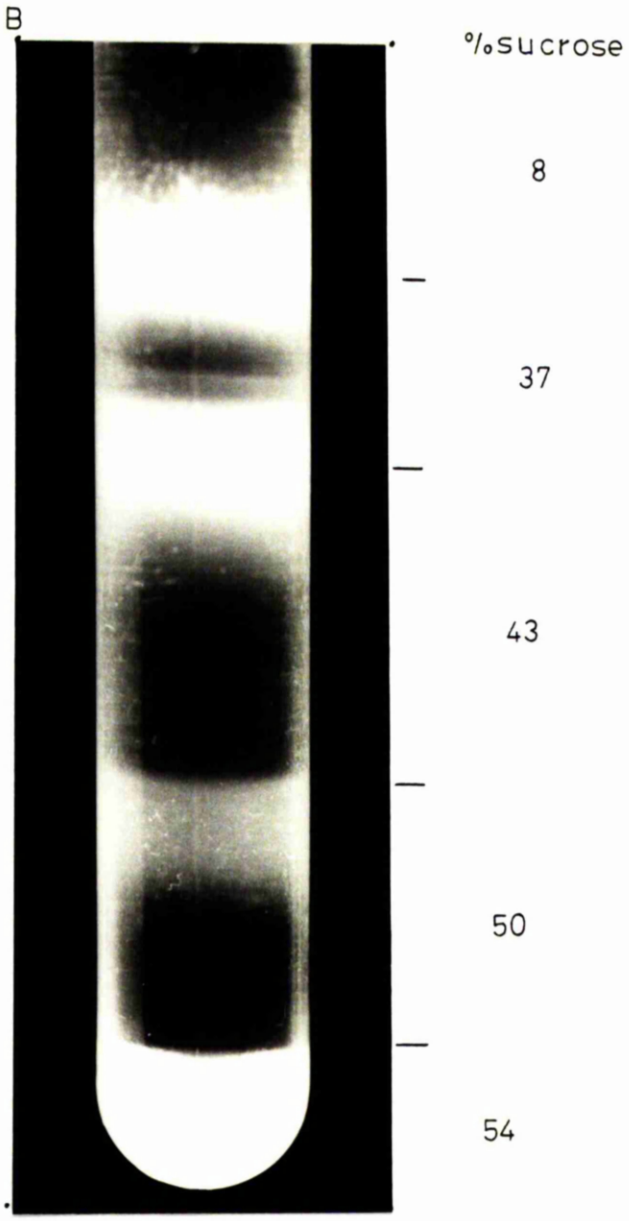

B
%sucrose
8
37
43
50
54

Fig. 28.

Iodination Profiles of Expanded and Contracted Nuclei produced by Soluble and Immobilised Lactoperoxidase

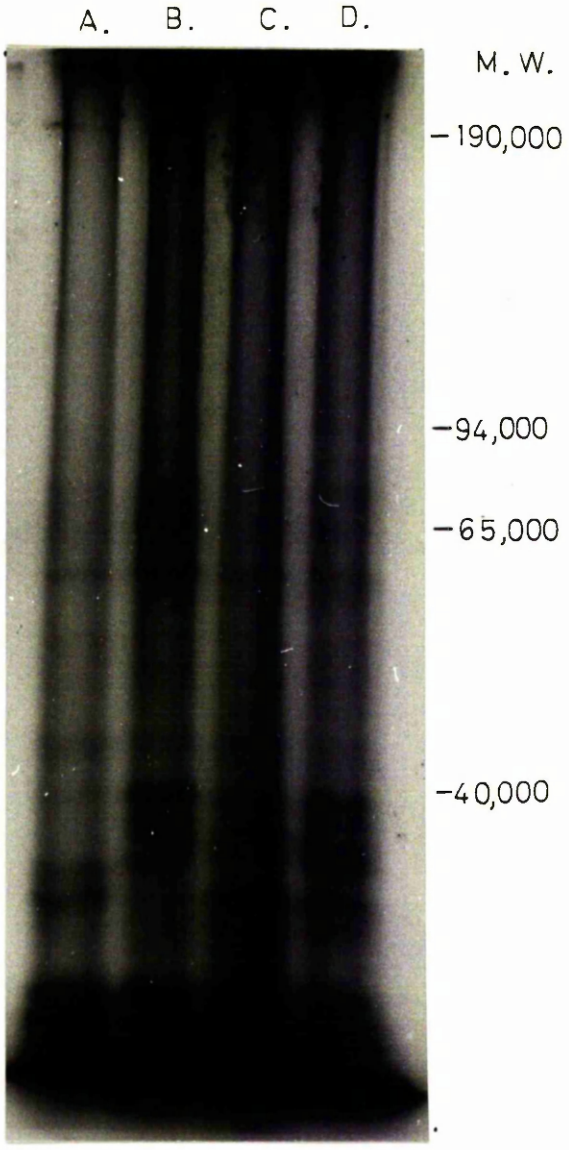
Freshly purified C_{13} nuclei were extracted with Triton-X100 and pelletted as described previously. They were then resuspended either in 0.25M sucrose/5mM Tris-HCl, 5mM $CaCl_2$, pH 7.4 or 0.25M sucrose/5mM Tris-HCl, 1mM EDTA 11mM KCl pH 7.4, and washed 3 times in these solutions. The nuclei were then iodinated with either soluble or immobilised lactoperoxidase as described in Section II.2.k. and electrophoresed on standard slab gels as described in Section II.2.g. The iodinated proteins were then identified by autoradiography.

A = Expanded nuclei iodinated with Enzymobeads

B = Expanded nuclei iodinated with soluble lactoperoxidase

C = Contracted nuclei iodinated with Enzymobeads

D = Contracted nuclei iodinated with soluble lactoperoxidase


A.
B.
C.
D.
M. W.
190,000
94,000
65,000
40,000

DISCUSSION

IV. 1. The Preparations

a. Isolation of Nuclei

Nuclei were originally isolated by the method of Blobel and Potter (1966). Some modification of this method was necessary for its application to BHK cells. Eventually a major change was made to the procedure such that the homogenisation stage occurred in low ionic strength hypotonic conditions. Of all the modifications this was found to be the critical factor in the subsequent condition of the nuclei. The presence of either Ca^{2+} or Mg^{2+} during the lytic stage resulted in a much less satisfactory preparation in which the nuclei carried attached endoplasmic reticulum and mitochondria. Recently Porter (1980) observed a cytoplasmic lattice of micro-trabeculae, between the microtubules and 10nm filaments, using stereo electron microscopy. When cells were incubated with 5mM Ca^{2+} for 10 minutes at 4°C the structure of this lattice became very thick and firmly attached to the other cytoplasmic organelles (Porter, personal communication). Since the homogenisation of the original procedure was preceded by an incubation in which the cells were subjected to precisely these conditions, it is possible a similar thickening and attachment of the cytoskeleton was occurring. The relative success of the modified procedure for the preparation of nuclei may be attributable to this factor alone.

IV.1.b. Isolation of Nuclear Envelope

When preparing a complex structure such as the nuclear envelope, the concept of chemical purity is meaningless. The isolation procedure must be designed to provide the optimum preparation for the investigation in hand, because the conditions that preserve one aspect of the structure may, of necessity, destroy

another. This project required a preparation containing membrane, pore-complex-lamina and possibly some matrix in a physical condition as close as possible to that of the in vivo situation. Enzyme activities were not under investigation so disruption of the nuclei by freezing and thawing was acceptable. The presence of membrane components was imperative, therefore the use of detergents was precluded.

Electron microscopy of the final nuclear envelope preparation reveals that the double nuclear membrane is present, bearing ribosomes and occasionally connected by pore complexes. There is also a large quantity of amorphous material which may be the lamina or matrix. This is in agreement with the morphological requirements for nuclear envelope listed by Harris and Aguilter (1976), although the proportion of pores to other components is rather low. Harris listed various conditions which are liable to destroy pore complexes, e.g. high K^+ concentration, but these conditions were not present during the procedures. Harris also provided a summary of the chemical compositions of nuclear envelope reported by various workers. Most contained similar quantities of DNA and RNA to those found here, but have substantially higher phospholipid contents, and rather less protein. This may indicate that the BHK nuclear envelope prepared in this project contained more intranuclear protein that did the preparations reviewed by Harris. There are some proteins of low molecular weight which may be histones in the profile of BHK nuclear envelope, however these are not a major contaminant because the profile is dominated by proteins of Mr 47,000-60,000. The values reviewed by Harris are, with one exception, obtained from liver nuclei so that genetic and environmental differences may also account for the discrepancy.

The method of nuclear envelope preparation is based on that of Bornens (1973, 1977) in which heparin is used to solubilise the chromatin. This worker reports that incubating nuclei with heparin in the presence of PO_4^{2-} and Na^+ results in complete solubilisation of chromatin, possibly by removal of the histones. The presence of Mg^{2+} impairs this action causing the formation of "spherules" on the inner nuclear membrane. Such spherules were also reported by Cook and Aikawa (1973) who suggested that they were histone-heparin complexes surrounding chromatin strands attached to the nuclear envelope. During the development of the nuclear envelope preparation it was found that both Mg^{2+} and Ca^{2+} caused an increase in the density of the nuclear envelope with a concomitant increase in the histone content. It seems likely that both divalent cations are capable of interfering with the action of heparin. As heparin is capable of chelating Ca^{2+} (Harris, 1976) it is probable that it will also chelate Mg^{2+} and that these cations interfere with the dissociation of chromatin from the nuclear structure (Bornens and Courvalin, 1977). This effect is reversed if divalent cations are removed by EDTA. The effect of Ca^{2+} in increasing the density of nuclear envelope is therefore separate from its action in increasing the density of whole nuclei by reducing their volume.

Many workers report the use of a sucrose gradient to purify nuclear envelope. The densities to which BHK nuclear envelope migrates correspond to those reported by Kashnig and Kasper (1969) for rat liver nuclear envelope. Endoplasmic reticulum on this gradient banded at a much lower density, possibly due to the absence of an associated lamina. Gel electrophoresis of the preparations from the two nuclear envelope bands gave almost identical profiles. It was therefore considered valid to pool these fractions. Electron

microscopy showed some difference in the size distribution of the membrane vesicles and this may account for the heterogeneity in the densities of the envelope fragments. Kashnig and Kasper do report some difference in the chemical composition of the two hands. However, they suggested that the heterogeneity may relate to a variation in the structural integrity of the nuclear ghosts.

In spite of early suggestions to the contrary (Gurr et al., 1963; Smith et al., 1969; Virtanen et al., 1977) there is no satisfactory method of separating the two nuclear membranes. Triton-X100 has been reported to remove only the outer nuclear membrane (Sadowski and Howden, 1968). This seems unlikely when at least 95% of the phospholipids can be removed by this detergent, leaving the lipid-free lamina (Richardson and Maddy, 1980a; Dwyer and Blobel, 1976).

In biochemical isolations proteolytic degradations should be prevented if possible. During the early preparations of nuclear envelope, protein profiles revealed by Coomassie blue were often inconsistent. The use of PMSF during both nuclear and nuclear envelope isolation did not improve this, and the inconsistencies disappeared after the methods of gel and sample preparation for electrophoresis were standardised. Reproducible protein profiles were obtained which did not alter when PMSF was omitted from the procedures. However the lectin overlay methods employed subsequently, which are capable of detecting much smaller amounts of protein, may have revealed a small degree of proteolytic activity at work during the nuclear envelope preparation. The addition of an inhibitor specific for proteases other than serine proteases may have prevented this.

IV.2. The Fractions

a. Pore-complex Lamina

As discussed in the introduction the preparation procedure for pore-complex lamina is very similar to that for the matrix devised by Berezney (1974, 1977, 1980) so that a distinction between these fractions is hard to make. The procedure used here was designed for hepatocyte nuclei but appears to be directly applicable to BHK nuclei because the protein profile of the resulting preparation closely resembles that of hepatocyte pore-complex-lamina (Dwyer and Blobel, 1976). One major difference between the preparations, however, is that BHK pore-complex lamina appears to contain far fewer pore-complexes than does the hepatocyte preparation. This feature was also noted in the BHK nuclear envelope compared to that of liver. Stevens and André (1969) noted that the frequency of pore complexes in a nucleus was approximately proportional to its metabolic activity. The frequency of pore complexes in these preparations may reflect the activity of BHK nuclei as compared to liver nuclei. Although BHK nuclei divide more often than liver nuclei, the liver cell, being secretory, may require more transcriptive activity of its nucleus than the BHK cell. The preparation procedure obviously did not destroy the pore complexes of the hepatocyte nuclei. It is unlikely that BHK nuclear pores should differ so much from those of rat liver as to be extensively destroyed by the same procedure. It is therefore possible that the differences do reflect nuclear metabolic activity.

Although considerable advances have recently been made towards delineating the structure of the nuclear envelope, the disposition of proteins through the various compartments of this structure is still poorly understood. The topography of the envelope was investigated by subjecting nuclei to various conditions of iodination. This

was done both with whole nuclei and with nuclei from which the membranes had been removed, as described in section III.5.b. Although the resolution of these profiles was rather poor, similar overall outlines have been achieved several times. The autoradiographs were actually somewhat better in appearance than these photographs suggest. Firstly, with both "Enzymobeads" and soluble lactoperoxidase, the iodination profile is extended after removal of the membranes with Triton-X100. This suggests that more proteins are available when the lamina surface is exposed than when it is obscured with phospholipid. It was found that very little protein could be extracted from purified nuclear envelope by Triton, and that therefore most of the nuclear envelope protein resides in the pore-complex lamina fraction. Therefore these results suggest that although the lamina is possessed of most of the nuclear envelope protein it is not exposed to the cytoplasm. The proteins which are exposed, namely those of Mr 63,000, 52,000, 26,000 and 24,000 may either be in the pore-complex or in the nuclear membrane. The protein of Mr 52,000, heavily iodinated in the whole nuclei is much less heavily labelled by both soluble and immobilised lactoperoxidase after Triton extraction. This suggests that it is a membrane protein removed by the detergent. The protein of Mr 63,000 is much more heavily iodinated by "Enzymobeads" after extraction with Triton-X100. This suggests that it forms a part of the structure which is normally occluded by the membrane. This protein corresponds in molecular weight to the smallest of the lamina triplet (lamin C). It may be that this protein is situated in the cytoplasmic face of the lamina, or in a part of the pore complex not normally exposed to the cytoplasm.

Recently Lam and Kasper (1979 a) showed that this protein is specifically phosphorylated by an endogenous protein kinase in the nuclear membrane. After perturbation of the membrane the activity was reduced, indicating the existence of a topographic relationship between the protein and the enzyme. This implies that the protein is situated in the cytoplasmic surface of the lamina in intimate association with the membrane. The recent work of Richardson and Maddy (1980a) also suggests that this protein may be found in the deeper regions of the envelope. The smallest protein of the lamina triplet (N3) was found only to be accessible to immobilised lactoperoxidase after nuclei had been disrupted. These authors also noted that the larger of the two lamina (N1 and N2) were accessible to immobilised lactoperoxidase in intact nuclei, indicating their situation in the pore complex. This was not found in the BHK nucleus.

One explanation for such a discrepancy could be that there is a difference between BHK and liver nuclei in the abundance of the larger lamins, or in the availability of their tyrosine residues. However the protein profile of the BHK pore complex lamina fraction contains the triplet of protein (of Mr 72,000-60,000) which is characteristic of this fraction. Therefore these proteins are present in similar quantities to N3 in this fraction. They are capable of iodination because they are clearly labelled after Triton-X100 extraction.

An alternative explanation may be that these proteins exist in both the lamina and the pore complex but are not iodinated on the surface of the BHK nucleus because it differs from that of liver in some way which affects the iodination process. One difference could be that there are fewer pores on the BHK nucleus than on liver. The pore complex lamina fraction and the nuclear envelope from BHK

nuclei were isolated by different procedures. Both appeared to have far fewer pores as seen by electron microscopy than do similar preparations from liver nuclei (Dwyer & Blobel, 1976; Bornens & Courvalin, 1978). Another explanation could be that the pores of the BHK nucleus do not project as far beyond the outer membrane as do liver pores. The BHK outer nuclear membrane is often seen to bleb away from the nuclear surface. This could prevent a close interaction between immobilised lactoperoxidase and the pore complex proteins.

Carbohydrates of the Pore Complex Lamina Fraction

Overlay of the pore complex lamina fraction with $\left[^{125}\text{I-}\right]$-lectins revealed that it contains a considerable population of glycoproteins with affinities for ConA, WGA and lentil lectin. Dwyer and Blobel (1976) did not analyse their preparation for carbohydrate, but Berezney and Coffey (1977) reported that hepatocyte nuclear matrix contained neutral carbohydrate (5.5%) of non-nucleic acid origin. This consisted largely of glucose, with traces of mannose, galactose, an unidentified carbohydrate, and very small amounts ($< 0.1\%$) of sialic acid.

Intracellular glycoproteins are believed to exist throughout the endomembrane system, as integral membrane proteins, their carbohydrate moieties situated on the non cytoplasmic surface. (Bretcher & Raff, 1975; Hirano et al., 1972; Keenan et al., 1974). This arrangement has been shown to exist for nuclear membrane by Virtanen and Waarlo (1976) who used ferritin-conjugated Con A to reveal carbohydrates in the perinuclear space. Triton-X100 could reasonably be expected to remove these glycoproteins as this agent removes 95% of the membrane lipids (Richardson and Maddy, 1980a). One interpretation of the retention of carbohydrates by the lamina is that transmembrane glycoproteins on the inner nuclear membrane have a Triton resistant protein-protein interaction with the lamina

and are not extracted. Triton-X100 is frequently used to extract membrane proteins because it forms mixed miscelles with membrane lipids and can solubilise proteins in an undenatured state (Helenius and Simmons, 1975). It has been used to investigate the red cell membrane, in which it is found not to disrupt protein-protein interactions (Tanford and Reynolds, 1976). Sheetz (1979) used this detergent in various concentrations to prepare erythrocyte cytoskeletons. He found that if the extractions were performed in isotonic conditions, a constant fraction (40%) of the integral membrane protein band 3 could not be extracted but remained on the cytoskeleton even when more than 90% of the lipids were removed. Triton extraction of nuclei during the preparation of pore complex lamina is performed in isotonic conditions. It is possible therefore that similar protein-protein interactions are occurring between some of the inner nuclear membrane glycoproteins and the lamina.

This proposal depends upon the extractions procedure being effective. Pore-complex lamina, prepared by this procedure from rat liver "does not contain measurable phospholipid" (Dwyer and Blobel, 1976; Aaronsen and Blobel, 1975). It is likely that a similar preparation is obtained from BHK nuclei. Other support for the efficiency of Triton in this process is as follows. Sheetz expressed the quantities of Triton he used as mg detergent/10^{10} cells, 300mg/10^{10} cells being the highest concentration used. The Triton extractions of nuclei were performed with a concentration of 100mg/10^{8} nuclei so that the actual ratio of detergent/μm^2 membrane was higher than that of the highest concentration used by Sheetz. Berezney (1977) used a 1% solution of Triton to extract nuclei during the third stage of his matrix preparation. The nuclear suspension contained 2mg of nuclear protein/ml at the start of the procedure. This is approximately

equivalent to 7×10^7 nuclei/ml, giving a detergent/nuclei ratio of 100mg/7×10^7 nuclei. This again is lower than the ratio used on BHK nuclei. During the preparation of pore-complex lamina, extraction with 2M NaCl and two washes in isotonic sucrose/buffer follow the Triton extraction. These treatments would be likely to remove ionically or loosely bound proteins remaining on the lamina. Finally, a comparison of the ConA-binding carbohydrates retained by the lamina with those of the nuclear membrane shows that the extraction has been selective as there are many differences between the profiles. If the apparent retention of carbohydrates by the lamina was due to ineffective extraction one would expect the profile of extracted ConA affinities to resemble the profile of those retained (See Fig. 30).

The possibility of an interaction between the Triton-extractable proteins and the lamina was investigated, and no such interaction was found. This could have been so because only the proteins which had weak or non-existent interactions with the lamina were being extracted. The work of Kawasaki and Yamashina (1972a) in which glycoproteins were found in Triton - extracted nuclei may also be explained by the interaction of these glycoproteins with the lamina. They suggested that the detergent was removing only the outer membrane, and that the glycoproteins were remaining in the residual inner membrane. Bhavanandan and Davidson (1975) also found glycoproteins in Triton extracted nuclei isolated from cells grown in ^{3}H-glucosamine. Labelling with ^{35}S-sulphate indicated that at least some of the carbohydrates were attached to sulphated molecules and not glycoproteins. This discovery raises the question of whether the overlay technique was not actually detecting proteoglycans from the intranuclear regions. Furukawa and Terayama (1977), Margolis et al.(1976) and Stein et al.(1975) have all reported that glycoproteins and

mucopolysaccharides exist in association with chromosomal and non-histone chromatin proteins. The pore-complex lamina is essentially free of DNA (Dwyer and Blobel, 1976; Aaronsen and Blobel, 1975), so the detection of chromatin associated saccharides is unlikely.

The detection of carbohydrates in the lamina does not preclude the possibility of glycoproteins existing as structural components of the lamina as they have been reported to do in the chromosome (Stein et al., 1975). Such a possibility would oppose the findings of Virtanen and would run contrary to the accepted view of glycoproteins existing as integral proteins in membranes. The detection of glycoproteins in the chromosomal fraction may possibly be a reflection of the isolation procedure because Triton was also used in these reports to remove the nuclear membrane. If pore-complex lamina, prepared by Triton extraction of nuclei, contains nuclear membrane glycoproteins which resist such extraction, then the distinction between membrane glycoproteins interacting with the lamina, and structural glycoproteins as components of the lamina, cannot be resolved by this approach.

Effects of Calcium

When nuclei are subjected to different concentrations of divalent cations, they undergo reversible expansion and contraction. In order to examine the topography of the lamina during these changes, nuclei, denuded of their membranes with Triton, were iodinated in both the expanded and contracted states. Soluble and immobilised lactoperoxidase was used. It was found that contracted nuclei present the same range of proteins to soluble and immobilised lactoperoxidase. This implies that either:

a) all the nuclear proteins remaining after Triton extraction have some portion exposed on the surface;

b) the lamina has a wide enough mesh for "Enzymobeads" to enter and iodinate intranuclear proteins, or

c) the mesh of the contracted nucleus is so compact that even soluble lactoperoxidase is excluded and its iodination range restricted to that of the beads. It is not likely that the situation in b) should be true since the diameter of the beads is approximately 1/3 of that of the contracted nucleus, and soluble lactoperoxidase would still be expected to have greater penetration than the beads and a wider iodination range. After expansion, the iodination profiles of both systems were extended somewhat, in that high molecular weight proteins were included. This argues against the situation in a) since expansion would not alter the range if all the proteins were available on the surface. So the proteins of the contracted lamina must be very closely packed to exclude a soluble protein in the absence of a phospholipid barrier. This implies a strong interaction between the proteins which may be mediated by Ca^{2+} since this ion was most effective in inducing volume changes. These changes were first observed by Anderson and Wilber (1951b) who suggested that they were caused by expansion and contraction of the chromatin because the action of DNA-ase abolished them. This was further investigated by Leake et al. (1972) who investigated the effects of various cations on both chromatin and nuclei. They proposed that volume changes in nuclei were due to similar changes in the chromatin induced by the environment or cations. They also suggested that gene activation required chromatin expansion which was induced by a reduction in the cation concentration. However they were also able to isolate chromatin

fractions from liver nuclei which responded in different ways to the same cation environment. This implies the intervention of some regulatory system.

Extensive evidence has been accumulated for the presence of contractile proteins in the nucleus (Jockusch et al., 1973; Douvas et al., 1975; Le Stourgeon et al., 1975; Sanger, 1975). Actin and myosin have most often been detected, together with troponin and tropomyosin which are not so well established. Actin in nuclei has more recently been associated with the condensation of chromatin. (Goldstein et al., 1977a, b; Karsenti et al., 1978). This suggests an interaction between the contractile system and the chromatin. The involvement of Ca^{2+} in this system is suggested by the work of Douvas et al. (1975), and Le Stourgeon et al. (1975), who all reported Ca^{2+} stimulated ATP-ase activities associated with contractile nuclear proteins. Timourian et al. (1974) found that the Ca^{2+} and PO_4^{3-} content of mitotic asters in sea urchin eggs was higher than the surrounding cytoplasm, implying its involvement in the movement of chromosomes, possibly in a system of contractile proteins.

There is also extensive evidence for an association between the chromatin and the nuclear matrix in situ (Mizuno et al., 1971; Wise+Prescott, 1973; O'Brian et al., 1973; Comings & Okada, 1973). Comings and Wallack (1978) showed that an isolated matrix has a strong affinity for DNA from various sources, having a preferential interaction with the DNA which was removed from the matrix during its preparation. In connection with this, Long et al. (1979) have shown that the spatial arrangement of the matrix and the chromatin are intimately connected. The matrices of differentiated and undifferentiated Friend erythroleukaemia cells have the same characteristic spatial arrangement as their respective chromatins.

It therefore seems likely that a system of contractile proteins, responsive to the Ca^{2+} concentration, may be able to regulate the expansion and contraction of both the chromatin and the matrix. In vivo this may be responsible for chromosomal condensation and dispersion and movement during mitosis. The early observations of Anderson (1951b) that DNAase abolished nuclear volume changes may have been due to the inactivation of actin by DNAase (Sheetz, 1979b).

The nuclear matrix may be inherently contractile, but require regulation by a Ca^{2+}-responsive system. This is suggested by the work of Wunderlich and Herlan (1977) who found that a reversibly contractile nuclear matrix could be isolated from Tetrahymena macronuclei. These matrices behaved in an unphysiological way in that volume changes did not require ATP and were not abolished by salyrgan The actomyosin system was not involved therefore. In contrast to this Hauser (1980) found that in the presence of Ca^{2+} (2-3mM), ATP dependant contractions occurred in Plasmadium bursaria macronuclei. These contractions were abolished by salyrgan, and reversed by EGTA, indicating the involvement of an actomyosin like system. The behaviour of the matrices isolated by Wunderlich would be explained by the removal of one or more components of such a contractile system.

In this context, the lamina/matrix of the BHK nucleus does not seem to undergo a major topographic change on expansion or contraction. Both soluble and immobilised lactoperoxidase iodinated a similar range of proteins in either expanded or contracted nuclei. This suggests that the proteins bear the same relationship to one another, but lie farther apart in the expanded state. This may suggest that there is not a direct interaction between the lamina proteins, but that it is mediated by some other system. As pointed out by Hauser (1980), these volume changes are not due to osmosis.

In BHK nuclei such changes occur in response to Ca^{2+} concentration after removal of the membrane.

IV.2.b. The Membranes

Relationship Between the Outer Nuclear Membrane and Rough Endoplasmic Reticulum

The outer membrane of the nuclear envelope has often been described as being continuous with the rough endoplasmic reticulum (Watson, 1955; Wischnitzer, 1958; Whaley *et al.* 1960). During this project comparative studies of these two fractions were conducted alongside those of other fractions in an attempt to clarify this point. Due to the presence of the pore-complex lamina, a comparison of the whole nuclear envelope with rough endoplasmic reticulum is not acceptable. Nuclear membrane proteins were consequently isolated by extraction with Triton-X100 during the preparation of the pore complex lamina fraction, as described. This may not be completely satisfactory however, because the detergent may be able to extract some intranuclear proteins. Certainly there were no grounds for assuming that Triton abstracted only membrane proteins from the nucleus. Initially Triton extracts of whole nuclei were used for the preparation of membrane proteins. It was then found that a similar extraction of isolated nuclear envelope yielded very little protein. This raised the question of the origin of the extra protein extracted from whole nuclei.

Profiles of the proteins extracted from whole and DNA-ase treated nuclei are very similar, apart from the regions of 61,000 and 47,000-53,000 where there is a difference in the relative proportions of the extracted polypeptides. However in view of the

great difference in the quantity of protein which could be extracted from whole nuclei as opposed to envelope, DNAase treated nuclei were decided upon for this purpose.

When this preparation was compared with rough endoplasmic reticulum major differences were seen in the protein profiles, especially in the region of Mr 60,000 - 100,000. Extra proteins are present in the nuclear membrane in this region, which are not seen in rough endoplasmic reticulum. In the regions of lower molecular weight there may be some homologies e.g. the proteins of Mr 43,000 and 41,000, and those of less than Mr 25,000 although these common polypeptides display differences in their relative proportions. It is interesting that such similarities as there are, occur in the regions of Mr 45,000 because it is this region which contains the ribosomal proteins. The similarities may reflect the presence of ribosomes on both membranes. Richardson and Maddy (1980b) pointed out that a comparison of the Triton extract of nuclei with whole endoplasmic reticulum is not ideal. The best solution would be a Triton extract of both membrane systems, subjected to the same conditions of centrifugation to remove ribosomes so that only "true" membrane proteins are being compared.

As was previously discussed the WGA and lentil lectin affinities of these two membrane systems are very similar indeed, indicating that they may contain homologous glycoproteins. However the profiles of their affinities for ConA are somewhat different, especially in the region of molecular weight Mr 75,000-43,000. In this area the affinity of rough endoplasmic reticulum proteins falls dramatically,

Fig. 29. Protein profiles of C_{13} nuclear membrane (n.m) and rough endoplasmic reticulum (rER).

whilst nuclear membrane has some major ConA binding proteins. There are other inequalities in the high and low molecular weight regions, but these may be differences of relative proportion, rather than changes

in the glycoproteins. Modified areas have been discovered within the endoplasmic reticulum system (Depiére & Dallner, 1976) so inequalities in the glycoprotein profiles of the nuclear membrane and endoplasmic reticulum do not necessarily imply that they are not continuous. The similarities between the WGA and lentil lectin affinities of these membranes and the carbohydrates of the lamina may suggest that both were continuous at least for some time during the cell cycle.

These results may provide evidence for the reformation of the nuclear membrane from incoming vesicles of endoplasmic reticulum after karyokinesis. These vesicles may only be a subpopulation of the endomembrane system targetted to nuclear membrane reformation by the presence of transmembrane glycoproteins with a high affinity for lamina proteins. Such vesicles could attach to the newly reformed lamina and spread laterally over its surface to coalesce around the the pore complexes, forming the double nuclear membrane. During this process, segregation could occur between the putative inner and outer membranes in that proteins with a high affinity for the lamina would tend to remain in the inner membrane. It is known that in the newly reformed nuclear membrane significant proportions of both the protein (60%) and the phospholipid (50%) pre-existed mitosis (Conner et al., 1980). It has been shown that when nuclear envelope dissociates prior to mitosis, nuclear membrane vesicles leave the structure and become indistinguishable from the rest of the endomembrane system (Porter and Machado, 1960). A reversal of this process would be the most economical way of reforming the membrane. The extensive differences in the ConA affinities of nuclear membrane and rough endoplasmic reticulum may indicate that since the last

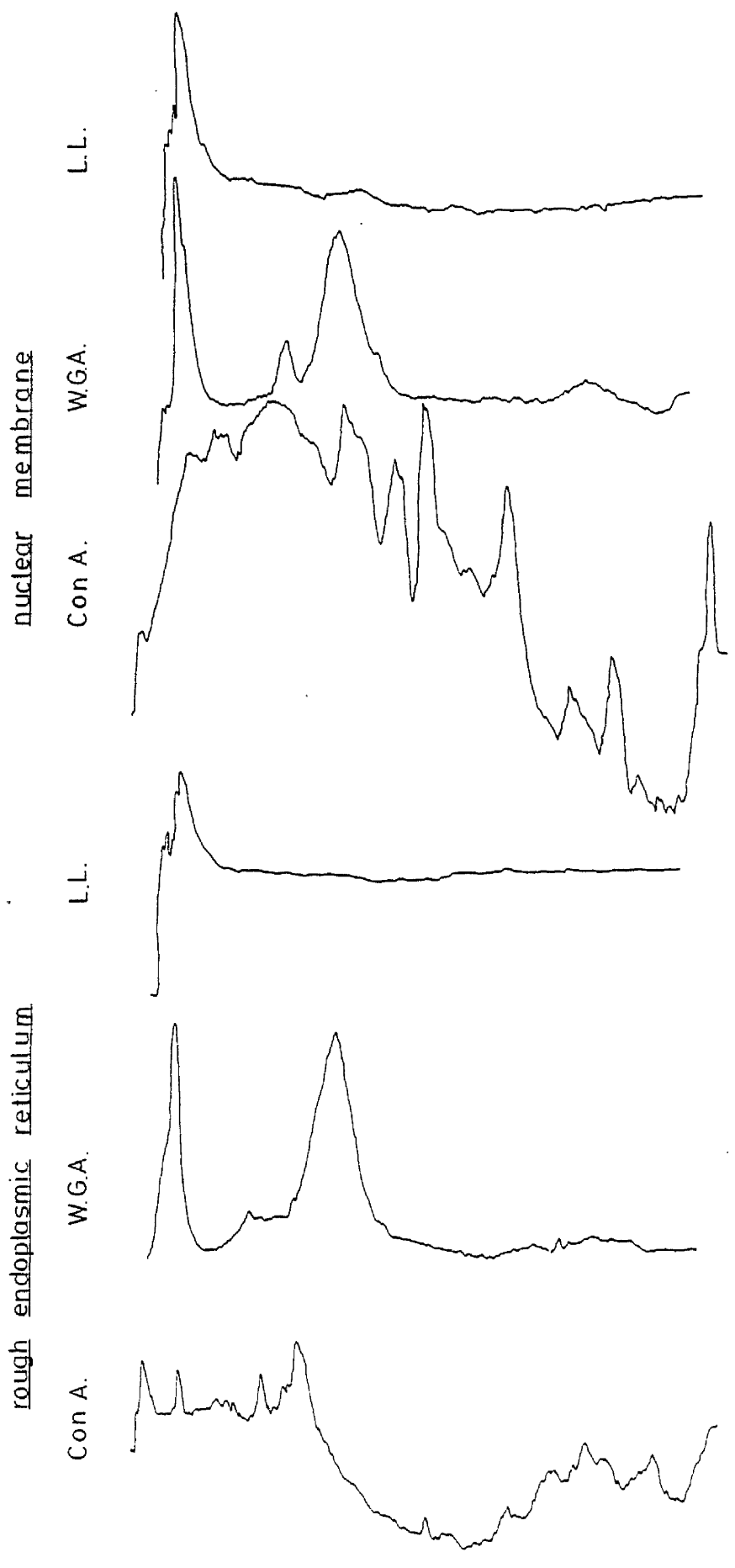


Fig.30. Lectin affinity profiles of C_{13} nuclear membrane (n.m) and rough endoplasmic reticulum (rER)

mitosis the systems have separated and their glycoproteins have been modified as suggested by Morré et al.(1979). This would also explain the paucity of reported cases in which the outer membrane is actually seen to be continuous with the rough endoplasmic reticulum, and the apparent lack of such continuities in liver cells. Liver cells do not divide very often. Ample time would have elapsed for the breakdown of continuities between the nuclear membranes and the endomembrane system to occur. After isolation they invariably appear to have complete outer membranes, and no attached endoplasmic reticulum. A strong and specific interaction between the nuclear membranes and the lamina would explain the apparent interaction between these structures reported by Wunderlich. He reported that changes in the lipid ordering and protein-lipid segregation of nuclear membranes occur in response to changes in the nuclear volume induced by concentrations of divalent cations. Such an interaction is also suggested by the work of Lam and Kasper (1979a) when they reported the phosphorylation of lamin C by a nuclear membrane kinase which was inactivated when the membrane was perturbed. The inner membrane often appears to be thicker and less convoluted than the outer membrane in electron microscopy. It is never seen to bleb into the cytoplasm as does the outer membrane. Such close apposition to the nuclear lamina could be due to a close interaction between these fractions.

IV.3. Transformation

One aim of the project has been to study the carbohydrate complement of the various nuclear envelope fractions before and after transformation. This has been done and, in most cases, only minor changes in the relative proportions of the various glycoproteins

were seen. For example, the ConA affinities of C_{13} and PyY nuclei are basically very similar, but with slight differences in the relative proportions of some peaks.

One qualitative change was found in the RCA affinities of nuclear and nuclear envelope of glycoproteins after transformation. The three small peaks of RCA affinity corresponding to Mr of 70,000, 58,000 and 33,000 disappear after transformation. Gurtler *et al.* (1979) reported two RCA affinities of Mr 70,000 and 60,000. These two glycoproteins were detected by incubating column gels with labelled lections. The gels were then sliced and counted. The use of $\left[^{125}\text{I}-\right]$-lectins with slab gels and autoradiography may provide a more sensitive method of detection. It is possible that the smaller glycoproteins detected by RCA were produced by proteolysis, since only one such peak was detected in whole nuclei. However this does not detract from the point that no RCA-binding glycoproteins are found in any PyY nuclear fraction.

There are, however, two points to consider before attributing any of these changes to transformation. Firstly, C_{13} cells are contact inhibited and when viewed by light microscopy show the characteristic "watered silk" morphology of normal fibroblasts. Consequently after confluence has been achieved their rate of division falls, and most of the population enter the resting phase. PyY cells are not inhibited after confluence. They appear as an untidy welter of interlacing cells and they continue to divide, albeit more slowly, to form piles of cells on the glass surface. Consequently the PyY population is at all stages of the cell cycle. If there are changes in the populations of glycoproteins of membranes throughout the cycle these differences

may be detected in a gross comparison of C_{13} and PyY populations. Without a similar comparison of synchronised populations the distinction between changes due to the cell cycle and changes due to transformation cannot be made.

Another consideration is that of clonal differences. The C_{13} line was originally transformed with Polyoma virus in 1964 (Stoker and Macpherson, 1964). As Burridge (1976) and Sakiyama et al. (1972) have pointed out, micromutations occur which could easily account for the minor changes seen, e.g. in the relative proportions of nuclear glycoproteins. In this context it should be noted that the C_{13} line will have changed somewhat since it was separated from the PyY line. These cells are no longer completely contact inhibited and so display a tendency towards the behaviour of a transformed line. A study of normal and recently transformed cells is needed to circumvent this difficulty.

IV.4. Speculation

In recent years the clarity of our understanding of the nuclear envelope has markedly improved. A major advance was made when the importance of the lamina in the nuclear envelope was appreciated. The topography of the lamina proteins is now the subject of much investigation. The nuclear matrix is also under examination, but the distinction between these fractions is somewhat arbitrary. A more realistic concept may be that of a nucleoskeleton of protein which is denser at its margin, in the lamina region, and more diffuse in the central matrix to accomodate the chromatin (Berezney et al., 1981; Eastment et al., 1981; Baglia and Maul, 1982).

As more is learned about the various nuclear components the importance of their interactions becomes more and more apparent. The matrix/lamina interacts with both DNA and hnRNA (Berezney et al., 1981; Baglia and Maul, 1982) and enzymes involved in processing the nucleic acids are found associated with the matrix (Bukhalov et al., 1981). The structure may therefore play a significant role in the ordering and/or expression of the genome. It is known that the structure undergoes a reversible depolymerisation to accomodate mitosis (Gerace and Blobel, 1980), and that after mitosis the structure is not synthesized de novo but is reformed from pre-existing components (Sieber-Blum and Berger, 1976; Conner et al., 1980). The dissociation of nuclear envelope proteins before mitosis, rather than their degradation, has been established (Ely et al., 1978; Gerace et al., 1978). It has been suggested that selective phosphorylation of the lamins are responsible for this (Lam and Kasper, 1979a). Recent work has also shown that there is a definite interaction between the nuclear membrane and both the lamina and the membrane-bound enzymes (Agutter and Suckling, 1981).

Although little is known of the biochemistry of the pore-complex two interesting observations have been made:

a) the number of pores per unit area seems to be related to nuclear activity (Stevens and Andre, 1969).

b) in the absence of a nuclear envelope, pores have been observed in a haphazard array on the surface of daughter chromatin masses (Peterson and Berns, 1978).

Thus the components of the pore complex also appear to be capable of reversible dissociations during mitosis and possibly also during interphase.

In the face of these diverse obserations a little speculation may now be justified. The complex nuclear envelope is a unique feature of the eukaryotic cell. Its evolution may have resulted from the need to organise an extensive genome, a large proportion of which was not being expressed. The primitive nucleus may not have had a membrane or lamina. Nucleo-cytoplasmic exchange may originally have occurred by diffusion. However, as the genome increased in complexity this may have been inadequate and a controlled route to the nucleo-cytoplasmic interface may have been necessary. Evidence for the controlled translocation of macromolecules in the nucleus was recently supplied by Feldherr (1980). The pore complex may then have evolved as the final mediator in the passage of molecules to and from the primitive nucleus. However, such a gateway would be useless in the absence of a membrane since uncontrolled exchanges could still occur. The evolution of a strong and specific interaction between endomembrane vesicles and the surface of the nucleus would markedly increase the effectiveness of the pore complex in regulating nucleo-cytoplasmic exchange. This would be a distinct evolutionary advantage, especially if such interactions could be broken down at the onset of mitosis to allow the dissociating structural proteins to leave the vi cinity of the chromatin.

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